NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS

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ARISTOTLE UNIVERSITY OF THESSALONIKI

SCHOOL OF SCIENCE

DEPARTMENT OF CHEMISTRY

INTERDISCIPLINARY INTERSECTIONAL POSTGRADUATE PROGRAMME
"CHEMICAL ANALYSIS – QUALITY CONTROL"

MASTER THESIS

Method development for the determination of emerging contaminants in biota by liquid chromatography coupled with high resolution mass spectrometry.

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Athens

October 2019

Master Thesis

Method development for the determination of emerging contaminants in biota by liquid chromatography coupled with high resolution mass spectrometry.

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DEFENDING DATE

October 2019

ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Ανάπτυξη μεθόδου προσδιορισμού αναδυόμενων ρύπων σε ζωϊκά δείγματα με υγροχρωματογραφία συζευγμένη με φασματομετρία μάζας υψηλής διακριτικής ικανότητας.

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ΗΜΕΡΟΜΗΝΙΑ ΕΞΕΤΑΣΗΣ
Οκτώβριος 2019

ABSTRACT

Overwhelming evidence over many decades has shown that many organic compounds, or “emerging contaminants”, have been released in the environment due to anthropogenic activities. Despite the fact that their chemical and physical properties allow them to enter marine, freshwater and/or terrestrial ecosystems, data for the potential bioaccumulation and biomagnification of these compounds though the food chains are missing, which increases the concern about their effects on the ecosystems, biota and human health. Apex predators play a key role in the monitoring of environmental contaminants and in risk assessment studies due to their unique characteristics.

This thesis reports the development of a generic protocol for the simultaneous determination of polar and semi-polar organic emerging contaminants in biota tissues. For samples treatment, an Accelerated Solvent Extraction protocol was used for the extraction of analytes followed by a clean-up step using Solid-Phase Extraction (SPE) with four different sorbent materials that covered a broad range of analytes with different physicochemical properties. Extracts were analyzed with reversed-phase liquid-chromatography coupled to quadrupole-time-of-flight mass spectrometry (RPLC-QTOF-MS) and the data were acquired through data dependent and data independent acquisition mode. The validation of this generic protocol was performed based on a representative group of compounds from different classes of emerging contaminants. A database of approximately 2,100 emerging contaminants was used for the wide-scope target screening and the detection was based on mass accuracy, retention time, isotopic pattern and fragmentation profile.

Following the aforementioned protocol, 20 samples of apex predators (common Buzzards, Harbour Porpoises, Eurasian Otters) and their prey (freshwater fishes; Roaches), which were gathered by the Center of Ecology and Hydrology (CEH) across the United Kingdom, were analyzed for the determination of emerging contaminants. The results indicate the presence of several plant protection products (including DEET, myclobutanil and isoprocarb), numerous pharmaceuticals and psychoactive drugs (including sertraline and quetiapine), stimulants (such as nicotine and its metabolites),

sweeteners, industrial chemicals (including benzotriazole and tolytriazole) in the tested samples. Furthermore, numerous transformation products (including propachlor-OXA, Nor-tramadol, 4-acetamido-antipyrine) were detected in livers from apex predators, underlying the power of wide-scope target screening and of high-resolution mass spectrometry. Moreover, more than 15 PFAS were detected in the samples with high detection frequency and at high concentration levels. Based on the results, significantly higher concentrations of chemicals (including perfluorodecanoic acid -PFDA- and propachlor-OXA) were detected in livers from apex predators than in the muscle samples from their prey, implying a potential bioaccumulation through the food chain.

SUBJECT AREA: Environmental Analytical Chemistry

KEYWORDS: Apex Predators; Emerging Contaminants; High Resolution Mass Spectrometry; Biomonitoring; Bioaccumulation.

ΠΕΡΙΛΗΨΗ

Τις τελευταίες δεκαετίες υπάρχουν πολλές ενδείξεις ότι δεκάδες οργανικές ενώσεις, ή αναδυόμενοι ρύποι, έχουν απελευθερωθεί στο περιβάλλον λόγω των ανθρωπογενών δραστηριοτήτων. Παρά το γεγονός ότι οι φυσικοχημικές τους ιδιότητες του επιτρέπουν να εισαχθούν στα υδάτινα και χερσαία οικοσυστήματα, δεδομένα για την πιθανή βιοσυσσώρευση και βιομεγέθυνση τέτοιων χημικών ενώσεων μέσα στην τροφική αλυσίδα λείπουν, γεγονός που αυξάνει την ανησυχία για την επίδραση τέτοιων ενώσεων στα οικοσυστήματα, στους οργανισμούς που ζουν μέσα σε αυτά και, κατά συνέπεια, στον άνθρωπο. Οι κορυφαίοι θηρευτές παίζουν έναν καθοριστικό ρόλο στις μελέτες παρακολούθησης περιβαλλοντικών ρύπων καθώς και σε μελέτες ανάλυσης επικινδυνότητας, λόγω των μοναδικών χαρακτηριστικών που διαθέτουν.

Σε αυτήν την εργασία ειδίκευσης περιγράφεται η ανάπτυξη ενός γενικευμένου πρωτόκολλου για ταυτόχρονο προσδιορισμό πολικών και μετρίως πολικών οργανικών αναδυόμενων ρύπων σε ιστούς ζωικών δειγμάτων. Κατά τη διάρκεια της προκατεργασία των δειγμάτων, χρησιμοποιήθηκε η τεχνική Accelerated Solvent Extraction (ASE) για την εκχύλιση των αναλυτών και στη συνέχεια σαν στάδιο καθαρισμού χρησιμοποιήθηκε η τεχνική Solid-Phase Extraction (SPE) με τέσσερα διαφορετικά πληρωτικά υλικά τα οποία καλύπτουν ένα ευρύ φάσμα αναλυτών με διαφορετικές φυσικοχημικές ιδιότητες. Τα εκχυλίσματα αναλύθηκαν με υγροχρωματογραφία αντίστροφης φάσης συζευγμένη με φασματομετρία μάζας με υβριδικό τετράπολο-αναλυτή χρόνου πτήσης (RPLC-QTOF-MS) χρησιμοποιώντας δύο λειτουργίες σάρωσης (DDA, DIA). Η επικύρωση ου γενικευμένου αυτού πρωτοκόλλου βασίστηκε σε μία αντιπροσωπευτική ομάδα αναδυόμενων ρύπων από διαφορετικές κατηγορίες. Μία βάση δεδομένων που περιείχε περισσότερους από 2.100 αναδυόμενους ρύπους χρησιμοποιήθηκε για τη στοχευμένη ανάλυση και η ανίχνευση βασίστηκε στην ακρίβεια μάζας, στο χρόνο ανάσχεσης, στο ισοτοπικό προφίλ και στα χαρακτηριστικά θραύσματα.

Ακολουθώντας το παραπάνω πρωτόκολλο, 20 δείγματα κορυφαίων θηρευτών (γερακίνες, φώκιες, ενυδρίδες) και η λεία τους (ψάρια του γλυκού νερού, τσιρόνια), τα οποία συλλέχθηκαν από το Center of Ecology and Hydrology (CEH) από διαφορετικές περιοχές του Ηνωμένου Βασιλείου αναλύθηκαν για τον προσδιορισμό αναδυόμενων ρύπων.

Τα αποτελέσματα υποδεικνύουν την ύπαρξη πολλών φυτοφαρμάκων (συμπεριλαμβανομένων των DEET, myclobutanil και terbuthylazine), φαρμακευτικών ενώσεων και ψυχοδραστικών ουσιών (συμπεριλαμβανομένων των sertraline και quetiapine), διεγερτικών ουσιών (όπως η νικοτίνη και οι μεταβολίτες της), γλυκαντικών, χημικών βιομηχανιών (benzotriazole και tolytriazole). Επιπροσθέτως, πολλά προϊόντα (βιο)μετατροπής (όπως propachlor-OXA, Nor-tramadol, 4-acetamido-antipyrine) ανιχνεύτηκαν σε ιστούς συκωτιού από τους κορυφαίους θηρευτές, υπογραμμίζοντας την δύναμη της στοχευμένης ανάλυσης σε συνδυασμό με την φασματομετρία μάζας υψηλής διακριτικής ικανότητας. Ακόμα περισσότερα από 15 PFAS ανιχνεύτηκαν στα δείγματα με υψηλή συχνότητα εμφάνισης και σε υψηλά επίπεδα συγκεντρώσεων. Βασισμένοι στα αποτελέσματα, πολλοί αναλύτες (όπως perfluorodecanoic acid -PFDA- και propachlor-OXA) ανιχνεύτηκαν σε σημαντικά υψηλότερες συγκεντρώσεις στους ιστούς συκωτιού από τους κορυφαίους θηρευτές σε σχέση με τον ιστό από τα θηράματά τους, υποδεικνύοντας μία πιθανή βιοσυσσώρευση δια μέσου της τροφικής αλυσίδας.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Περιβαλλοντική Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Κορυφαίοι Θηρευτές, Αναδυόμενοι Ρύποι, Φασματομετρία Μάζας Υψηλής Διακριτικής Ικανότητας, Βιοπαρακολούθηση, Βιοσυσσώρευση.

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PREFACE

This master thesis was performed at the laboratory of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens under the supervision of Professor Nikolaos S. Thomaidis. A part of the experimental procedure was carried out at the laboratory of “Chemical Analysis” in the Hellenic Research & Innovation Centre (HRIC).

I would like to thank my supervisor, Professor N. S. Thomaidis, for his advices, guidance, support during my master and for giving me the opportunity to work in LIFE APEX project as a member of TrAMS group. I am very happy to be part of his research group and grateful for all the opportunities and trust that I received. Also, I especially thank the members of the examination committee, Professor Anastasios Economou and Assistant Professor Evagelos Gikas, for their comments and remarks in regards with my master thesis.

These past two years were very important in order to develop and improve my theoretical knowledge and practical skills in the field of Chemical Analysis and Quality Control. I would like to thank all the professors for teaching and imparting their knowledge during my master. Moreover, I am extremely grateful to post-doc researcher Dr. Maria-Christina Nika for her advices, guidance, support during my master thesis, for sharing with me her expertise, knowledge and for our harmonic collaboration during the past one year. I would like to say a big thank you to each and every member of TrAMS group I met in the lab during my master for our collaboration in a friendly and creative environment in the lab, for sharing our ideas and knowledge and for creating many beautiful memories. Also, I would like to acknowledge all the people I met in the Hellenic Research & Innovation Centre (HRIC) for the friendly environment and our collaboration within the framework of the collaboration between our Laboratory and HRIC.

Last but not least, I would like to deeply thank my family, my parents, Othon and Dimitra, my friends and of course Maria for their enormous support, care and encouragement during these two years of my master and, in general, in every decision and every challenge in my life.

ACKNOWLEDGMENTS

This research has received funding from the European Union through the program LIFE17 ENV/SK/000355 (LIFE APEX) "Systematic use of contaminant data from apex predators and their prey in chemicals management" (https://lifeapex.eu/).

CHAPTER 1
INTRODUCTION

1.1 Emerging Contaminants

Over the last decades, many scientific researches have shown that a big number of chemical compounds (pollutants) have been released in the environment from various anthropogenic sources [1]. In the past, a big percentage of the worldwide environmental research focused on the determination of so-called persistent organic pollutants (POPs). POPs include many toxic and bioaccumulative chemical compounds, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCB), and dioxins. Many of these compounds were, thus, recognized as priority pollutants and, therefore, subjected to environmental regulations [2].

However, due to the development of new and more sensitive analytical techniques, evidence is existed for the presence of a large number of chemical compounds in the environment. The distribution of these potentially dangerous chemical compounds, which are globally known as emerging contaminants (ECs) or emerging pollutants (EPs), throughout the environment and organisms, causes adverse ecological and health effects [2]. In this way, the research interest has been shifted from the determination of priority and conventional pollutants, to the determination of emerging contaminants.

The term “emerging contaminants” (ECs) has been established for any chemical that is not subjected to marketing restrictions, to national or international monitoring programs and is not previously included in existing environmental-quality regulations, but is continually being introduced into the environment due to anthropogenic activities [1,3,4]. These chemicals need not, necessarily, be new, although their environmental fate and (eco)toxicological study have not yet been evaluated [5]. Therefore, ECs are candidates for future regulation, due to their frequent detection in environmental samples and their potential hazardous properties for aquatic and terrestrial ecosystems and human health [3,5].

Although dozens of thousands of chemicals are marketed in Europe, so far only a 600 chemicals have been screened and identified as PBT (persistent, bioaccumulative, toxic), ED (endocrine disrupting) and/or CMR (carcinogenic, mutagenic, toxic for reproduction), because human and environmental hazard assessment is laborious and often obstructed due to the lack of data (ECHA's 2016 General Report, ECHA 2017). Due to their large number, only a small percentage of these is toxicologically evaluated. There is an increasing number of emerging contaminants, which need to be identified and quantified in different environmental samples and biological tissues [1].

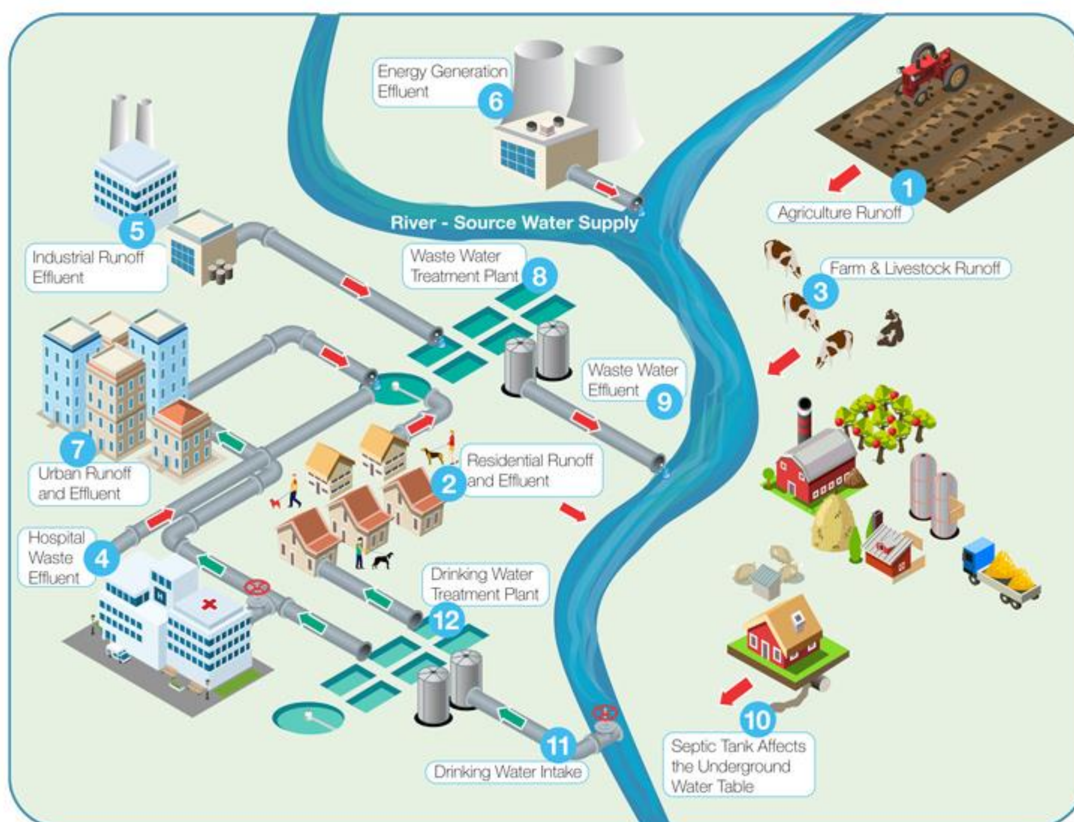


Figure 1: Sources of Emerging Contaminants
(source: https://www.thermofisher.com/gr/en/home/industrial/environmental/environmental-learning-center/contaminant-analysis-information/emerging-contaminants-analysis.html).

1.2 Classes of Emerging Contaminants

ECs encompass a diverse group of compounds, with different physicochemical properties, belonging to different chemical classes and with different applications. Into this group are included pharmaceuticals, illicit drugs

and drugs of abuse, personal-care products (PCPs), steroids, surfactants, per- and polyfluoroalkyl substances (PFAS), brominated and organophosphate flame retardants and plasticizers, industrial additives and agents, food additives (e.g. artificial sweeteners), pesticides, siloxanes as well as their (bio)transformation products [(bio)TPs] [6,7].

1.2.1 Pharmaceuticals

Over the last decades, pharmaceuticals have been receiving increasing attention as potential bioactive chemicals in the environment [8]. Pharmaceuticals are a large and diverse group of organic compounds with various physicochemical properties, which are used for the prevention and treatment of diseases in humans and animals [9]. The rapid advances in medical science lead to the development of new medications and treatments and an increased production of drugs that are consumed [10]. Among ECs, pharmaceuticals belong to a group of increasing interest due to their pharmacological activity and rising consumption deriving from their use in human and veterinary medicine. Pharmaceuticals and their metabolites have been detected at trace concentrations (µg/L or ng/L levels) in a wide variety of environmental water samples including sewage flows, rivers, lakes, groundwater aquifers and drinking water [10]. Although the concentrations of these compounds in water bodies are very low, they are considered as ‘‘pseudo’’ persistent pollutant (EU 2013) similarly to other micropollutants, because they are omnipresent in the environment from continual input into the aquatic bodies and they can affect water quality and potentially the impact drinking water supplies, ecosystem and human health [8,10–13]. The presence of pharmaceuticals in water is attributed to personal hygiene products, pharmaceutical industry raw and treated effluents, runoff from agricultural fields fertilized with treated sewage sludge, hospital waste and therapeutic drugs [8–10].

1.2.2 Illicit Drugs & Drugs of Abuse

Illicit drugs are those whose nonmedical use is prohibited by the national or international law. There are many categories of compounds, which are included in the class of illicit drugs, such as opioids, cocaine, cannabis, amphetamine type substances (ATSs), and ecstasy-group substances [14]. This heterogeneous group includes many compounds with different structures and physicochemical properties [15]. In analogy with pharmaceuticals, the main source of contamination for illicit drugs is the legal or illegal human consumption or the accidental/ deliberate disposal from surreptitious drug laboratories. The residues of drugs of abuse persisting in consumers' urine can reach sewage treatment plants (STPs) in detectable amounts, escaping degradation, and can be released into surface water. Illicit drugs were recently indicated as emerging contaminants since they have been detected in waste, surface and drinking water and in the airborne particulates in several European countries and USA [14,15].

1.2.3 Personal Care Products (PCPs)

Personal care products (PCPs) are a big group of various well-known household substances, which are used for health, beauty and cleaning purposes (e.g. disinfectants, fragrances, insect repellents, preservatives and UV filters). These compounds are used mainly to improve the quality of daily life [16,17]. Some of them are considered ECs, due to their presence and negative impact on aquatic ecosystems, specially related to endocrine disruption and reproductive disorders. These chemicals end up to aquatic ecosystem through the sewage effluents (releasing via cleaning, showering and bathing) from wastewater treatment plants due to their incomplete or inefficient removal [16,18]. An increasing number of studies has confirmed the presence of various PCPs in different environmental compartments at concentrations capable of causing detrimental effects to the aquatic organisms, which raises concerns about the potential adverse effects to environment, wildlife and humans health [17].

1.2.4 Surfactants

A diverse group of ECs comprises of surfactants. The chemical compounds which are included in this class are consisted of a polar, hydrophilic head group and a non-polar hydrophobic hydrocarbon tail group [19]. A surfactant (or Surface Active Agent), according to the International Union of Pure and Applied Chemistry (IUPAC), is "*a substance which lowers the surface tension of the medium in which it is dissolved, and/ or the interfacial tension with other phases, and, accordingly, is positively adsorbed at the liquid/ vapor and/or at other interfaces*" [20]. The surfactants are used mainly in the manufacture of household and industrial detergents, laundry products, and cleaners (e.g. soaps, dishwashing liquids, shampoos). After their massive use, surfactants and their metabolites are released directly or through sewage systems into aquatic ecosystems. They are, also, high bioaccumulated compounds and their high concentrations in the environmental samples import that surfactants can greatly affect the ecosystem; their toxicity to organisms is, already, well known [19,21]. Although, a lot of data exists on the presence and distribution of Linear Alkylbenzene Sulfonates (LAS) and Nonylphenol Ethoxylates (NPEOs), respective data on aliphatic surfactants (such as Alcohol Ethoxy Sulphates - AES - and Alkyl Ethoxylates - AEOs-) are more limited, despite the fact that their production volumes being similar [21].

1.2.5 Per- and Poly- Fluoroalkyl Substances (PFAS)

Per- and Poly- Fluoroalkyl Substances (PFAS) are a class of anthropogenic chemicals that have been used to make household and industrial products resistant to heat, oil, stains, grease and water [22,23]. Until now, there are more than 4700 already known PFAS that have found many commercial uses.[24] Despite the fact that PFAS do not occur naturally in the environment, a big percentage of them is widely dispersed in the environment. PFAS are not readily biodegradable, they are persistent (long environmental lifetimes) and are bioaccumulated through aquatic food webs, because they contain one of the strongest chemical bonds (C-F) known [22,23,25]. Human exposure to PFAS, through consumption of food and water, has been

associated with metabolic disruption, immunotoxicity, and cancer [25]. Through the past years, the scientific interest was focused only on the detection of few perfluoroalkyl acids (PFAAs), especially in the detection of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). PFOS and its salts are listed under Annex B of the Stockholm Convention for Persistent Organic Pollutants and PFOA and perfluorohexane sulfonate (PFHxS) have been proposed for inclusion. These compounds are replaced from the industries with structurally similar PFAS, which are currently not routinely monitored or present on regulatory guideline lists. To conclude, there is a continuous need for the quantitation of the unregulated and emerging PFAS in aqueous environmental matrices.[24]

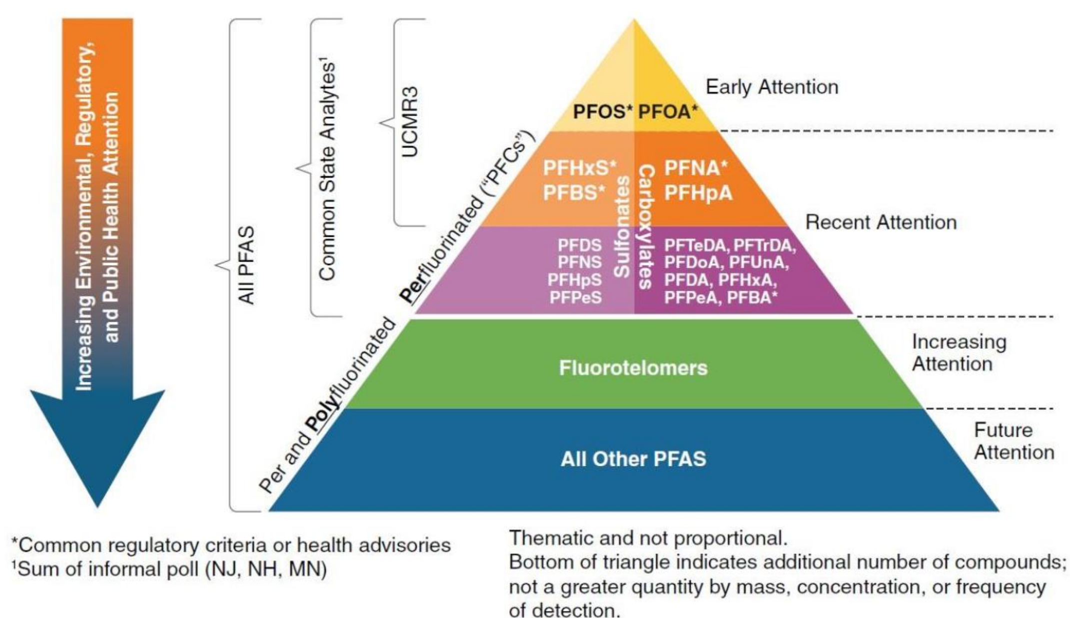


Figure 2: Emerging awareness and emphasis on PFAS occurrence in the environment *(source:https://waterinstitute.unc.edu/files/2018/11/03_VP27_Technological_Public_Health_Implications_Emerging_Contaminants_USA_Water.pdf).*

1.2.6 Artificial Sweeteners

Sucralose and other artificial sweeteners have recently been identified as persistent emerging pollutants. Artificial sweeteners are used worldwide as sugar substitutes in remarkable amounts in food, beverages. They are widely used in the human diet, because, in contrast to sugar, they do not cause any

glycemic effect/ insulin response or calorie intake once digested, and do not adversely affect the microflora of dental plaque [26,27]. Artificial sweeteners are water contaminants that are highly specific to wastewater. Many artificial sweeteners, especially acesulfame and sucralose, were detected in the aquatic ecosystems (surface water, groundwater, and drinking water) in concentrations, which reached levels of micrograms per liter. These concentrations are much higher in contrast to most pharmaceuticals' concentrations [26–28]. The research interest has shifted, recently, to the parallel determination of other artificial sweeteners, such as acesulfame, saccharin, cyclamate, and aspartame [29].

1.2.7 Pesticides

Pesticides are a diverse group of ECs, which are used in order to prevent, destroy, or control a harmful organism/disease, or to protect plants/plant products during production, storage and transport [30]. Many chemical compounds are included in that group such as herbicides, fungicides, insecticides, acaricides, nematicides, molluscicides, rodenticides, growth regulators, repellents, rodenticides and biocides. According to Regulation (EC) no 1107/2009 of the European Parliament and of the Council (2009) plant protection products are "pesticides" which protect crops or desirable or useful plants. They are primarily used in the agricultural sector but also in forestry, horticulture, amenity areas and in-home gardens. Pesticides enter aquatic ecosystems via spray drift, evaporation and deposition, and after rain events as runoff and erosion or drainage [30]. In recent years, the additional challenges of the presence of illegal and counterfeit pesticides on the market have become an increasing global problem and monitoring data of pesticides in environmental samples are very useful for the review of the authorization and the regulations [30–32].

1.2.8 (Bio) Transformation Products [(bio) TPs]

When ECs end up to aquatic bodies, they are subjected to both biotic and abiotic transformation processes that are responsible for their

transformation and/or elimination. Formation of transformation products (TPs) takes place mainly through oxidation, hydroxylation, hydrolysis, conjugation, cleavage, dealkylation, methylation and demethylation. The ECs and their TPs can move vertically through the groundwater away from the source site [33].

TPs of ECs can be detected in wastewater treatment plants (WWTPs) or in the environment as a result of a multitude of abiotic (the outcome of hydrolysis, photolytic and photocatalytic degradation in the natural environment as well as water treatment processes, such as chlorination, ozonation and advanced oxidation processes) and biotic (human, animal and microbial metabolites) processes acting on the parent compounds or the metabolites [33,34]. TPs are of environmental concern particularly if they are biologically active or resistant to biodegradation. However, there is only limited information in the literature on the fate of these TPs, and many of them remain undiscovered [35]. TPs can differ from the parent compounds either regarding their bioconcentration (toxicokinetics) and/or their mode of toxic action (toxicodynamics) [36].

1.3 Emerging Contaminants through the food chain – Apex Predators

Consumers living in terrestrial and aquatic ecosystems are capable of accumulating ECs to toxic concentrations. There are some properties which are critical for the accumulation of ECs in an aquatic food chain; (a) a high octanol-water partition coefficient, (b) chemical and metabolic stability in the water and aquatic organisms in the food chain, and (c) a low toxicity in the food chain, in order to not broke by loss of an intermediate consumer [37].

There are two terms which are mainly used for the description of the distribution of ECs in the environment. The first term is the bioaccumulation, which describes *an increase in the concentration of a substance obtained from the abiotic environment in one or more tissues of an organism*. Bioaccumulation occurs within a given trophic level. The second term is the biomagnification, which describes *the increase in the concentration of a substance obtained from organisms at lower trophic levels by an organism at a higher trophic level* [38].

The determination of ECs is of great ecotoxicological significance, especially in biota, because it shows the distribution within the organisms (bioaccumulation), through food chain (biomagnification) and provides information for understanding and quantifying effects of this exposure in human. The determination of ECs in animal tissues is demanding, because of the variety of biological matrices and the low concentrations (parts per trillion levels) in biota, which demand highly sensitive analytical techniques [4].

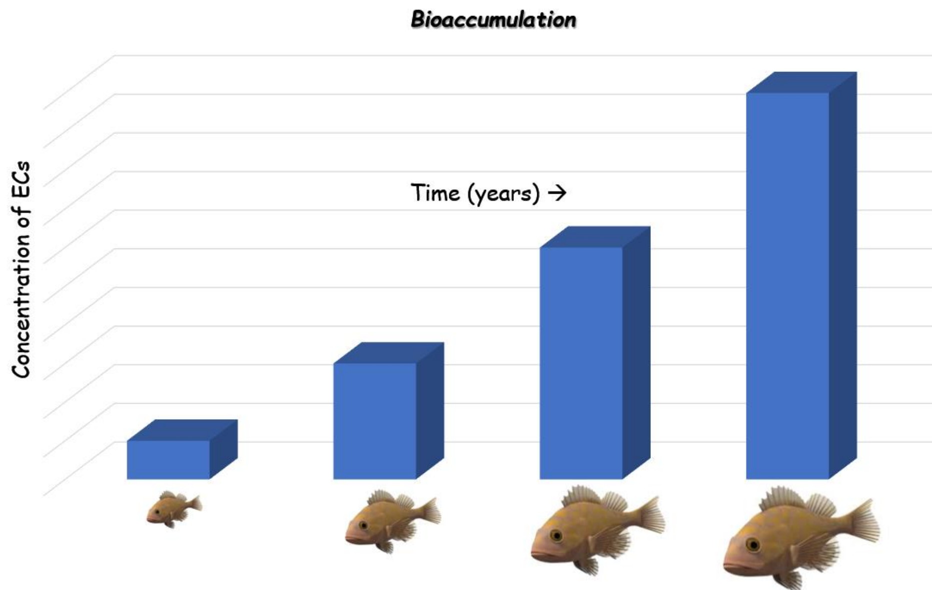


Figure 3: Bioaccumulation of ECs in a trophic level.

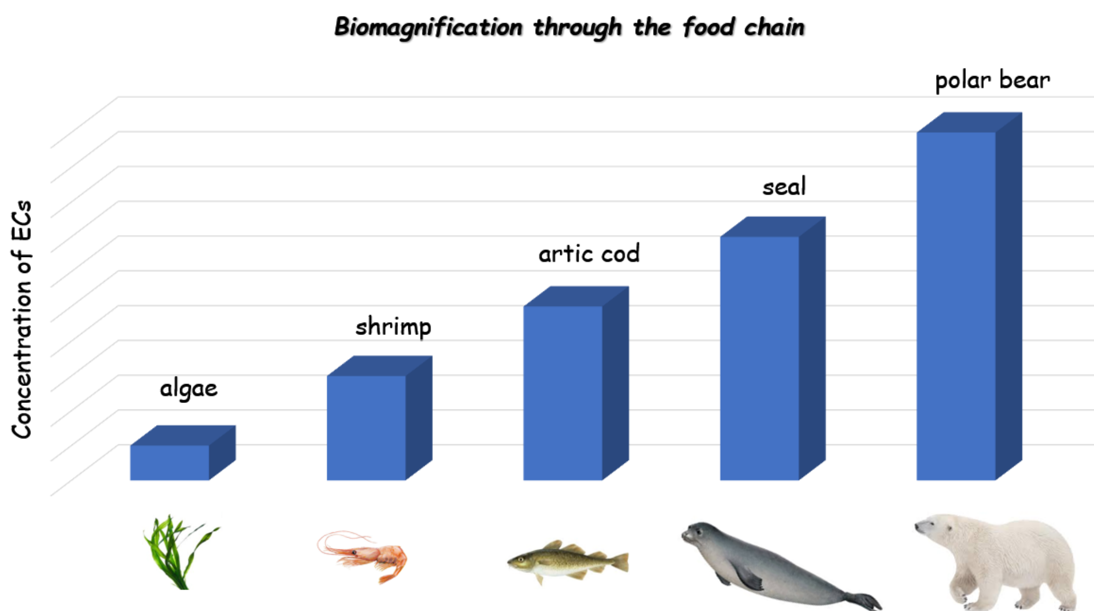


Figure 4: Biomagnification of ECs through the food chain.

The determination of contaminants in apex predators and their prey (AP&P) allows for the identification of the emergence of chemicals, including substitutes of regulated compounds which may present similar levels of hazard. Apex predators play a key role in the monitoring of environmental contaminants and in risk assessment studies, for a number of reasons, including: their position at the top of food webs, a relatively long lifespan over which to accumulate contaminants, their integration of exposure both over time and relatively large spatial areas, relative ease with which samples can be obtained, and relative ease with which populations can be quantified and monitored [39].

1.4 European Specimen Banks – Natural History Museums

Environmental Specimen Banks (ESBs) are facilities that engaged in the systematic long-term preservation of representative environmental specimens for future research and monitoring purposes [40,41]. Environmental samples can be used to study the quality of environment, environmental processes and how they change through time [40]. The sample materials are provided so that they can be used for analyzing temporal trends in exposure to known hazardous substances, previously unrecognized pollutants, or for substances for which analytical techniques were inadequate at the time of sampling. ESBs collect and process samples for archiving without altering their original chemical composition and preserve them in a stable environment for future analysis [40]. Specimens from ESBs are used for retrospective analysis and evaluation for regulatory decision making. As such, a well-designed ESB can be a valuable resource of specimens for real-time and retrospective monitoring [41]. Moreover, the use of archived biological samples allows the fast analysis of samples from different years and regions under comparable conditions. Thus, the results of retrospective monitoring could help to assess the relevance of compounds in question with respect to concentration levels and temporal trends (exposure monitoring) [42].

Most specimens covered by existing banks are biota, e.g. fish, birds' eggs, mussels and plant material. Some environmental specimen banks

(ESBs) also archive sewage sludge, sediments and soil. The specimens must be preserved in such a way that they can still be analyzed many years after they were collected - either because new techniques have become available or because a new interest has arisen in substances that were not considered important in the past. Mostly, specimens are stored at very low temperatures (e.g. −80 or −170 °C) to avoid loss of chemical information within the samples during storage. As a prerequisite for ESBs, sampling, processing and archiving are strictly standardized to ensure consistent samples [43].

Natural History Museums (NHMs) provide a rich source of data, which can be used in various studies. The samples provide an outstanding (and largely untapped) resource for comparison with modern survey data. NHMs, which collect the samples using a coherent and systematic sampling strategy, are inevitably more likely to provide research-quality material than ad-hoc or point samples [44].

CHAPTER 2
DETECTION OF EMERGING CONTAMINANTS IN BIOTA – LITERATURE REVIEW

2.1 Introduction

The detection of ECs and priority pollutants (PPs) in biota is a promising approach to understand the extent of their use by population and their movement within the organisms and through the food chain, but meantime it is a difficult task and a major analytical challenge. The low concentrations (parts per billion) of ECs and PPs in biota and the complexity of biological matrices (e.g. fish, eggs, livers), which include a large number of other substances with different physicochemical properties make it difficult to identify them reliably [4]. The usage of advanced analytical techniques is crucial, so liquid chromatography coupled to high resolution mass spectrometry is the technique of choice for polar and semi-polar compounds because of its excellent selectivity and sensitivity.

2.2 Sample treatment

A big variety of extraction techniques is reported in environmental studies, such as liquid-solid extraction (LSE), which is combined with microwave-assisted extraction (MAE) or ultrasound assisted extraction (UAE), solvent reduced techniques, [e.g. matrix solid-phase dispersion (MSPD), and liquid-phase micro extraction (LPME)], supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) [7,45]. One of the most powerful and inexpensive techniques, which is used for the extraction of chemical compounds from biota matrices is Accelerated Solvent Extraction (ASE), or Pressurized Liquid Extraction. ASE is an automated extraction technique that uses organic solvents at elevated temperatures above the boiling point and at high pressures in order to extract analytes from solids and semisolids matrices [46–50].

This technique has various benefits; it's fast, provides cleaner extracts (filtration is not needed), uses a small percentage of extraction solvents, provides a more efficient analyte – solvent contact, gives the opportunity to use different sample masses [49]. The disadvantages are the restricted selectivity, which means that a further clean-up step is required, and the high purchasing cost in comparison with other extraction techniques.

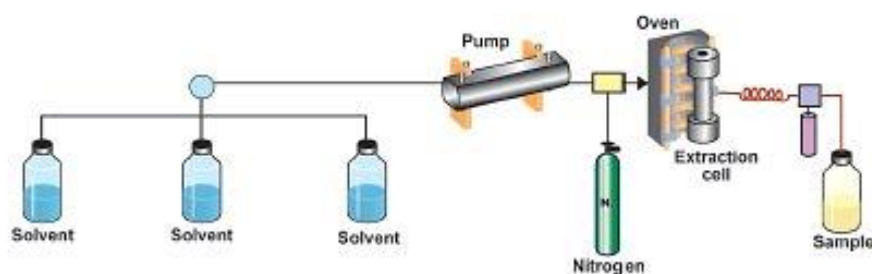


Figure 5: Accelerated Solvent Extraction
(source: https://www.americanlaboratory .com/914-Application-Notes/743-Accelerated-Solvent-Extraction-With-Acid-Pretreatment-for-Improved-Laboratory-Productivity/)

Temperature is the most crucial parameter in that extraction technique, because, at high temperatures, extraction solvent can get into the pores of the sample in an easier way and that helps with the target analytes solubilization. Furthermore, at increased temperatures molecules have a bigger amount of kinetic energy, capable to disrupt analytes - matrices interactions. Therefore, the extraction of analytes from matrices is faster in comparison with other extraction techniques [47–49].

The role of elevated pressures is just to maintain the organic extraction solvents in their liquid state, which ensures safe and rapid extraction. Furthermore, high pressure forces solvents into the pores of the sample matrix, helping with the extraction due to the closer contact with analytes in these pores [47–49].

The common technique that is used as a clean-up step during the determination of ECs and PPs in biota matrices is Solid Phase Extraction (SPE). The usual steps of SPE include the conditioning of the sorbent in the cartridge, the loading of the sample, where analytes interact with the sorbent

and impurities pass through, the wash-up, the drying and finally the elution of the analytes.

SPE is the most suitable technique for isolation of the target compounds from biota matrices. The interference of matrix components during the analytical measurement causes signal suppression or enhancement due to co-eluting matrix compounds of samples during ionization in Liquid Chromatography coupled to Mass Spectrometry (LC-MS), and mainly when using Electrospray Ionization (ESI) as source.

The need for the determination of a big variety of analytes with different physicochemical properties demands the usage of a generic clean-up step using SPE. For this purpose, but in different environmental matrices, Kern et al. [51], Diamanti et al. [52] used 4 different sorbents as SPE cartridge materials in order to have broad enrichment efficiency during the SPE step.

2.3 Analytical techniques – Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

LC–MS is a sophisticated hyphenation of analytical techniques, which enables the determination of organic emerging contaminants in complex environmental matrices. A range of different LC-MS technologies have been put forward in recent years for the analysis of mixtures of many known and unknown compounds at low concentrations in complex matrices [53,54].

2.3.1 Reversed Phase Ultra High Performance Liquid Chromatography (RP-UHPLC)

In UHPLC, short chromatographic columns, which include small-diameter particles in the stationary phase. UHPLC fast and high resolution separation is provided, which increases LC-MS sensitivity and minimizes matrix interference arising from minimal sample preparation [55,56]. UHPLC is mainly performed in reversed-phase (RP) mode, using C_{18} columns. The mobile phase consists of an aqueous and an organic solvent. Methanol and Acetonitrile are commonly used as organic solvents. In some methods, the mobile phase is

acidified with small percentages by volume of acetic or formic acid in order to improve ionization of the compounds in the positive ionization mode [57]. Gradient elution programs are preferred for better and faster separations.

2.3.2 High-resolution mass spectrometry (HRMS)

Many scientific groups, which are dealing with the determination of organic contaminants in biota, develop analytical methods that include liquid chromatography coupled to tandem mass spectrometry using low resolution mass analyzers, usually triple quadrupole (QqQ), because this technique is reliable for qualitative and quantitative determination of selected/known biomarkers.

On the other hand, the use of liquid chromatography coupled to high-resolution mass spectrometry allows the wide-scope screening of parent compounds and (bio)TPs, which may be already known, suspect or unknown. Consequently, it can be used for the determination of the continuous growing and diverse group of ECs [58–60].

Among the possible ionization techniques in LC-MS, ESI is the most widely used, compared with atmospheric pressure chemical ionization (APCI) or the more recent atmospheric pressure photoionization (APPI) [54].

LC-HRMS has an excellent performance on qualitative applications thanks to the high mass accuracy and the selectivity in full-scan acquisition mode that ensure reliable detection and identification, while more and more studies use LC-HRMS for complete analysis, both identification and quantification [58–60]. With full-spectrum accurate-mass data, a theoretically unlimited number of analytes which are present in a sample can be identified, because the acquisitions have been made as “all ions all the time” [53]. The simultaneous determination of a broad number of compounds in one injection, with a corresponding reduction of time and costs, and even when reference standards are not available, make LC-HRMS one of the current trends in environmental analytical chemistry [61]. Moreover, investigation can be performed in a retrospective way in order to detect compounds that initially were not considered, even after years, without additional analysis of the samples.

This ability is advantageous, because in some occasions, samples might already have been discarded or the analytes have been degraded [59,60].

Time-of-flight (TOF) is one of the most used HRMS analyzers and it is easily coupled to ultra high performance liquid chromatography (UHPLC). Mass resolution typically ranges from 20,000 up to 80,000 Full Width at Half Maximum (FWHM) and mass accuracy is lower than 2 ppm. Hybrid configurations, such as Quadrupole-Time-of-flight (QTOF), increase the potential of the analyzer for screening purposes and provide relevant structural information by obtaining accurate-mass product-ion spectra after MS/MS experiments [61]. Product-ion spectra can be obtained with either data dependent acquisition or data independent acquisition, where the instrument automatically switches after a full-scan-mode acquisition to a product-ion scan mode as the second scan event in the scan cycles [53].

2.3.2.1 Data Dependent Acquisition (DDA)

In this acquisition, there is firstly a full scan which is defined as the survey scan and data are processed “on-the-fly” to determine the candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. If the selection criteria are met, MS/MS analysis is then triggered and MS/MS scans (data-dependent) are performed [54,60]. With this acquisition, ‘clean’ spectra with structural information are obtained in one injection. However, if the number of candidates of interest is big, the number of scans is decreased, so there are less data points that affect the detectability of the chromatographic peak [54].

2.3.2.2 Data Independent Acquisition (DIA)

With this acquisition, there is no need to pre-select the precursor ion. Full-scan spectra at different collision energies are obtained in one injection. This acquisition provides simultaneously accurate mass data of parent compounds and fragment ions in a single run using two scans, one at low and one at high collision energy. By applying low energy (LE) in the collision cell, no fragmentation is performed. A full-scan spectrum is obtained that provides

information for the parent ion (the (de)-protonated molecule) and, in some cases, the adduct ions and the in-source fragments. By applying high energy (HE) in the collision cell, fragmentation is performed and a spectrum similar to MS/MS experiments is obtained. This approach is called all-ions MS/MS, MS^E or bbCID, according to the QTOF manufacturer [54].

2.4 Data Treatment

After the sample preparation and the LC-HRMS analysis, raw data can be treated with three different approaches, target, suspect and non-target screening. A systematic workflow for all three approaches is shown in the following Figure.

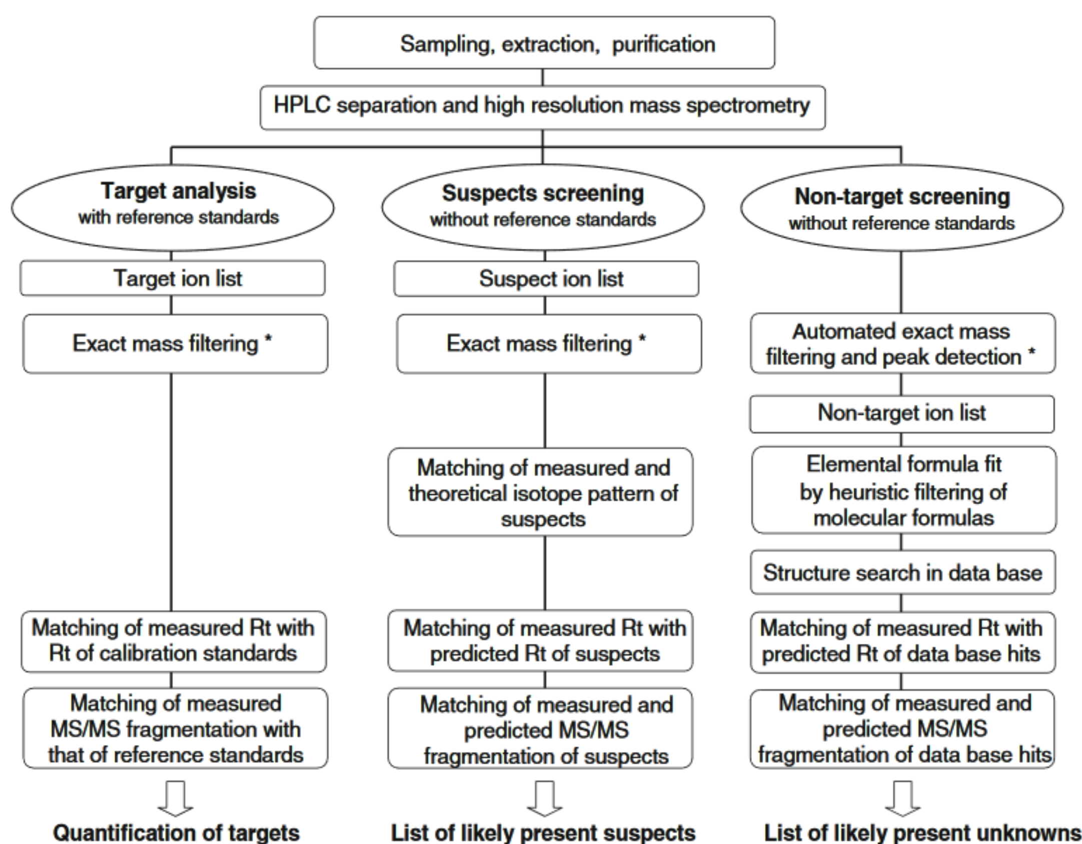


Figure 6: Systematic workflow for target, suspect and non-target screening by LC-HRMS/MS *(source:* [54]*).*

2.4.1 Target screening

In this approach, an in-house developed database is used for the screening of a large number of compounds. The information included in the database is based on the analysis of the available reference standards [61]. The reference standard is necessary for comparison of the retention time, the MS spectrum profile (precursor ion, adducts, in-source fragments), as well as the MS/MS spectrum (fragment ions and ion ratios) [62]. Quantitation can be performed in full-scan mode, but requires greater effort than in LC-LRMS methods where Single Reaction Monitoring (SRM) mode is used [61,62].

2.4.2 Suspect screening

In this approach, a list of suspect compounds that are possible to be found in specific samples is built. The screening is based only on the exact m/z of the expected ions, which, in case of the ESI source, are usually the pseudomolecular ions $[M+H]^+$ and $[M-H]^-$, except for some compounds which exclusively show adduct formation. Molecular formula and structure are known, so this information can be efficiently used in the identification and confirmation process [54]. Absence from blank samples, mass accuracy, isotopic pattern, retention time prediction, ionization efficiency and information on fragment ions reported in the literature are parameters that can facilitate tentative identification of suspect candidates [54,63].

2.4.3 Non-target screening

In non-target methodologies, samples are searched for compounds without any previous information on them. These unknown compounds are actually new, unexpected or not searched ones in specific samples. Identification is a challenge in this approach, as more than one elemental formula and several plausible structures are obtained for a given unknown compound detected in a sample [59,60]. Except for the elucidation of unknowns, non-target screening is used for the identification of (bio)TPs, arising from in vivo and in vitro experiments, in-silico modeling and degradation laboratory studies [53]. In this case, the number of chemically meaningful

structures, which can be assigned to an unknown peak, is limited to structures that show a close relationship with the parent compound and also, an adequate control sample or time series is available [54].

2.4.4 Confidence in the identification procedure

2.4.4.1 Confidence in target screening

The confirmation of positive findings in target screening can be performed by attributing identification points (IPs). According to the 2002/657/EC guideline, 4 IPs are required for unequivocal confirmation, and for HRMS instruments with resolution higher than 10,000, the precursor ion earns 2 IPs and the product ions earn 2.5 IPs [64]. This means that one single HRMS/MS transition can confirm the detection of a substance, which is risky when there are several co-eluting isomers. Another fact is that resolving power may largely vary between HRMS instruments, which makes the definition of general criteria difficult [53]. More precise criteria for the use of mass accuracy and mass resolution have to be implemented to define clearly the requirements for a reliable confirmation in LC-HRMS [54]. Bletsou et al. [62] proposed an identification points system for HRMS analysis in order to take full advantage of the capabilities of HRMS instruments.

2.4.4.2 Confidence in suspect and non-target screening

An identification strategy through five levels of confidence has been proposed for HRMS screening by Schymanski et al. [65], as described in the following Figure. Level 1 corresponds to the confirmed structure by the use of a reference standard, level 2 to a probable structure using literature or diagnostic data, level 3 to tentative candidate(s) with possible, not exact, structures, level 4 to an unequivocal molecular formula and level 5 to the exact mass. Non-target screening starts from level 5 and suspect screening from level 3 and, as identification confidence increases, they reach ‘better’ levels up to level 1. Target screening starts by definition from level 1. If the evidence of the sample and the evidence of the reference standard (target) or the tentative candidate (suspect) do not match, then the component associated with the

target or suspect should become a 'non-target of interest' and 'downgrade' to level 5 [66].

Generally, in both suspect and non-target screening, reference standards are required for ultimate and unambiguous confirmation, but should be purchased in a final stage, when solid well-found evidence exists on the presence of the compound in the sample [54].

Moreover, complementary techniques can be used for evaluation of possible candidates, such as NMR, a powerful structure elucidation technique, although this requires sufficiently high concentrations and often an isolation of the unknown compound [54].

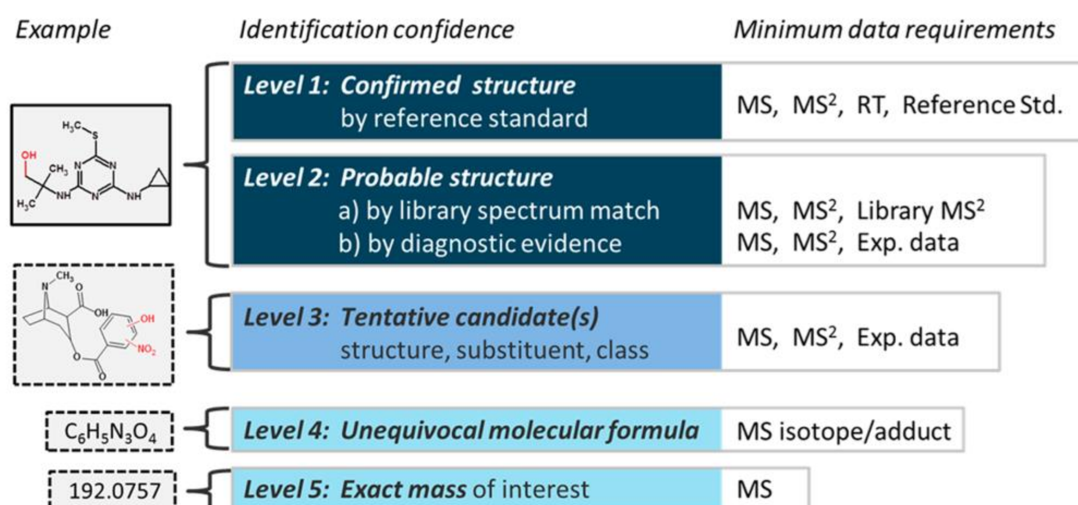


Figure 7: Identification confidence levels in HRMS *(source:*[65]*).*

2.5 Literature Review

So far, there are a lot of studies which focus on the determination of a specific class of ECs or PPs in biota [67–84]. In the same time, there are only few studies which deal with the determination of a broad range of ECs and PPs in biota from different classes, using HRMS [58–60].

In the following text, an overview of the analytical procedures and methods that have been applied in ECs and PPs detection in biota is performed.

For the extraction of ECs and PPs from biota matrices using ASE, diatomaceous earth [69,71,72,75,76,78,79,81] and sodium sulfate are mainly used as dispersant [73,74,82,84]. The temperature which is used during the

extraction depends on the analytes, but in most studies temperatures above 70°C are used. The pressure during the ASE is 1500 psi, in the majority of studies, the heating time is 300s, the static time is 300s, the extraction cycles are 2 or 3 and the flush volume is 60%. The extraction solvent depends on the analyte and its physicochemical properties, but, mainly, (acidic) milli-Q water, Methanol and Acetonitrile, or combinations of them are used for the polar and semi-polar analytes' extraction [67,68,75–78,80–84]. All the information for ASE is included in the following table.

As clean-up step Solid Phase Extraction (SPE) [68–70,73,75,76,82,84] is mainly used, while in a few studies Gel Permeation Chromatography (GPC) [71,72] is used. In the other hand, there are many scientific groups which omit the clean-up step [67,77–81,83]. The sorbents, which are used mainly, in the SPE cartridges are Oasis MCX [70,73] (Waters, Milford Massachusetts, USA), which is a mixed-mode strong cation-exchange reversed-phase sorbent, Oasis HLB [68,76] (Waters, Milford Massachusetts, USA), which is a strongly hydrophilic, reversed-phase sorbent with unique hydrophilic-lipophilic balance, aminopropyl [69,84] (Waters, Milford Massachusetts, USA), which is a polar sorbent with different selectivity for acidic/basic analytes or as a weak anion exchanges in aqueous medium below pH=8 and C18 bond-Elute [75,82] (Agilent, Santa Clara California, USA), which is the most hydrophobic, bonded silica sorbent available in the Bond Elute range.

For the separation of ECs and PPs in the biota, reversed phase columns, in particular C8 [74] and C18 columns [58,59,60,67,69–71,76–79,82,83] have mainly been used. Regarding with the LC-MS analysis, the most developed methods for the determination of a specific category of polar and semi-polar ECs and PPs use low resolution mass spectrometry (LRMS), in particular quadrupole [67,74,82], quadrupole-ion trap (QIT) [75] and, mainly, triple quadrupole (QqQ) [68–73,76–79,80,81,83,84] as mass analyzers.

Table 1: Biota Matrices, Analytes, Clean-Up Techniques, Analytical Techniques for polar and semi-polar compounds.

#	Matrices	Analytes	Clean-Up Technique	Analytical Technique	Reference
1	Blue Mussels (*Mytilus edulis*), Dungeness Crabs (*Cancer magister*), White-Spotted Greenling (*Hexogrammos stelleri*)	Phthalate Ester Metabolites	Solid Phase Extraction (SPE)	LC-ESI(-)-MS/MS (QqQ)	[68]
2	Eel, Salmon	Hexabromocyclododecane (HBCD), Tetrabromo-Bisphenol A (TBBPA)	Gel Permeation Chromatography (GPC)	LC–ESI(-)-MS/MS (QqQ)	[71]
3	Mosquito Fish (*Gambusia holbrooki*)	Pharmaceuticals	Solid Phase Extraction (SPE)	LC-MS/MS (QqQ)	[73]
4	Osprey Eggs Carp, Lake Trout and Walleye Fish	Alkylphenol, Alkylphenolethoxylates	Solid Phase Extraction (SPE)	LC-ESI(+)-MS/MS (QqQ)	[84]
5	Rainbow Trout (*Oncorhynchus mykiss*)	Brominated Flame Retardants (BFRs)	Gel Permeation Chromatography (GPC)	LC-MS/MS (QqQ)	[72]
6	Beef, Chicken, Pork, Sea Bream and Trout filets	Macrolide Antibiotics	-	LC-ESI-MS (Q)	[67]
7	Meat (Cattle, Pig) samples	Antimicrobials	-	LC-MS/MS (QqQ)	[77]
8	muscles and livers of Swine, Cattle, Sheep and Chicken	Benzimidazole, Metabolites of Albendazole, Fenbendazole and Mebendazole	-	LC–ESI(+)-MS/MS (QqQ)	[78]
9	Bovine liver	Corticosteroid Drugs	-	LC–APCI(-)-MS/MS (QqQ)	[79]
10	Bovine (tissues of Veal, Tender Beef, And Beef), Porcine and Poultry raw meat	Sulfonamide Residues	-	LC-ESI(+)-MS/MS (QqQ)	[80]
11	Table Eggs	Fluoroquinolones (FQs)	-	LC-ESI(+)-MS/MS (QqQ)	[81]

#	Matrices	Analytes	Clean-Up Technique	Analytical Technique	Reference
12	kidney fat	Anabolic Steroids	Solid Phase Extraction (SPE)	LC–ESI(+)-MS (Q)	[82]
13	Bivalve Mollusk	Alkylphenols, Bisphenol A	-	LC-ESI(-)-MS/MS (QqQ)	[83]
14	Pork, Fish, Rabbit, Duck, Chicken	Alkylphenols, Bisphenol A	Solid Phase Extraction (SPE)	LC–ESI(-)–MS/MS (QqQ)	[69]
15	Salmon, Shrimp	Malachite Green, Gentian Violet, Leuco Malachite Green, Leuco Gentian Violet	Solid Phase Extraction (SPE)	LC-ESI-MS/MS (QqQ)	[70]
16	Fish liver	Alkylphenols, Bisphenol A	Column Chromatography	LC-ESI(-)-MS (Q)	[74]
17	Fish (17 different species)	Pacific ciguatoxin-1 (P-CTX-1)	Solid Phase Extraction (SPE)	LC-MS/MS (q-linear ion-trap)	[75]
18	Bovine muscle and liver	Neonicotinoid Insecticides residues	Solid Phase Extraction (SPE)	LC-ESI(+)-MS/MS (QqQ)	[76]

Table 2: Accelerated Solvent Extraction Conditions for polar and semi-polar compounds.

#	*dispersant*	*temperature (°C)*	*pressure (psi)*	*heating time (s)*	*static time (s)*	*flush volume*	*purge time (s)*	*# static cycles*	*extraction solvent*	*Reference*
1	Silica	70	700	300	300	60%	-	3	ultrapure Water	[68]
2	Diatomaceous Earth	100	1500	300	300	40%	300	3	Hexane: Acetone (1:1, v/v)	[71]
3	Sodium Sulfate	80	1500	300	600	60%	120	1	Dichloromethane	[73]
4	Sodium Sulfate	25	1000	-	600	90%	200	3	Acetonitrile	[84]
5	Diatomaceous Earth	100	2000	300	-	75%	-	3	Dichloromethane	[72]
6	Alumina	80	1500	300	900	150%	300	2	Methanol	[67]
7	EDTA-washed sand	70	1500	300	600	60%	60	1	Methanol: Water (25:75, v/v)	[77]
8	Diatomaceous Earth	60	1500	-	300	60%	-	1	Acetonitrile: Hexane (80:20, v/v)	[78]
9	Diatomaceous Earth	50	1450	300	300	60%	100	1	Hexane: Ethyl Acetate (1:1, v/v)	[79]
10	C_{18}	160	1470	480	300	-	60	1	ultrapure Water	[80]
11	Diatomaceous Earth	70	1500	300	300	50%	60	3	Phosphate (50mm pH=3.0): Acetonitrile (50:50, v/v)	[81]

#	dispersant	temperature (°C)	pressure (psi)	heating time (s)	static time (s)	flush volume	purge time (s)	# static cycles	Elution Solvent	Reference
12	Sodium Sulphate	50	1500	300	300	60%	300	1	Acetonitrile	[82]
13	Silica	40	1500	300	300	60%	60	2	Methanol	[83]
14	Diatomaceous Earth	100	1500	300	180	60%	120	3	Dichloromethane	[69]
15	Basic Alumina	60	1500	300	-	60%	-	1	McIlvaine buffer (pH=3.0): Acetonitrile: Hexane	[70]
16	Sodium Sulfate	100	1500	300	-	60%	-	1	Acetone: n-Hexane (1:1, v/v)	[74]
17	Diatomaceous Earth	75	1500	300	300	60%	100	2	Methanol	[75]
18	Diatomaceous Earth	80	1450	-	300	-	-	2	ultrapure Water	[76]

CHAPTER 3
SCOPE

In the last decade, many scientific groups are dealing with the occurrence of ECs in various environmental matrices. Many of these chemical compounds, through various ways, end up in aquatic ecosystems, and are, thus, detected at various concentrations in environmental samples, like surface waters and aquatic organisms. The biomagnification through the food chain of a big percentage of ECs, makes the monitoring of the occurrence of these chemicals in top consumers/predators and their prey an important demand.

Recent advances and improvements in analytical techniques, and especially in high resolution mass spectrometry, have given the opportunity to scientific groups to detect and identify a huge number of chemical compounds, even in complex matrices, like biota. LC-HRMS allows the wide-scope screening of ECs and their (bio)TPs with an acquisition of accurate-mass full spectrum data. These data can be used for target, suspect and non-target screening, as well as retrospective screening, years after the treatment of samples without additional analysis of them.

Recent studies focus on the determination of selected ECs in biota by LC-LRMS and available reference standards. However, efforts for screening of a wide range of ECs in biota by LC-HRMS are very limited.

The aim of this study is the target screening of ECs in top consumers/predators and their prey from United Kingdom, which are gathered by EBSs and NHMs, which is a part of the European Union funded project LIFE APEX (LIFE17 ENV/SK/000355, 2018-2022, https://lifeapex.eu/). For this purpose, a generic sample preparation was developed for the enrichment of the extracts with a wide number of analytes with different physicochemical properties. Furthermore, a data independent acquisition by LC-HRMS was used, in order to obtain information for both parent and fragment ions with only one injection and no pre-selection of analytes. Consequently, using this

developed generic protocol of sample preparation, there are existed extracts of biota for future target, suspect, non-target, as well as retrospective screening of ECs.

CHAPTER 4
MATERIALS AND METHODS

4.1 Chemicals and Materials

Regarding with the sample preparation, for the extraction using Accelerated Solvent Extraction (ASE), cellulose ASE Extraction Filters and Diatomaceous Earth for ASE were obtained from Thermo Scientific (Waltham, Massachusetts, USA). For the clean-up step using Solid Phase Extraction (SPE), the empty solid phase extraction polypropylene tubes (6 mL) and the cartridge sorbent materials Sepra ZT (Strata-X), Sepra ZT-WCX (Strata-X-CW) and ZT-WAX (Strata-X-AW) were purchased from Phenomenex (Torrance, USA), while the Isolute ENV+ sorbent material and the frits (20 μm, 6 mL) were purchased from Biotage (Ystrad Mynach, UK). Regenerated cellulose (RC) syringe filters (diameter 15 mm, pore size 0.2 μm) were obtained from MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany). Regarding the chemicals of the sample preparation; Methanol, Acetonitrile and Hexane were HPLC grade and were purchased from Fischer Scientific (Loughborough, UK), Ethyl Acetate ≥ 99.5% (GC) was purchased from Sigma Aldrich (Steinheim, Germany), Formic Acid 98-100% for analysis was purchased from CARLO ERBA Reagents S.A.S. (Barcelona, Spain), while Ammonia solution 25% for analysis was purchased from CHEM-LAB NV (Zedelgem, Belgium).

All the solvents for the chromatographic analysis were hypergrade for LC-MS. Methanol and Acetonitrile were obtained from Merck (Darmstadt, Germany) and the eluent additives ammonium formate, ammonium acetate and formic acid 99% were purchased from Sigma Aldrich (Steinheim, Germany). Ultrapure water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA).

The reference standards, which were used for method development and validation; Albendazole, Albendazole sulfone, Ambroxol, Amitriptyline, Amlodipine, Amphetamine, Atenolol, Atorvastatin, desethyl-Atrazine, Azithromycin, Benzenesulfonamide, 2-hydroxy-Benzothiazole, 2-amino-Benzothiazole, 2-amino-6-chloro-Benzothiazole, Benzotriazole (BTR), 5-

methyl-Benzotriazole, 1-hydroxy-Benzotriazole, 5,6-dimethyl-Benzotriazole, Bisphenol A, Boscalid, Bromazepam, Caffeine, Carbamazepine, Carbendazim, Chloramphenicol, Chlorbufam, Chlorpromazine, Cimaterol, Cimetidine, Ciprofloxacin, Citalopram amide, Citalopram carboxylic acid, Clarithromycin, Clenbuterol, Clofibric acid, Clomipramine, Colchicine, Closantel, Cortisol F, Cortisone E, Danofloxacin, Dapsone, Decoquinate, Diaveridine, Diclazuril, Diclofenac, Difloxacin, Dimethoate, Dimetridazole, 2-4-Dinitrophenol (DNP), Doxepin, Emamectin B1a, Enrofloxacin, Erythromycin, Ethopabate, Etrimfos, Fenbendazole, Fenoxycarb, Fenthion-sulfoxide, Florfenicol, Fluazuron, Flubendazole, Flufenamic acid, Flumequine, Flunixine, Fluoxetine, Furosemide, Gemfibrozil, Haloperidol, Hydrochlorothiazide, Imidacloprid, Imipramine, Ketoprofen, Levamisol, Lincomycin, Lorazepam, Lufenuron, Mabuterol, Marbofloxacin, Mebendazole, Meclofenamic Acid, Mefenamic acid, Meloxicam, Metformin, Methomyl, Metoprolol, Metribuzin, Metronidazole, Morantel, Nalidixic acid, Naproxen, Niflumic acid, Nigericin, Nonylphenol (4-NP), Novobiocin, Olanzapine, Omethoate, Oxamyl, Oxfendazole, Oxprenolol, Paroxetine, Perfluoro butane sulfonic acid (PFBuS), Perfluoro decanoic acid (PFDeA), Perfluoro dodecanoic acid (PFDoA), Perfluoro Heptanoic acid (PFHpA), Perfluoro hexanoic acid (PFHxA), Perfluoro nonanoic acid (PFNA), Perfluoro octanoic acid (PFOA), Perfluoro undecanoic acid (PFUnA), Phenylbutazone, di-n-octyl-Phthalate, diethyl-Phthalate, diphenyl-Phthalate, Prednisolone, Primidone, Progesterone, Prometryn, Propranolol, Ractopamine, Ranitidine, Rifaximin, Ronidazole, Salbutamol, Salicylic acid, Sarafloxacin, Sertraline, Simvastatin, Spiroxamine, Sulfachloropyridazine, Sulfaclozine, Sulfadimethoxine, Sulfadimidine (Sulfamethazine), Sulfadoxine, Sulfamerazine, Sulfameter (Sulfumetin), Sulfamethizole, Sulfamethoxazole, Sulfamethoxypyridazine, Sulfamonomethoxine, Sulfamoxole, Sulfapyridine, Sulfaquinoxaline, Sulfathiazole, Sulfisoxazole, Terbutaline, Terbuthylazine, Ternidazole, Theophylline, Thiabendazole, Thiacloprid, Thiamphenicol, Tiamulin, Tilmicosin, Toltrazuril, Toluenesulfonamide, Topiramate, Tramadol, Triamterene, Triclabendazole, Trimethoprim, Valsartan, Vedaprofen and Venlafaxine, were purchased from Sigma Aldrich (Steinheim, Germany), Toronto Research Chemicals (Ontario, Canada), LGC (Mercatorstrass,

Germany), Acros Organics (Morris Plains, NJ) and Alfa Aesar GmbH & Co KG (Karlsruhe, Germany).

Regarding with the internal standards (IS), which were used for the analysis and the method validation, Flunixin-d_3, Meloxicam-d_3, Bisphenol A (BPA)-d_{16}, Diuron-d_6, Atrazine-d_5, Diazepam-d_5 and (±) Amphetamine-d_6 were obtained from Sigma Aldrich (Steinheim, Germany), while Sulfadiazine-d_4, Sulfadimidine-d_4, Sulfadimethoxine-d_4, Cetirizine-d_3, Mefenamic Acid-d_3, Diethyl Phthalate-d_4, Aspartame-d_3 and Sucralose-d_6 were obtained from Toronto Research Chemicals (Ontario, Canada).

4.2 Sample Pretreatment

Top consumers'/predators' livers (Buzzard, Eurasian Otter and Harbour Porpoise) and muscles from their prey (freshwater fish: Roach) were gathered by Center of Ecology and Hydrology (CEH) from different locations in United Kingdom. Details for the samples are included in the following table and the sampling locations are shown in the following figure. These wet samples were sent in Laboratory of Analytical Chemistry (National and Kapodistrian University of Athens) with dry ice, according to the strict protocols of CEH. All samples were lyophilized, using the Telstar's Freeze-Dryer LyoQuest, before analysis, in order to enhance extraction efficiency, improve the precision and achieve lower detection limits. After lyophilization, the %humidity of each sample was calculated and the freeze-dried samples were homogenized using pestle and mortar. After homogenization, the samples were storage in brown glass bottles in the freezer (-80 °C) till the analysis.

Figure 8: Lyophilized and homogenized LIFE APEX samples.

Figure 9: Freeze-Dryer, Telstar
(source: https://www.telstar.com/lab-hospitalsequipment/laboratory -freeze-dryers/lyoquest/).

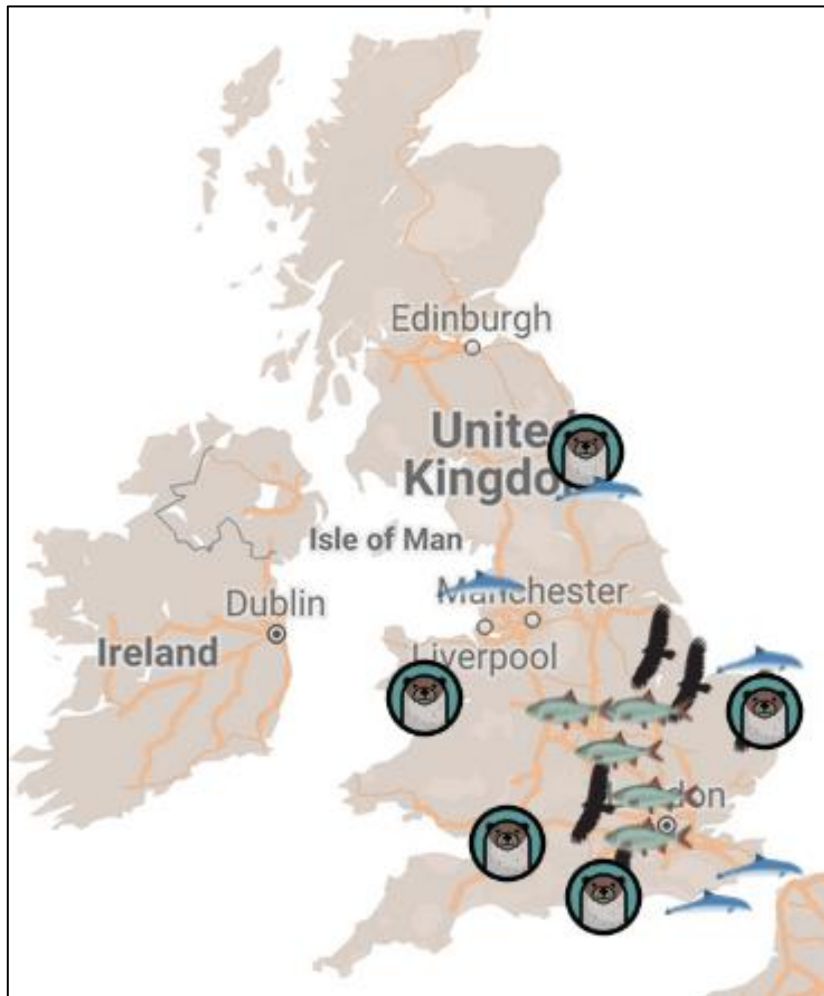


Figure 10: Sampling Locations *(source: https://tinyurl.com/y2u2xqpk, ©Alygizakis N.).*

Table 3: Samples details and coding.

Short Coding	*Sample Code*	*Species*	*Matrices*	*Sampling Location*	*Sampling Year*
LIFE APEX 11	**1-Bu(Lp)-GB(6)-17**	Buzzard	liver (pooled)	The Wash	2017
LIFE APEX 12	**1-Bu(Lp)-GB(7)-17**	Buzzard	liver (pooled)	South Lincolnshire	2017
LIFE APEX 13	**1-Bu(Lp)-GB(8)-17**	Buzzard	liver (pooled)	East Anglia	2017
LIFE APEX 14	**1-Bu(Lp)-GB(9)-17**	Buzzard	liver (pooled)	Sussex/Hampshire	2017
LIFE APEX 15	**1-Bu(Lp)-GB(10)-17**	Buzzard	liver (pooled)	South Wales/Somerset/Hertfordshire	2017
LIFE APEX 16	**1-R(Mp)-GB(1)-16**	Roach	muscle (pooled)	River Thames (Shepperton-Sunbury)	2016
LIFE APEX 17	**1-R(Mp)-GB(2)-16**	Roach	muscle (pooled)	River Lee (Wheathampstead)	2016
LIFE APEX 18	**1-R(Mp)-GB(3)-17**	Roach	muscle (pooled)	River Anker (Stationfields)	2017
LIFE APEX 19	**1-R(Mp)-GB(4)-17**	Roach	muscle (pooled)	River Thames (Standford-Abington)	2017
LIFE APEX 20	**1-R(Mp)-GB(5)-17**	Roach	muscle (pooled)	River Welland (Stamford Meadows)	2017
LIFE APEX 21	**1-O(Lp)-GB(11)-16**	Otter	liver (pooled)	Solent and South Downs	2016
LIFE APEX 22	**1-O(Lp)-GB(12)-16**	Otter	liver (pooled)	North East	2016
LIFE APEX 23	**1-O(Lp)-GB(10)-16**	Otter	liver (pooled)	South Wales/Somerset/Hertfordshire	2016
LIFE APEX 24	**1-O(Lp)-GB(8)-16**	Otter	liver (pooled)	East Anglia	2016
LIFE APEX 25	**1-O(Lp)-GB(13)-17**	Otter	liver (pooled)	North Wales	2017
LIFE APEX 26	**1-HP(Lp)-GB(14)-17**	Harbour Porpoise	liver (pooled)	Hartlepool	2017
LIFE APEX 27	**1-HP(Lp)-GB(15)-17**	Harbour Porpoise	liver (pooled)	Kent	2017
LIFE APEX 28	**1-HP(Lp)-GB(16)-19**	Harbour Porpoise	liver (pooled)	Blackpool	2019
LIFE APEX 29	**1-HP(Lp)-GB(17)-17**	Harbour Porpoise	liver (pooled)	East Sussex	2017
LIFE APEX 30	**1-HP(Lp)-GB(18)-17**	Harbour Porpoise	liver (pooled)	Norfolk	2017

Table 4: Freeze-Drying Details.

LIFE APEX Code	mass of wet sample (g)	mass of freeze-dried sample (g)	% humidity
1-R(Mp)-GB(1)-16	32.1139	6.5263	80
1-R(Mp)-GB(2)-16	36.4843	7.6105	79
1-R(Mp)-GB(3)-17	29.5738	6.0866	79
1-R(Mp)-GB(4)-17	38.0349	7.3738	81
1-R(Mp)-GB(5)-17	33.5530	6.7562	80
1-HP(Lp)-GB(18)-17	27.8849	8.4364	70
1-HP(Lp)-GB(17)-17	21.1315	6.5274	69
1-HP(Lp)-GB(15)-17	36.0547	9.8862	73
1-HP(Lp)-GB(14)-17	27.2009	8.2211	70
1-HP(Lp)-GB(16)-19	67.9134	20.3984	70
1-Bu(Lp)-GB(6)-17	48.9085	14.4346	70
1-Bu(Lp)-GB(7)-17	36.7551	10.3893	72
1-Bu(Lp)-GB(8)-17	41.5095	12.2413	71
1-Bu(Lp)-GB(9)-17	35.2658	10.7161	70
1-Bu(Lp)-GB(10)-17	36.9289	10.8992	70
1-O(Lp)-GB(13)-17	53.2738	16.2208	70
1-O(Lp)-GB(12)-16	40.2140	13.2133	67
1-O(Lp)-GB(11)-16	36.7873	10.5584	71
1-O(Lp)-GB(8)-16	31.7987	10.3030	68
1-O(Lp)-GB(10)-16	50.4635	14.6748	71

4.3 Sample preparation

All samples were lyophilized (temperature: -55°C, vacuum: 5×10-2 mbar) before analysis, in order to enhance extraction efficiency, improve the precision and achieve lower detection limits. In addition, the concentrations of the analytes can be expressed in both dry weight and wet weight.

Samples' weighting was the first step of the protocol; 0.2 g of apex predators' liver or 1g of fish muscle were mixed with anhydrous Sodium Sulfate (Na_2SO_4), which was used as dispersant in order to improve recovery of analytes during or after extraction, with pestle and mortar in a ratio sample: dispersant 1:4. An IS mix solution was spiked in each sample before the extraction with ASE. The conditions which were used for the extraction are summarized in the table 5.

Table 5: Conditions which were used in Accelerated Solvent Extraction.

Temperature (°C)	50
Pressure (psi)	1500
Heating Time (s)	300
Static Time (s)	420
# Static Cycles	3
Flush Volume (%)	60
Purge Time (s)	180
Extraction Solvent	Methanol: Acetonitrile (67:33)

Before the clean-up step the extract was evaporated till 3-4 mL (if the extract was not transparent, it was filtered through filter paper). Then milli-Q water was added till 15 mL.

Figure 11: Dionex™ ASE™ 350 Accelerated Solvent Extractor
(source: https://www.thermofisher.com/order/catalog/product/083114)

In an effort to obtain more clear extracts a defatting step with hexane was used as the first step of sample clean-up. 5 mL of n-hexane were added, then the sample was stirred with vortex for 1 minute, it was centrifuged in 4000 rpm for 10 minutes, the two layers were separated, the hexane layer (upper) was rejected and in the water layer milli-Q water was added till 50 mL. This milli-Q water addition was taken place in order to reduce the percentage of methanol content. To achieve sufficient enrichment for a broad range of compounds, SPE with mixed bed multilayer cartridges was used for sample clean-up. These in-house SPE cartridges consisted of 200 mg of Strata-X (polymeric reversed phase sorbent for extraction of neutral and aromatic compounds) and a mixture of 100 mg of Strata-X-AW (weak anion exchanger for extraction of acidic compounds with pKa<5), 100 mg of Strata-X-CW (weak cation exchanger for extraction of basic compounds with pKa>8) and 150 mg of Isolute ENV+ (polymeric reversed phase sorbent for extraction of polar compounds). The conditioning of the cartridges was performed with 3 mL methanol and 3 mL water. The samples were loaded to the SPE cartridges and then they were dried under vacuum at a flow rate of 10 mL/min for 0.5 to 1 h. The elution of the analytes from the adsorbent material was performed by a basic solution (4 mL of ethyl acetate: methanol (50:50 v/v) containing 2%

ammonia hydroxide (v/v)), followed by an acidic solution (2 mL of ethyl acetate: methanol (50/50 v:v) containing 1.7% formic acid (v/v)).

The extracts were evaporated under a gentle nitrogen stream (40-45°C) till dryness and finally reconstituted to a final volume of 250 μL methanol: water 50:50. Every extract was filtered directly into a 2 mL vial using a syringe fitted with a 0.2 μm RC membrane filter in order to remove the solid particles that were still present and may cause blockage of the column filter, and then they were ready for LC-HRMS/MS analysis.

4.4 Instrumentation

An Ultra-High Performance Liquid Chromatography (UHPLC) system (UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a Quadrupole-Time of Flight Mass Spectrometer (QTOF-MS) (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for the analysis of the samples. The UHPLC apparatus consists of a solvent rack degasser, a binary pump with solvent selection valve (HPG-3400), an auto-sampler and a column. The QTOF-MS apparatus consists of an Electrospray Ionization (ESI) source operating in positive and negative mode.

Figure 12: UHPLC-QTOF-MS, Maxis Impact, Bruker Daltonics
(source: http://www.directindustry.com/prod/bruker-daltonics/product-30029-991983.html).

In the analysis, two separate reversed-phase chromatographic runs were performed for positive and negative ESI mode. An Acclaim RSLC 120 C_{18} column (2.1 × 100 mm, 2.2 μm) (Dionex Bonded Silica Products, Thermo Scientific, Dreieich, Germany), preceded by an ACQUITY UPLC BEH C_{18} 1.7 μm guard column of the same packaging material (VanGuard Pre-Column, Waters, Dublin, Ireland), and thermostated at 30°C, was used. In the positive ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5 mM $HCOONH_4$, 0.01% HCOOH and the organic mobile phase consisted of CH_3OH, 5 mM $HCOONH_4$, 0.01% HCOOH. In the negative ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5 mM CH_3COONH_4 and the organic mobile phase consisted of CH_3OH, 5 mM CH_3COONH_4. The gradient elution program was the same for both ionization modes and applied changes in mobile phase and in flow rate. It started with 1.0% of organic phase (flow rate 0.200 mL/min) for 1 min, increasing to 39.0% by 3 min (flow rate 0.200 mL/min), and then to 99.9% (flow rate 0.400 mL/min) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.480 mL/min) and then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.200 mL/min for the last minute. The gradient elution program which is used in the chromatographic analysis is also presented in the following table. The injection volume was set to 5 μL.

The operating parameters of the ESI interface were the following: capillary voltage 2500 V for positive and 3000 V for negative mode, end plate offset 500 V, nebulizer pressure (N_2) 2.0 bar, drying gas (N_2) 8.0 L/min, drying temperature 200°C.

Data were acquired through a Data Independent Acquisition (DIA) scan mode, called broad-band Collision Induced Dissociation (bbCID), which provided both MS and MS/MS spectra simultaneously using two different collision energies with a scan rate of 2 Hz and a mass range of 50-1000 Da. Low collision energy (4 eV) provided a full scan spectrum (MS) and high collision energy (25 eV) provided a spectrum where all ions were fragmented (bbCID MS/MS).

Table 6: The gradient elution program

Time (min)	*Flow Rate (mL/min)*	*Aqueous Solvent (%)*	*Organic Solvent (%)*
0	0.2	99.0	1.0
1	0.2	99.0	1.0
3	0.2	61.0	39.0
14	0.4	0.1	99.9
16	0.48	0.1	99.9
16.1	0.48	99.0	1.0
19.1	0.2	99.0	1.0
20	0.2	99.0	1.0

An external calibration of the QTOF mass spectrometer was performed with a sodium formate solution before analysis. Also, a calibrant injection was performed automatically at the beginning of each run and the segment of 0.1-0.25 min was used for internal calibration. The calibrant solution of sodium formate consisted of 10 mM sodium formate clusters in a mixture of water: isopropanol 1:1. The theoretical exact masses of calibration ions with formulas $Na(NaCOOH)_{1-14}$ in the range of 50−1000 Da were used for calibration. The instrument provided a typical resolving power of 36,000-40,000 during calibration.

Bruker’s software that was used for raw data analysis was Data Analysis 5.1, TASQ Client 2.1.

4.5 Method Development

The aim of this study was the development of a novel, generic protocol for the determination of polar and semi-polar organic compounds of ECs and PPs. The generic protocol for sample preparation was of crucial importance, because the usage of HRMS permits the use of wide-scope target, suspect, non-target methodologies for the screening and also the retrospective analysis.

For the method development a mix with 174 standards of ECs from different classes (pharmaceuticals and veterinary drugs, illicit drugs, industrial chemicals, pesticides, PFAS) was used. As criteria for the evaluation of each test 3 parameters were used; % Recovery of each one of the spiked compounds, which was assessed by the equation: *%Recovery* = [(Spiked Sample – Sample) / (Matrix Matched – Sample)] × 100, % Factor of Matrix Effect, which was assessed by the equation *%FME* = [(Matrix Matched - Sample) / (Standard – Solvent)] × 100, and the *number of detected compounds*.

The first step of the method development was the planning of the protocol. In accordance with the bibliographic review, for the extraction of the analytes from the biota matrices, the usage of the ASE was considered as the best option, due to its unique benefits. The crucial parameters, which have to be tested, were the initial mass of freeze-dried sample in both tissues and livers, the temperature during the ASE extraction, the extraction solvents and the clean-up step of the samples.

For the method development tests, two pooled samples consisted of different matrices were composed. The first one was consisted of tissues from different fish (*Sparus aurata*, *Engraulis encrasicolus*, *Sardina pilchardus*, *Solea*, *Galeorhinus galeus*, *Dicentrarchus labrax*) and the second one was consisted of bovine livers. These pooled samples were freeze-dried, homogenized and stored in glass bottles in -80°C.

In the first test, the importance of the clean-up step was tested, because in many studies this step was omitted. In samples from the same pooled fish sample using the same conditions in the extraction step, a clean-up step was performed in half of samples, using mixed-mode SPE cartridges, which were previously used in the laboratory in different matrices (Diamanti et al, [52]), whereas in the same time in the other half of samples the clean-up step was omitted.

In the second test, different temperatures (50, 80 and 100°C) in the extraction and different extraction solvent mixtures (Methanol: Acetonitrile: milli-Q water 50:25:25 and Methanol: Acetonitrile 67:33) were tested.

In the third test, different masses of freeze-dried fish tissue (1g, 2.5g and 5g) and bovine liver (0.2g and 0.5g) were tested.

Finally, in the last test, a comparison of the final developed method with an already developed generic method (Dasenaki et al 2015, [85]), which was used in the laboratory for the determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs was taken place.

4.6 Method Validation

A representative validation dataset of 60 compounds from different classes of ECs was used in order to evaluate linearity, accuracy, precision, matrix effects and detectability of the screening method. The compounds of the validation dataset and some of their fragments in positive and in negative ESI mode are shown in Table 7. These selected compounds represented almost all the classes of ECs in the database and had several physicochemical properties, so they eluted all over the chromatogram.

Linearity was studied for each compound by analyzing spiked samples (standard addition curve) in each one of the two matrices at 6 different concentrations ranging from 10 μg/L till 250 μg/L, as well as standard solutions in the same levels. Using these calibration curves, the limits of detection (LOD) were calculated by multiplying the standard error by 3 and dividing it by the slope.

Accuracy was assessed with recovery experiments. Method recovery was calculated by dividing the peak area of the spiked samples by the peak area of the matrix-matched samples at 25, 70 and 150 μg/L. The initial samples were analyzed for determination of the analytes of the validation dataset and if the sample already contained the analyte, its peak area was subtracted from the peak area of the spiked sample and the peak area of the matrix-matched sample. Precision was expressed as method repeatability in terms of relative standard deviation (%RSD) in 6 spiked samples at 150 μg/L. After the calculation of the matrix factor by dividing the peak area of matrix-matched samples by the peak area of the standard solutions, matrix effect was assessed by the equation: %Matrix Effect = (Matrix Factor - 1) × 100.

Table 7: Validation Dataset.

Compound Name	CAS Number	Molecular Formula	Calculated m/z precursor ion	Retention Time (min)	Fragment 1	Fragment 2	Fragment 3	Fragment 4
Methamphetamine	537-46-2	C10H15N1	150.1277	4.21	91.0542	65.0386	119.0855	150.1278
Cathine	492-39-7	C9H13NO	152.1070	3.68	91.0542	115.0542	17.0699	134.0964
3,4-Methylenedioxyamphetamine (MDA)	4764-17-4	C10H13N1O2	180.1019	4.19	105.0699	79.0542	135.0441	133.0648
3,4-Methylenedioxymethamphetamine (MDMA)	42542-10-9	C11H15N1O2	194.1176	4.18	105.0699	135.0441	79.0542	133.0648
Codeine	76-57-3	C18H21N1O3	300.1594	3.4	215.1067	243.1016	199.0754	58.0651
Lysergic acid diethylamide (LSD)	50-37-3	C20H25N3O1	324.2070	5.48	223.1230	208.0758	281.1648	74.0964
3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	82801-81-8	C12H17N1O2	208.1332	4.39	105.0699	135.0441	133.0648	136.0754
Dihydro-Codeine	125-28-0	C18H23N1O3	302.1751	3.36	199.0754	245.1172	227.1067	302.1777
Nor-Fentanyl	1609-66-1	C14H20N2O1	233.1648	4.68	84.0808	55.0542	56.0495	57.0335
D L-N O-Didesmethyl-Venlafaxine	135308-74-6	C15H23N1O2	250.1802	4.91				
2-Amino-Benzothiazole	136-95-8	C7H6N2S	151.0324	5.84	151.0325	124.0217	109.0116	152.0342
2-OH-Benzothiazole	934-34-9	C7H5NOS	152.0165	6.53	124.0226	152.0169	119.0364	134.0049
4-Me-Benzotriazole	136-85-6	C7H7N3	134.0713	5.83	134.0715	79.0543	77.0386	106.0651
2-4-Dinitrophenol (DNP)	51-28-5	C6H4N2O5	164.9942	4.5	95.0146	123.0085	109.0174	183.0048
Perfluorodecanoic acid (PFDeA)	335-76-2	C10F19O2H	512.9589	11.11	218.9862	468.9702	268.9830	168.9894
Perfluorododecanoic acid (PFDoA)	307-55-1	C12F23O2H	612.9526	12.19	268.9830	218.9862	568.9627	
Perfluorooctanoic acid (PFOA)	335-67-1	C8HF15O2	412.9653	9.68	418.9734	168.9894	218.9862	118.7511
Perfluorooctanesulfonamide (PFOSA)	754-91-6	C8F17SO2NH2	497.9451	11.59	268.9830	218.9862	525.9764	118.9920
Perfluorotetradecanoic acid (PFTeDA)	376-06-7	C14F27O2H	712.9462	13.01	368.9766	318.9787	668.9563	
Perfluoroundecanoic acid (PFUnA)	2058-94-8	C11F21O2H	562.9557	11.69	268.9830	218.9862	518.9659	
Perfluorobutanesulfonic acid (PFBuS)	375-73-5	C4F9SO3H	298.9419	6.64	298.9432	79.9574	98.9558	82.9609
Perfluoroheptanoic acid (PFHpA)	375-85-9	C7F13O2H	362.9685	8.79	368.9766	168.9894	112.9856	118.9925
Perfluorohexanoic acid (PFHxA)	307-24-4	C6F11O2H	312.9717	7.73	368.9766	168.9894	118.9926	268.9833
Perfluorononanoic acid (PFNA)	375-95-1	C9F17O2H	462.9621	10.44	218.9862	468.9702	268.9830	168.9892
Perfluorooctanesulfonic acid (PFOS)	1763-23-1	C8F17SO3H	498.9291	10.43	499.0186	168.7352	498.9575	98.7875
Perfluoropentanoic acid (PFPeA)	2706-90-3	C5F9O2H	262.9749	6.39	268.9830	118.9926	196.9834	
Perfluoroheptanesulfonic acid (PFHpS)	375-92-8	C7F15SO3H	448.9323	9.69	418.9734	168.9894	498.9291	
Perfluorohexanesulfonic acid (PFHxS)	355-46-4	C6F13SO3H	398.9355	8.84	368.9766	168.9894	448.9323	
Levamisol	16595-80-5	C11H12N2S	205.0794	3.68	205.0766	178.0663	123.0263	129.0625

Compound Name	CAS Number	Molecular Formula	Calculated m/z precursor ion	Retention Time (min)	Fragment 1	Fragment 2	Fragment 3	Fragment 4
Propranolol	525-66-6	C16H21N1O2	260.1645	6.59	74.0600	56.0495	116.1070	183.0804
Ketoprofen	22071-15-4	C16H14O3	255.1016	8.53	105.0335	209.0961	95.0491	177.0546
Metformin	657-24-9	C4H11N5	130.1087	1.39	71.0604	85.0509	60.0557	130.1087
Oxprenolol	6452-71-7	C15H23N1O3	266.1751	5.66	72.0808	116.1070	98.0964	266.1756
Tramadol	27203-92-5	C16H25N1O2	264.1958	4.88	58.0651	159.0795	246.1839	121.0635
Trimethoprim	30806-86-1	C14H16N4O4	305.1244	5.49	137.0458	244.0968	259.0826	275.0775
Flufenamic acid	530-78-9	C14H10F3NO2	280.0591	9.31	280.0591	236.0693	216.0630	234.0536
Meloxicam	71125-38-7	C14H13N3O4S2	350.0275	6.41				
Citalopram	59729-33-8	C20H21N2O1F1	325.1711	6.59	109.0448	262.1027	116.0495	234.0714
Clomipramine	303-49-1	C19H23N2Cl1	315.1623	9.21	86.0964	58.0651	242.0731	315.1624
Fluoxetine	54910-89-3	C17H18N1O1F3	310.1413	8.6	44.0495	148.1114	117.0696	91.0543
Fosinopril	98048-97-6	C30H46NO7P	564.3085	12.43	436.2247	390.2193	418.2142	152.2142
Quetiapine	111974-69-7	C21H25N3O2S1	384.1740	7.26	253.0794	279.0950	221.1073	158.1176
Timolol	26839-75-8	C13H24N4O3S1	317.1642	4.88	74.0600	261.1016	244.0750	188.0488
Mepivacaine	96-88-8	C15H22N2O	247.1805	4.66	98.0964	70.0651	247.1809	
Meptazinol	54340-58-8	C15H23NO	234.1852	4.74	107.0491	133.0648	121.0648	126.1274
4-Acetamido-Antipyrine	83-15-8	C13H15N3O2	246.1237	3.94	83.0604	104.0495	56.0495	94.0651
O-Desmethyl-Tramadol	73986-53-5	C15H23N1O2	250.1802	4.06	58.0651			
4-Formylamino-Antipyrine	1672-58-8	C12H13N3O2	232.1081	3.96	56.0495	83.0604	104.0495	94.0651
N-bisdesmethyl-Tramadol (dinor-tramadol)	73806-40-3	C14H21NO2	236.1645	5.19	121.0648	189.1274	218.1539	81.0699
DEET (Diethyltoluamide)	134-62-3	C12H17NO	192.1383	8.20	119.0491	91.0542	109.0648	72.0444
Spiroxamine	118134-30-8	C18H35NO2	298.2741	9.32	144.1383	100.1121	298.2740	72.0811
Bromoxynil	1689-84-5	C7H3Br2NO	273.8498	5.35	273.8510	78.9188	193.9246	166.9377
Dinoterb	1420-07-1	C10H12N2O5	239.0662	8.13	239.0673	207.0410	179.0712	176.0351
Methiocarb-sulfoxide	2635-10-1	C11H15NO3S	242.0845	4.86	185.0631	122.0728	170.0382	168.0604
Desethyl-Atrazine	6190-65-4	C6H10ClN5	188.0697	5.73	146.0228	104.0010	79.0058	188.0691
3-Hydroxy-Carbofuran	16655-82-6	C12H15NO4	238.1074	5.09	163.0754	181.0859	220.0968	135.0808
Ethiofencarb-sulfoxide	53380-22-6	C11H15NO3S	242.0845	4.7	185.0631			
Metamitron-desamino	36993-94-9	C10H9N3O1	188.0818	5.29				
Cotinine	486-56-6	C10H12N2O1	177.1022	3.75	80.0495	98.0600	70.0651	177.1014
Nor-Nicotine	5746-86-1	C9H12N2	149.1073	3.14	130.0645	132.0798	149.1065	106.0651

4.7 Target screening for the determination of emerging contaminants

An in-house database (https://www.norman-network.com/nds/SLE/, S21 - UATHTARGETS, last visit 08/10/2019) of more than 2100 ECs and priority pollutants was used for the target screening of the biota (top predators and their prey) samples in both positive and negative ESI mode. The database contained compounds from different classes; personal care products, steroids & hormones, pharmaceuticals (>450), antibiotics (>50), illicit drugs and new psychoactive substances (>500), industrial chemicals (>100), pesticides (>900), sweeteners, surfactants, biocides as well as their (bio)TPs. The database contained information for the precursor ions, retention time, adducts, in-source fragments and bbCID MS/MS fragments, as well as identifiers for the compounds (CAS number, InChI). This information was acquired from the analysis of the standard solutions of these compounds, which were available in the laboratory, with the bbCID method, or was part of the manufacturer's database, Bruker's ToxScreener 2.1, which was built with the same bbCID method.

The raw data were processed with Bruker's TASQ Client 2.1 and Data Analysis 5.1. The TASQ method in TASQ Client 2.1 created in all samples the Extracted Ion Chromatogram (EIC) of the precursor ion of the compounds included in the database with a mass error window of ±0.005 Da.

Every peak, which was detected for a target compound was evaluated according to some parameters that were set to the method and after manual inspection. The first one was the mass accuracy, which refers to the difference between the accurate mass (measured) and the exact mass (theoretical) and is expressed in mDa or ppm. The second one was the retention time shift, which refers to the difference between the measured retention time and the one that is recorded to the database. The last parameter was the isotopic fitting, which refers to the correlation between the theoretical and the experimental isotopic pattern. Its calculation is based on the standard deviation of the masses and the intensities for all isotopic peaks and is expressed by the mSigma value. Lower mSigma value indicates better isotopic fitting.

The screening parameters that were set to the method in both positive and negative ESI mode were an area threshold of 1000 counts and an intensity threshold of 500 counts. Regarding the mass accuracy, peaks having this value higher than 2.0 mDa and 5 ppm were rejected. Regarding the retention time, peaks having this value higher than 0.4 min were also rejected. The mSigma threshold was set to 200. However, this value was only considered as a positive confirmation and not for rejecting peaks, because strong matrix effects combined with low concentration levels of analytes may affect the isotopic pattern results and give a bad mSigma value, although the compound may be present.

In order to confirm the screening results, bbCID MS/MS fragments were examined, as well as adducts and in-source fragments in full scan MS.

Apart from the EIC of the precursor ion of a compound, the TASQ method created with the same mass error window the EICs of its adducts, in-source and bbCID MS/MS fragments, so the fitting of their chromatographic profiles were inspected and evaluated. Except for TASQ Client 2.1, Data Analysis 5.1 was used for the inspection and evaluation of the bbCID mass spectra.

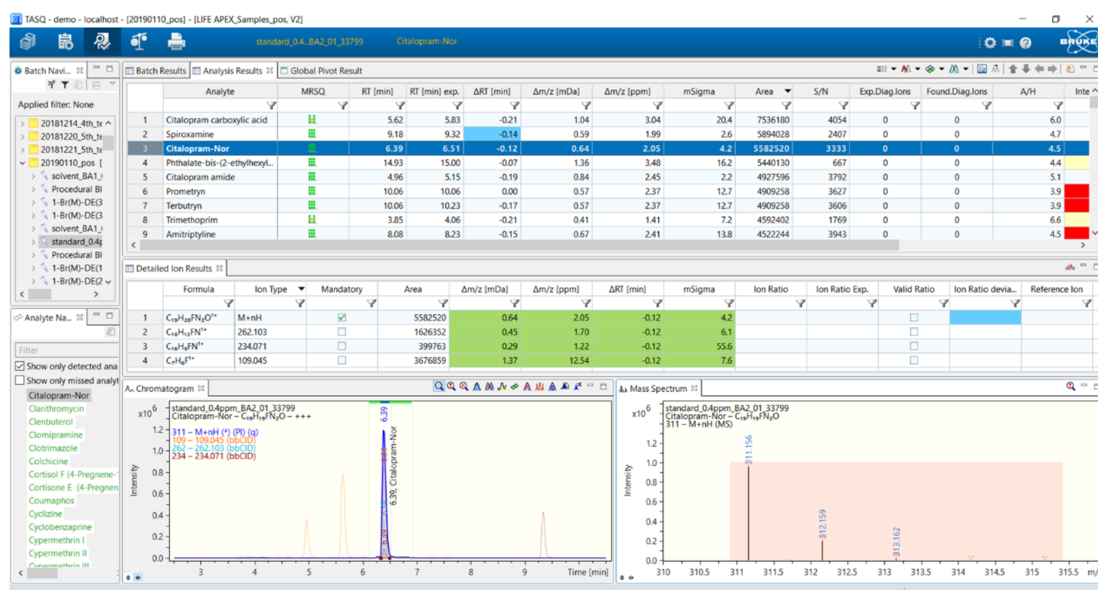


Figure 13: Data Treatment Interface - Tasq Client 2.1 (Bruker Daltonics)

For the identification and confirmation of the analytes, the Identification Points (IPs) system that has been proposed for HRMS analysis by Bletsou et al. [62] was used. Precursor ion (mass accuracy) and retention time earn together 2 IPs, while isotopic fitting earns 0.5 IP. Furthermore, each of the in-source and bbCID MS/MS fragments (mass accuracy) earns 2.5 IPs.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Method Development Results

As mentioned in the chapter 4.5, for the method development several tests took place and were tested the importance of the clean-up step, different solvents and temperatures during the extraction, different masses during the extraction for each matrix and finally took place a comparison of the developed protocol with an already protocol which was developed in the laboratory.

In the Box-Plot diagram (figure 14), a comparison of the % Recoveries protocols with and without clean-up step is presented. In all the classes of compounds, which were used in the method development, the recoveries of the spiked compounds were higher in the protocol with the clean-up step in contrast with the protocol without the clean-up step. Also, the % Factors of Matrix Effect (%FME) were higher in the protocol without the clean-up step, which indicates that in that protocol the matrix effect during the analysis affects the determination of the analytes.

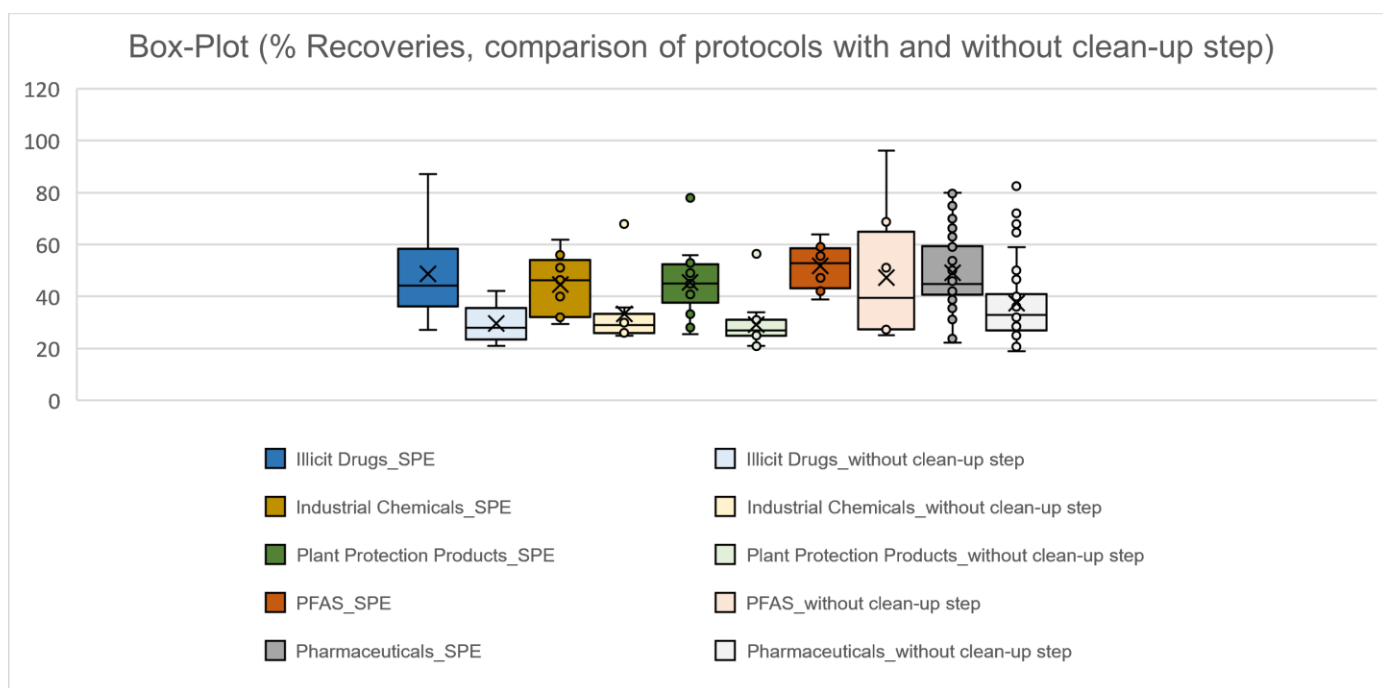


Figure 14: Box-Plot (% Recoveries, comparison of protocols with and without clean up step.

In respect to the extraction solvents, two different mixtures of extraction solvents were tested; Methanol: Milli-Q water: Acetonitrile (2:1:1) and Methanol: Acetonitrile (2:1). When the mixture of extraction solvents, in which the milli-Q water was included, was used, intense foaming during the rotary evaporation was observed (figure 15). That foaming led to sample loss during that step and the use of the extraction mixture of Methanol: Acetonitrile (2:1) was the best option.

Figure 15: Foaming during the rotary step.

In the different temperatures, slight variation in the Factors of Matrix Effect (%FME) was observed and the evaluation of the tests took place using the %Recoveries and the number of the total compounds. The number of the detected compounds, in total, as well as the number of the detected compounds with %Recovery above of 50%, 60%, 80% and 120% respectively are presented in the table 8 and in figure 16. Although the differentiation in the number of total compounds is almost the same in the tests, in the test with extraction temperature 50°C higher recoveries were observed.

Table 8: Number of detected compounds and %Recoveries in different temperatures.

	detected compounds	***%R<50***	***%R>50***	***%R>60***	***%R>80***	***%R>120***
Protocol_50°C	157	94	63	32	7	0
Protocol_80°C	155	100	54	20	1	1
Protocol_100°C	140	95	45	15	0	0

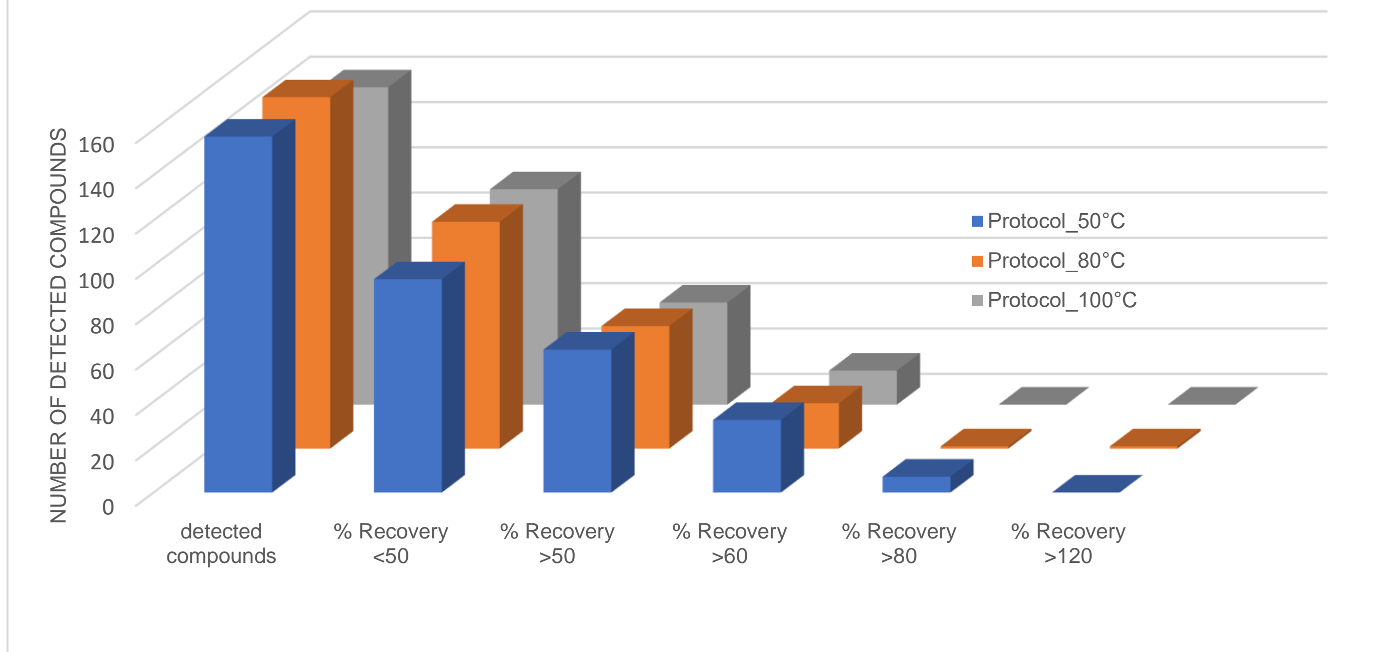


Figure 16: Comparison of protocols different temperatures.

For the evaluation of the different masses, Box-Plot diagrams (% Recoveries) were constructed for the different classes of compounds, due to the small variation between the % Factors of Matrix Effect (%FME). In almost all categories higher recoveries were observed in the test with 1g mass of fish and 0.2g mass of liver.

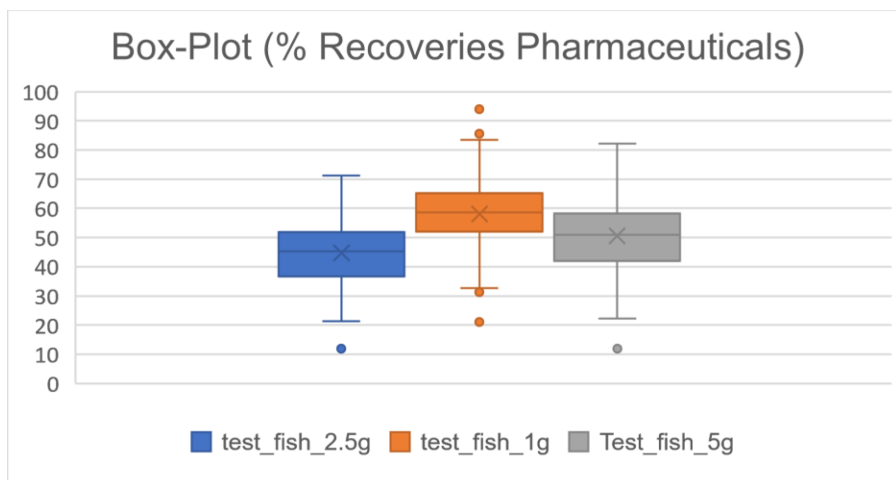


Figure 17: Box-Plot (%Recoveries Pharmaceuticals, fish)

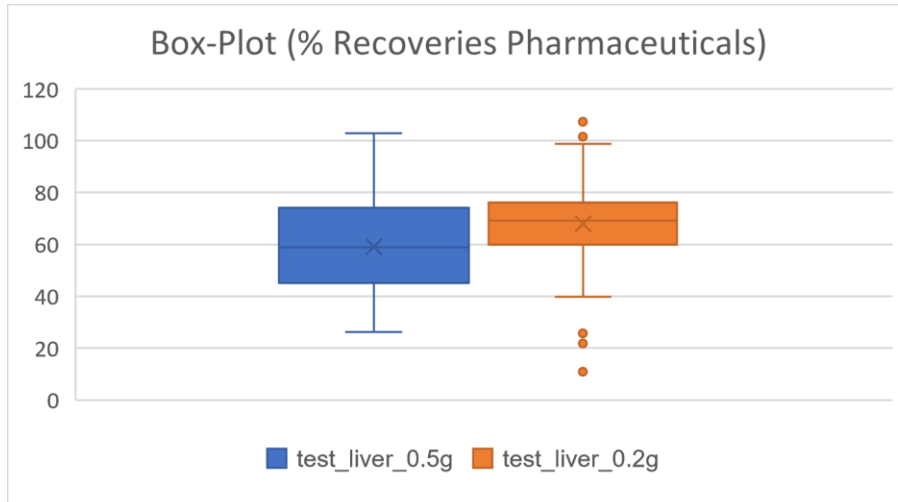


Figure 18:Box-Plot (%Recoveries Pharmaceuticals, liver)

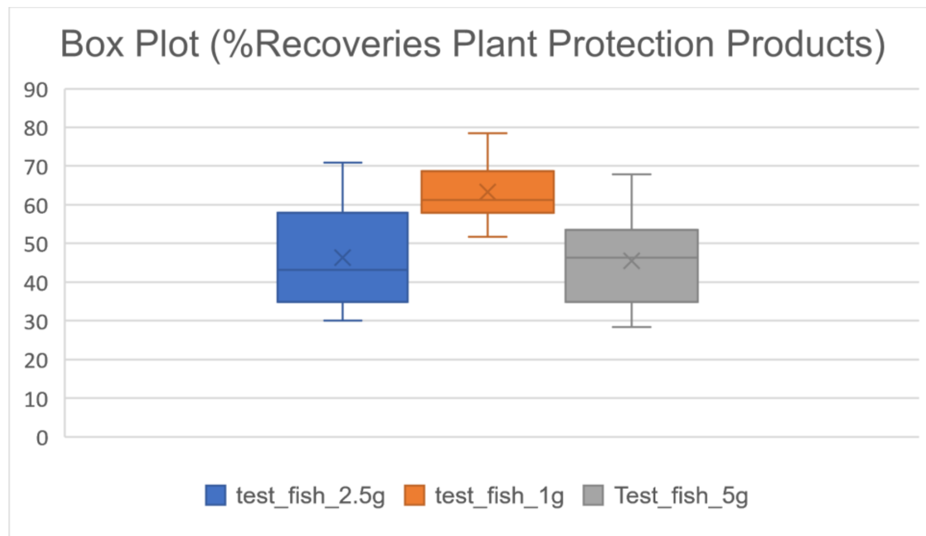


Figure 19:Box-Plot (%Recoveries, Plant Protection Products, fish)

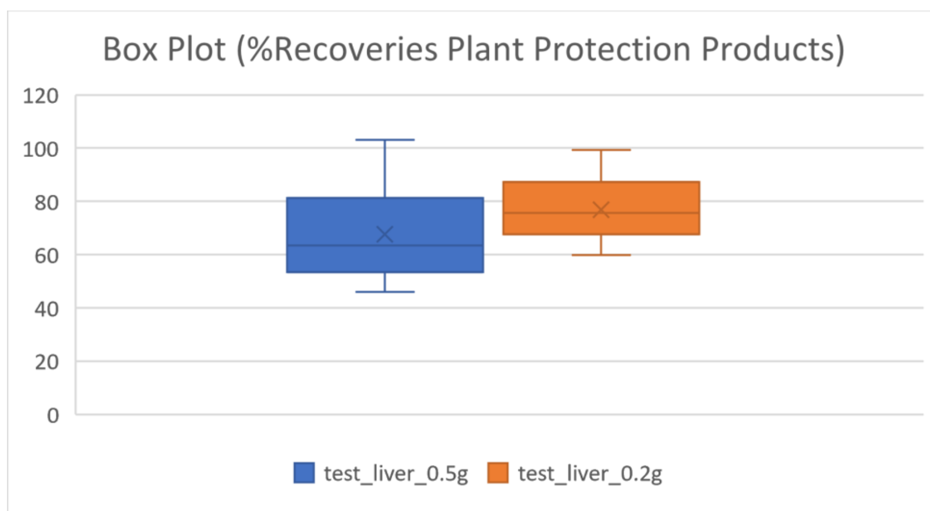


Figure 20: Box-Plot (%Recoveries, Plant Protection Products, liver)

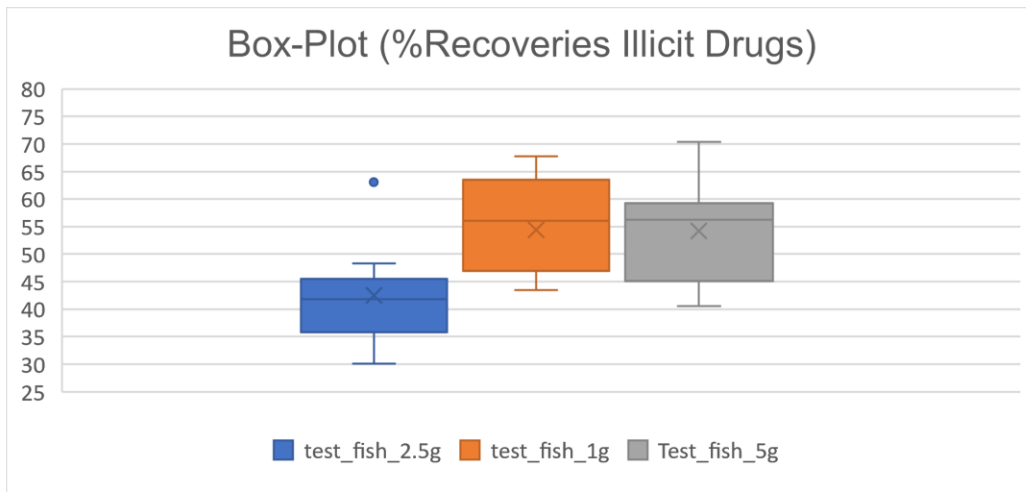


Figure 21: Box-Plot (%Recoveries, Illicit Drugs, fish)

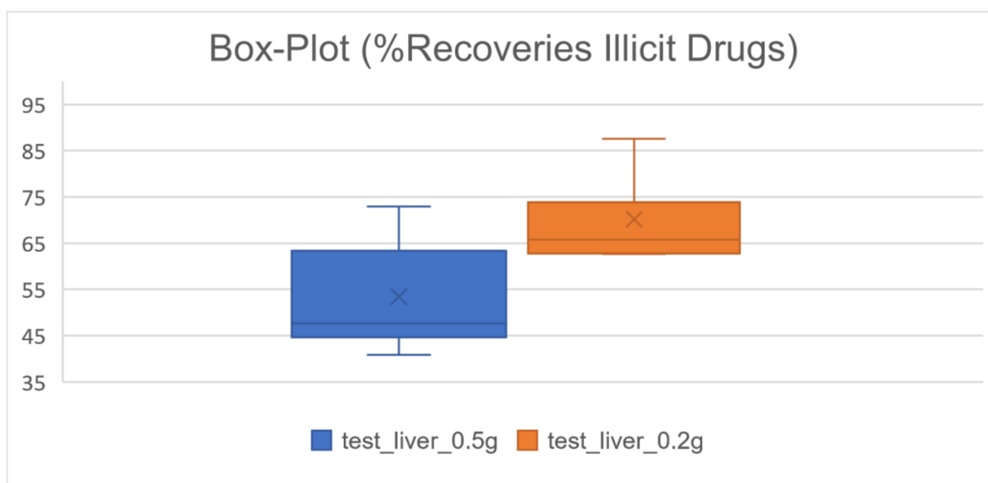


Figure 22: Box-Plot (%Recoveries, Illicit Drugs, liver)

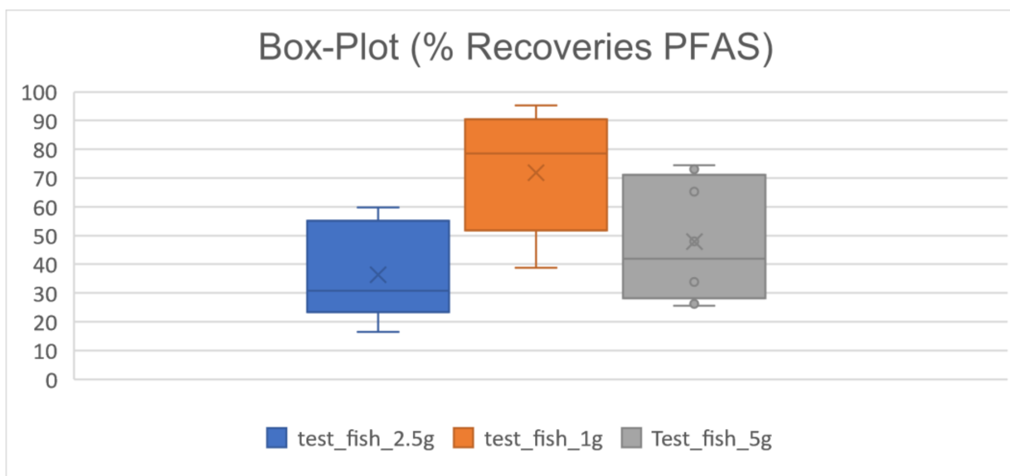


Figure 23: Box-Plot (%Recoveries, PFAS, fish)

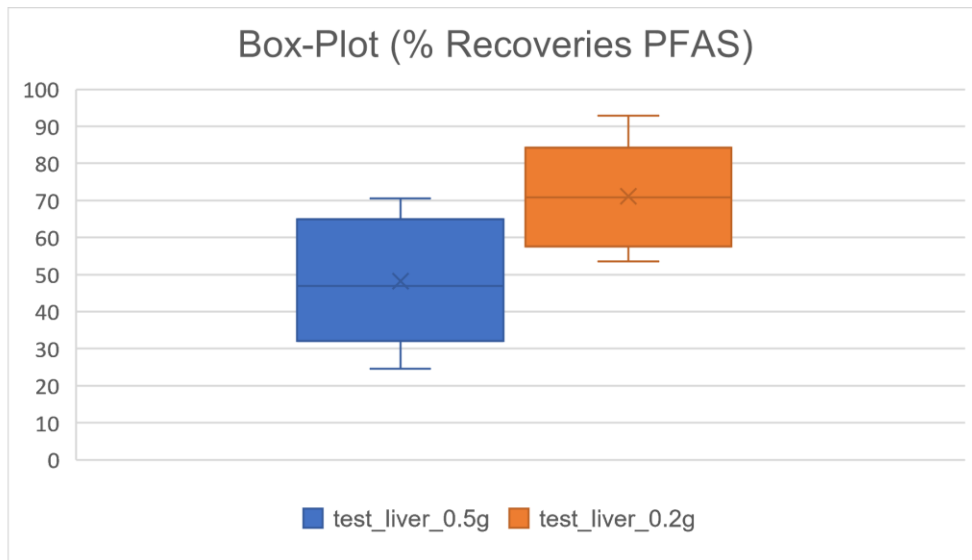


Figure 24: Box-Plot (%Recoveries, PFAS, liver)

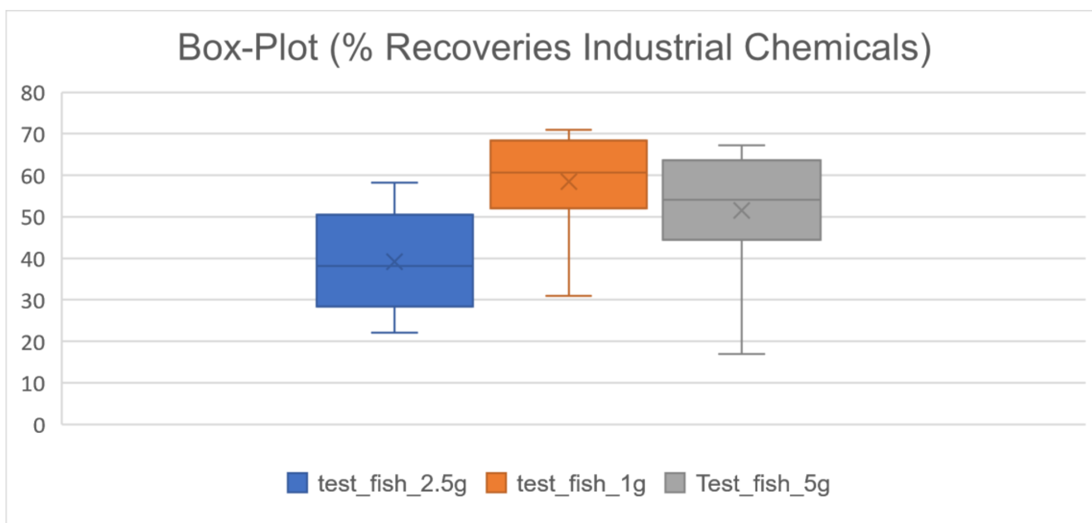


Figure 25: Box-Plot (%Recoveries, Industrial Chemicals, fish)

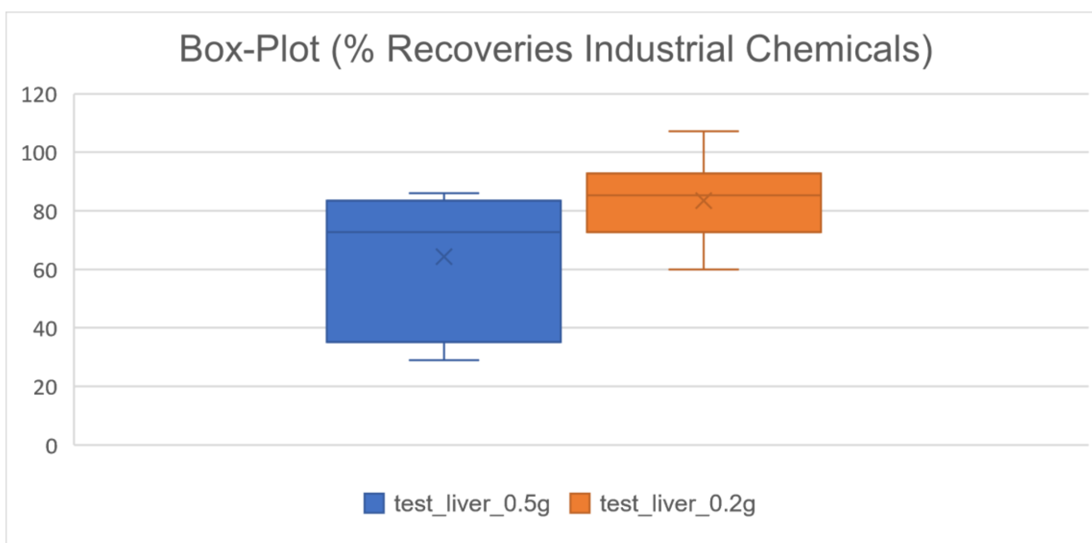


Figure 26: Box-Plot (%Recoveries, Industrial Chemicals, liver)

In the final step of the method development, %Recoveries for different classes of compounds were evaluated in the two different protocols. The %Recoveries in the already developed protocol (Dasenaki et al 2015) were higher in pharmaceuticals. This is reasonable because that protocol was developed for the simultaneous determination of pharmaceuticals in biota matrices. In the other classes of the spiked compounds, %Recoveries were higher in the developing protocol.

Consequently, the developing protocol was ideal for the simultaneous determination of polar and semi-polar compounds from various categories with different physicochemical properties.

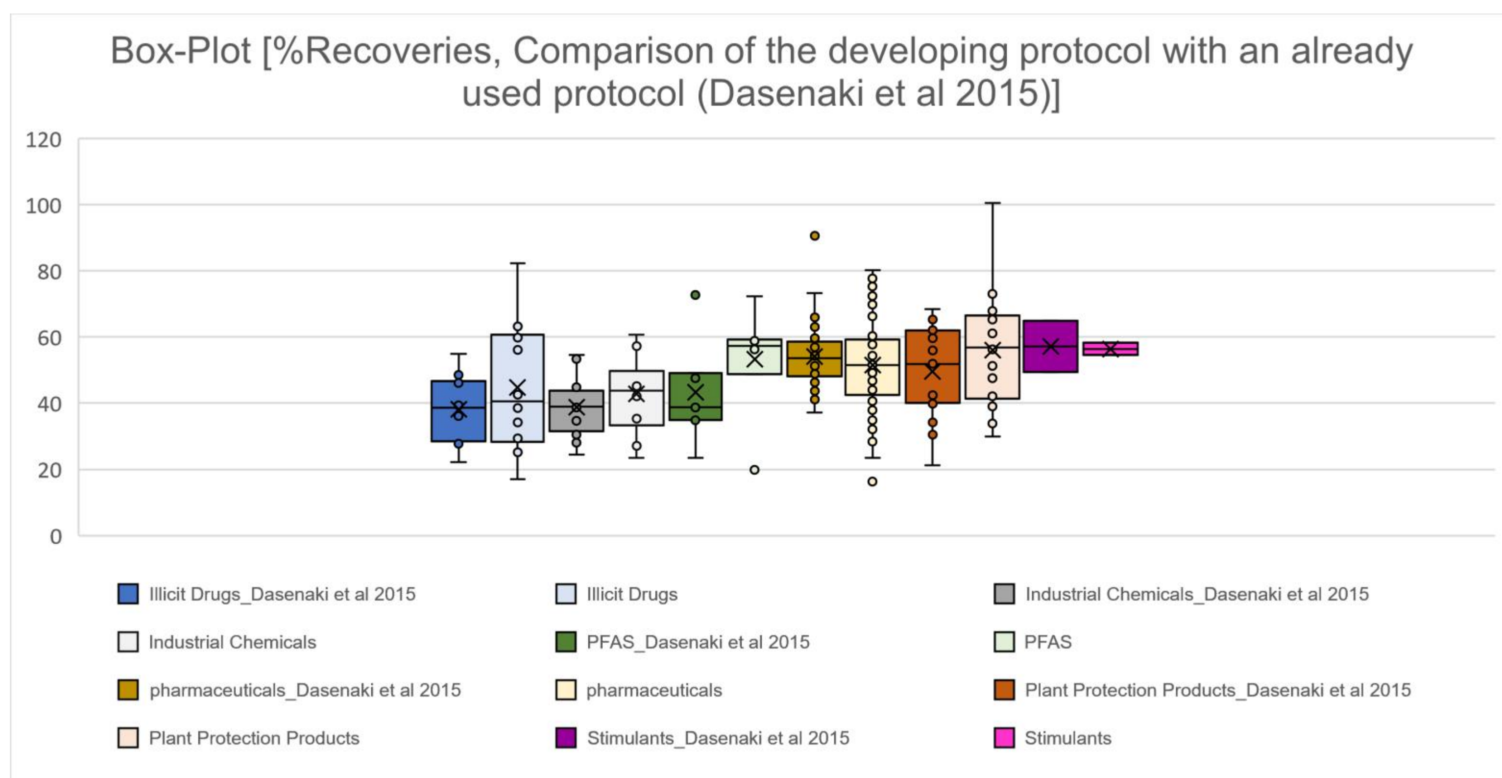


Figure 27: Box-Plot [%Recoveries, Comparison of the developing protocol with an already used protocol (Dasenaki et al 2015)]

5.2 Method Validation Results

As mentioned in chapter 4.6, for the evaluation of linearity, accuracy, precision, matrix effects and detectability of the generic developed protocol, a representative validation dataset of 60 compounds with different physicochemical properties from different classes of ECs was used.

Regarding linearity, the slope, the intercept and the correlation coefficient (R^2) of the standard addition curve in each one of the two matrices for each compound are presented in the Tables 9 and 10 for livers and muscles respectively.

The method limits of detection (LODs) that were calculated from the data of the calibration curves are presented in the Tables 9 and 10 for livers and muscles respectively. The method LODs for the analytes were 4-14 μg/Kg for the muscles and 13-80 μg/Kg for the livers.

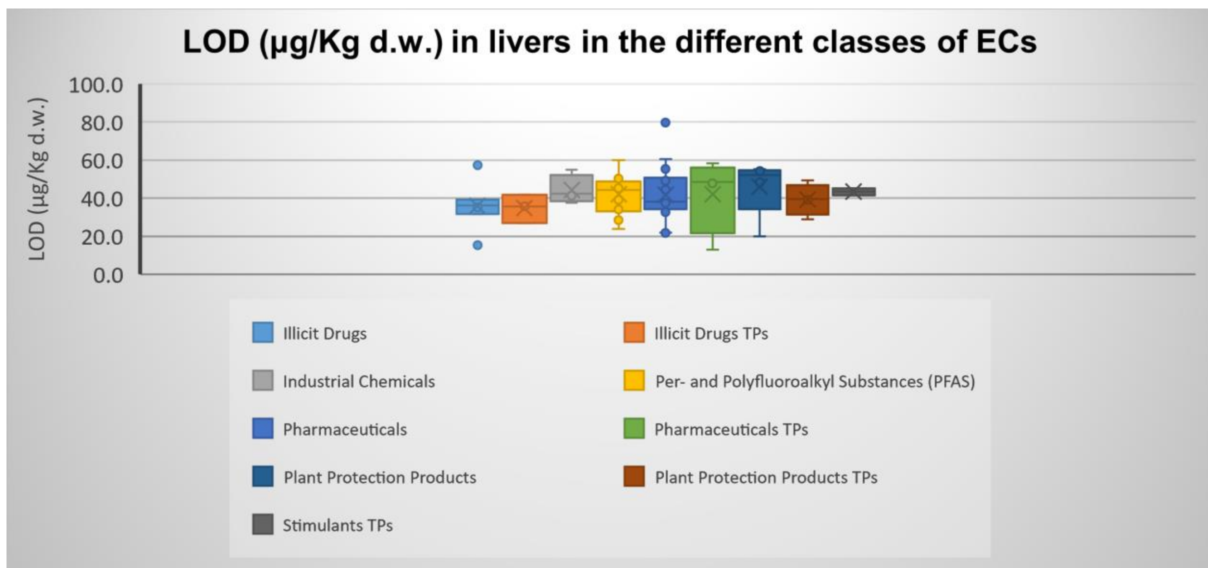


Figure 28: LOD (μg/Kg d.w.) in livers in the different classes of ECs.

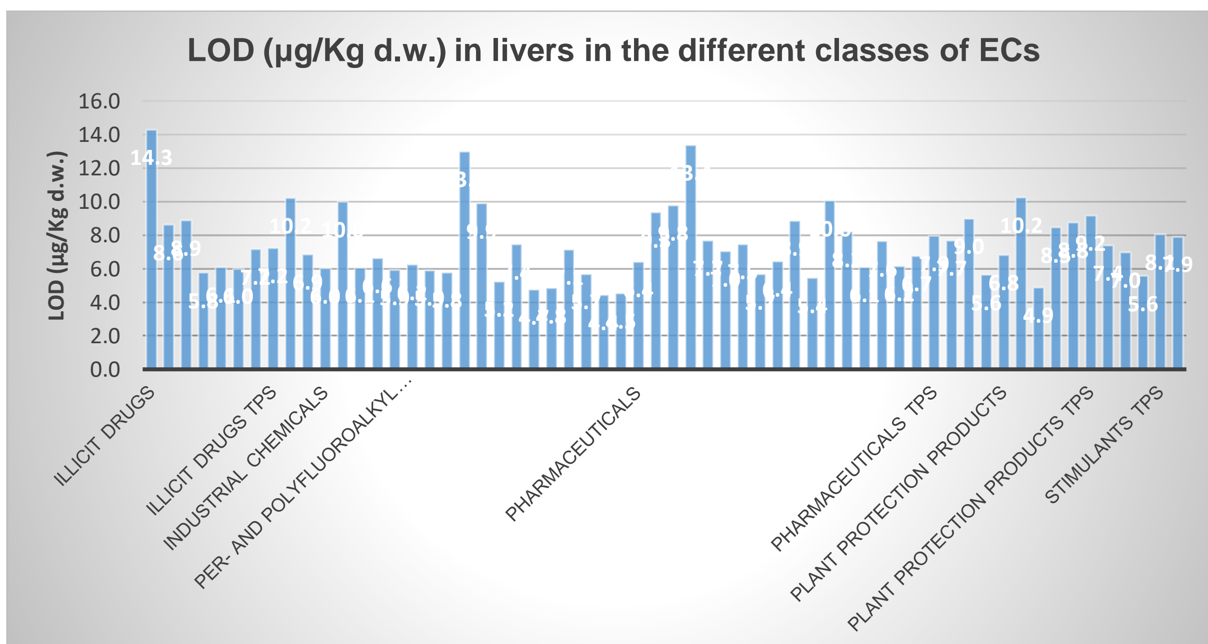


Figure 29: LOD (μg/Kg d.w.) in muscles in the different classes of ECs.

Table 9: Validation results in livers - Linearity: Slope, intercept and correlation coefficient, Detectability: LODs

Class	*Compound*	*livers*						
		IS	*a*	s_a	R^2	*b*	s_b	*LOD (μg/Kg) dry weight*
Illicit Drugs	***Methamphetamine***	Amphetamine-D6	0.00803	0.00056	0.95	-0.111	0.077	39.4
	Cathine	Amphetamine-D6	0.00615	0.00040	0.96	-0.103	0.055	36.7
	3,4-Methylenedioxyamphetamine (MDA)	Amphetamine-D6	0.01723	0.00050	0.996	-0.261	0.064	15.3
	3,4-Methylenedioxymethamphetamine (MDMA)	Cetirizine-D8	0.01628	0.00091	0.97	-0.22	0.12	31.6
	Codeine	Cetirizine-D8	0.00297	0.00021	0.98	-0.052	0.026	36.3
	Lysergic acid diethylamide (LSD)	Cetirizine-D8	0.0223	0.0014	0.96	-0.287	0.191	35.3
	3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	Diazepam-d5	0.144	0.015	0.91	-2.5	2.0	57.3
Illicit Drugs TPs	***Dihydro-Codeine***	Sulfadiazine-d4	0.0861	0.0054	0.97	-0.85	0.74	35.6
	Nor-Fentanyl	Diuron-d6	0.000630	0.000046	0.95	-0.0061	0.0064	41.8
	D L-N O-Didesmethyl-Venlafaxine	Cetirizine-D8	0.00694	0.00033	0.98	-0.033	0.045	26.8
Industrial Chemicals	***2-Amino-Benzothiazole***	Cetirizine-D8	0.00663	0.00044	0.96	-0.069	0.060	37.6
	2-OH-Benzothiazole	Cetirizine-D8	0.000232	0.000022	0.91	-0.0050	0.0031	55.0

	4-Me-Benzotriazole	Cetirizine-D8	0.00143	0.00010	0.95	0.000	0.015	43.2
	2-4-Dinitrophenol (DNP)	Bisphenol A-d16	0.00462	0.00033	0.97	-0.040	0.046	41.3
Per- and Polyfluoroalkyl Substances (PFAS)	***Perfluorodecanoic acid (PFDeA)***	Bisphenol A-d16	0.00831	0.00055	0.96	-0.248	0.079	39.2
	Perfluorododecanoic acid (PFDoA)	Bisphenol A-d16	0.00676	0.00054	0.95	-0.160	0.078	47.6
	Perfluorooctanoic acid (PFOA)	Bisphenol A-d16	0.00587	0.00047	0.94	-0.049	0.065	45.4
	Perfluorooctanesulfonamide (PFOSA)	Bisphenol A-d16	0.00735	0.00048	0.96	-0.098	0.066	37.3
	Perfluorotetradecanoic acid (PFTeDA)	Bisphenol A-d16	0.00224	0.00011	0.98	-0.034	0.015	28.3
	Perfluoroundecanoic acid (PFUnA)	Bisphenol A-d16	0.00841	0.00068	0.94	-0.251	0.098	48.3
	Perfluorobutanesulfonic acid (PFBuS)	Bisphenol A-d16	0.01376	0.00058	0.98	-0.084	0.079	23.8
	Perfluoroheptanoic acid (PFHpA)	Bisphenol A-d16	0.00550	0.00052	0.93	-0.064	0.071	53.0
	Perfluorohexanoic acid (PFHxA)	Bisphenol A-d16	0.00333	0.00020	0.97	-0.053	0.027	34.0
	Perfluorononanoic acid (PFNA)	Bisphenol A-d16	0.00349	0.00035	0.92	0.239	0.051	60.0
	Perfluorooctanesulfonic acid (PFOS)	Bisphenol A-d16	0.00595	0.00045	0.95	0.058	0.062	43.3
	Perfluoropentanoic acid (PFPeA)	Bisphenol A-d16	0.00249	0.00014	0.97	-0.018	0.019	30.8

	Perfluoroheptanesulfonic acid (PFHpS)	Bisphenol A-d16	0.0262	0.0021	0.94	0.03	0.29	45.8
	Perfluorohexanesulfonic acid (PFHxS)	Bisphenol A-d16	0.0331	0.0029	0.93	-0.24	0.40	50.3
Pharmaceuticals	***Levamisol***	Sulfadiazine-d4	0.0755	0.0051	0.96	-1.36	0.70	38.2
	Propranolol	Atrazine-d5	0.00745	0.00050	0.96	-0.16	0.07	38.0
	Ketoprofen	Cetirizine-D8	0.000740	0.000072	0.93	0.005	0.011	60.5
	Metformin	Diazepam-d5	0.00097	0.00012	0.91	-0.028	0.019	79.6
	Oxprenolol	Sulfadimethoxin-d4	0.0146	0.0011	0.94	-0.20	0.16	44.4
	Tramadol	Sulfadimethoxin-d4	0.0331	0.0028	0.94	1.19	0.39	49.2
	Trimethoprim	Sulfadimidine-d4	0.00613	0.00034	0.97	-0.075	0.049	32.7
	Flufenamic acid	Flunixin d3	0.000686	0.000046	0.96	-0.0130	0.0062	37.5
	Meloxicam	Bisphenol A-d16	0.000430	0.000027	0.96	-0.0024	0.0038	36.0
	Citalopram	Diazepam-d5	0.00894	0.00060	0.96	-0.148	0.083	38.2
	Clomipramine	Diazepam-d5	0.01238	0.00053	0.993	-0.160	0.065	21.8
	Fluoxetine	Atrazine-d5	0.00896	0.00064	0.95	-0.157	0.089	40.8

	Fosinopril	Diazepam-d5	0.001024	0.000043	0.993	-0.0132	0.0054	21.8
	Quetiapine	Diazepam-d5	0.0371	0.0016	0.989	-0.47	0.21	23.5
	Timolol	Amphetamine-D6	0.0185	0.0013	0.95	-0.34	0.18	39.5
	Mepivacaine	Cetirizine-D8	0.0379	0.0037	0.91	-0.88	0.51	55.2
	Meptazinol	Cetirizine-D8	0.0268	0.0025	0.92	-0.62	0.34	52.2
Pharmaceuticals TPs	***4-Acetamido-Antipyrine***	Sulfadimidine-d4	0.0408	0.0033	0.95	1.00	0.47	47.9
	O-Desmethyl-Tramadol	Sulfadimidine-d4	0.0139	0.0014	0.91	-0.19	0.20	58.3
	4-Formylamino-Antipyrine	Cetirizine-D8	0.00452	0.00039	0.93	-0.118	0.054	49.2
	N-bisdesmethyl-Tramadol	Amphetamine-D6	0.002412	0.000061	0.997	-0.0307	0.0076	13.0
Plant Protection Products	***DEET (Diethyltoluamide)***	Cetirizine-D8	0.0112	0.0011	0.91	-0.16	0.15	55.2
	Spiroxamine	Cetirizine-D8	0.0127	0.0012	0.92	-0.08	0.16	52.2
	Bromoxynil	Bisphenol A-d16	0.001734	0.000062	0.990	-0.0197	0.0084	20.0
	Dinoterb	Cetirizine-D8	0.01167	0.00099	0.93	-0.17	0.14	48.3
	Methiocarb-sulfoxide	Cetirizine-D8	0.00380	0.00036	0.92	-0.072	0.050	54.3

Plant Protection Products TPs	***Desethyl-Atrazine***	Atrazine-d5	0.00178	0.00013	0.95	0.005	0.017	40.1
	3-Hydroxy-Carbofuran	Cetirizine-D8	0.00267	0.00023	0.93	-0.026	0.032	49.2
	Ethiofencarb-sulfoxide	Cetirizine-D8	0.00408	0.00029	0.96	-0.070	0.039	39.2
	Metamitron-desamino	Diuron-d6	0.001746	0.000089	0.97	0.004	0.012	29.0
Stimulants TPs	***Cotinine***	Sulfadiazine-d4	0.0483	0.0035	0.95	-0.21	0.49	41.6
	Nor-Nicotine	Cetirizine-D8	0.001128	0.000090	0.94	-0.024	0.012	45.2

Table 10:Validation results in muscles - Linearity: Slope, intercept and correlation coefficient, Detectability: LODs

Class	***Compound***	***muscles***						
		IS	***a***	s_a	R^2	***b***	s_b	***LOD (µg/Kg) dry weight***
Illicit Drugs	***Methamphetamine***	Amphetamine-D6	0.00686	0.00092	0.92	0.28	0.12	14.3
	Cathine	Amphetamine-D6	0.00873	0.00066	0.95	0.020	0.091	8.6
	3,4-Methylenedioxyamphetamine (MDA)	Amphetamine-D6	0.00729	0.00056	0.94	-0.051	0.079	8.9
	3,4-Methylenedioxymethamphetamine (MDMA)	Cetirizine-D8	0.01602	0.00081	0.98	-0.27	0.11	5.8
	Codeine	Cetirizine-D8	0.00255	0.00015	0.986	0.012	0.019	6.1

	Lysergic acid diethylamide (LSD)	Cetirizine-D8	0.01341	0.00078	0.987	-0.17	0.10	6.0
	3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	Diazepam-d5	0.1156	0.0073	0.96	-1.4	1.0	7.2
Illicit Drugs TPs	***Dihydro-Codeine***	Sulfadiazine-d4	0.0888	0.0059	0.97	-0.41	0.78	7.2
	Nor-Fentanyl	Diuron-d6	0.001044	0.000094	0.93	-0.016	0.013	10.2
	D L-N O-Didesmethyl-Venlafaxine	Cetirizine-D8	0.01305	0.00079	0.96	-0.21	0.11	6.9
Industrial Chemicals	***2-Amino-Benzothiazole***	Cetirizine-D8	0.00620	0.00033	0.97	-0.067	0.045	6.0
	2-OH-Benzothiazole	Cetirizine-D8	0.000315	0.000028	0.93	0.0102	0.0038	10.0
	4-Me-Benzotriazole	Cetirizine-D8	0.00489	0.00026	0.97	-0.118	0.036	6.1
	2-4-Dinitrophenol (DNP)	Bisphenol A-d16	0.00650	0.00038	0.97	-0.077	0.052	6.6
Per- and Polyfluoroalkyl Substances (PFAS)	***Perfluorodecanoic acid (PFDeA)***	Bisphenol A-d16	0.01830	0.00091	0.98	-0.41	0.13	5.9
	Perfluorododecanoic acid (PFDoA)	Bisphenol A-d16	0.00801	0.00042	0.98	-0.232	0.060	6.2
	Perfluorooctanoic acid (PFOA)	Bisphenol A-d16	0.01179	0.00061	0.97	-0.231	0.084	5.9
	Perfluorooctanesulfonamide (PFOSA)	Bisphenol A-d16	0.01055	0.00051	0.98	-0.265	0.074	5.8
	Perfluorotetradecanoic acid (PFTeDA)	Bisphenol A-d16	0.00142	0.00015	0.92	-0.075	0.022	13.0

	Perfluoroundecanoic acid (PFUnA)	Bisphenol A-d16	0.01171	0.00097	0.94	-0.35	0.14	9.9
	Perfluorobutanesulfonic acid (PFBuS)	Bisphenol A-d16	0.02005	0.00092	0.98	-0.26	0.13	5.2
	Perfluoroheptanoic acid (PFHpA)	Bisphenol A-d16	0.01111	0.00073	0.96	-0.24	0.10	7.4
	Perfluorohexanoic acid (PFHxA)	Bisphenol A-d16	0.00545	0.00023	0.98	-0.094	0.031	4.7
	Perfluorononanoic acid (PFNA)	Bisphenol A-d16	0.01151	0.00047	0.987	-0.224	0.067	4.8
	Perfluorooctanesulfonic acid (PFOS)	Bisphenol A-d16	0.00749	0.00045	0.97	-0.109	0.065	7.1
	Perfluoropentanoic acid (PFPeA)	Bisphenol A-d16	0.00421	0.00021	0.98	-0.091	0.029	5.7
	Perfluoroheptanesulfonic acid (PFHpS)	Bisphenol A-d16	0.0375	0.0015	0.985	-0.28	0.20	4.4
	Perfluorohexanesulfonic acid (PFHxS)	Bisphenol A-d16	0.0453	0.0018	0.98	-0.35	0.25	4.5
Pharmaceuticals	***Levamisol***	Sulfadiazine-d4	0.0669	0.0038	0.97	-1.03	0.52	6.4
	Propranolol	Atrazine-d5	0.00686	0.00054	0.95	-0.015	0.078	9.3
	Ketoprofen	Cetirizine-D8	0.00154	0.00012	0.95	-0.049	0.018	9.8
	Metformin	Diazepam-d5	0.00098	0.00010	0.92	-0.041	0.016	13.4
	Oxprenolol	Sulfadimethoxin-d4	0.0184	0.0012	0.96	-0.37	0.17	7.7

	Tramadol	Sulfadimethoxin-d4	0.0448	0.0028	0.96	0.74	0.38	7.0
	Trimethoprim	Sulfadimidine-d4	0.01051	0.00069	0.96	-0.10	0.10	7.5
	Flufenamic acid	Flunixin d3	0.001265	0.000063	0.98	-0.025	0.009	5.7
	Meloxicam	Bisphenol A-d16	0.001462	0.000083	0.97	-0.027	0.011	6.4
	Citalopram	Diazepam-d5	0.00647	0.00050	0.94	-0.115	0.070	8.9
	Clomipramine	Diazepam-d5	0.00275	0.00015	0.989	-0.043	0.018	5.4
	Fluoxetine	Atrazine-d5	0.00614	0.00054	0.93	-0.104	0.075	10.0
	Fosinopril	Diazepam-d5	0.00165	0.00012	0.95	-0.018	0.016	8.2
	Quetiapine	Diazepam-d5	0.0275	0.0015	0.97	-0.45	0.20	6.1
	Timolol	Amphetamine-D6	0.0212	0.0014	0.96	-0.14	0.20	7.6
	Mepivacaine	Cetirizine-D8	0.0314	0.0017	0.97	-0.46	0.23	6.2
	Meptazinol	Cetirizine-D8	0.0217	0.0013	0.97	-0.31	0.18	6.7
Pharmaceuticals TPs	***4-Acetamido-Antipyrine***	Sulfadimidine-d4	0.01324	0.00089	0.96	-0.32	0.13	7.9
	O-Desmethyl-Tramadol	Sulfadimidine-d4	0.0203	0.0014	0.96	-0.27	0.19	7.7

	4-Formylamino-Antipyrine	Cetirizine-D8	0.00641	0.00049	0.95	-0.170	0.070	9.0
	N-bisdesmethyl-Tramadol	Amphetamine-D6	0.00292	0.00015	0.98	-0.020	0.020	5.6
Plant Protection Products	***DEET (Diethyltoluamide)***	Cetirizine-D8	0.01358	0.00081	0.97	-0.13	0.11	6.8
	Spiroxamine	Cetirizine-D8	0.00359	0.00030	0.95	0.102	0.045	10.2
	Bromoxynil	Bisphenol A-d16	0.00257	0.00011	0.985	-0.074	0.015	4.9
	Dinoterb	Cetirizine-D8	0.0363	0.0026	0.96	-1.02	0.37	8.5
	Methiocarb-sulfoxide	Cetirizine-D8	0.00182	0.00014	0.94	-0.024	0.019	8.8
Plant Protection Products TPs	***Desethyl-Atrazine***	Atrazine-d5	0.00299	0.00024	0.94	-0.021	0.033	9.2
	3-Hydroxy-Carbofuran	Cetirizine-D8	0.00285	0.00019	0.96	-0.036	0.026	7.4
	Ethiofencarb-sulfoxide	Cetirizine-D8	0.001306	0.000089	0.98	0.010	0.011	7.0
	Metamitron-desamino	Diuron-d6	0.001742	0.000085	0.98	-0.010	0.012	5.6
Stimulants TPs	***Cotinine***	Sulfadiazine-d4	0.0681	0.0046	0.96	-0.20	0.66	8.1
	Nor-Nicotine	Cetirizine-D8	0.00186	0.00013	0.95	-0.013	0.018	7.9

Table 11: Validation results in livers - Repeatability, Reproducibility, Recoveries, Factors of Matrix Effect.

Compound	Instrumental %RSD	% Repeatability	% Reproducibility	% R (25 μg/L)	% R (70 μg/L)	% R (150 μg/L)	% FME (25 μg/L)	% FME (70 μg/L)	% FME (150 μg/L)
Methamphetamine	4.3	12	12	71	53	58	1.4	3.4	1.0
Cathine	10	12	13	51	74	82	2.6	2.2	0.9
3,4-Methylenedioxyamphetamine (MDA)	3.4	1.2	1.2	63	91	113	4.8	5.0	3.0
3,4-Methylenedioxymethamphetamine (MDMA)	2.1	6.8	6.3	81	123	111	1.1	1.9	1.4
Codeine	4.2	7.5	12	85	98	74	1.8	2.1	2.4
Lysergic acid diethylamide (LSD)	6.2	10	8.4	72	87	104	1.1	2.5	1.6
3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	5.3	21	21	119	95	82	1.0	2.2	1.4
Dihydro-Codeine	5	13	12	53	61	87	2.8	4.9	2.9
Nor-Fentanyl	5.9	7.9	8.2	84	92	86	0.6	0.8	0.6
D L-N O-Didesmethyl-Venlafaxine	6.4	10	9.0	59	61	72	0.9	1.5	1.0
2-Amino-Benzothiazole	3.4	12	13	123	84	122	0.7	2.8	0.9
2-OH-Benzothiazole	18	14	13	67	74	88	1.5	2.6	1.3
4-Me-Benzotriazole	7.8	10	10	57	51	64	1.5	5.3	1.7
2-4-Dinitrophenol (DNP)	6.1	14	14	97	75	84	0.4	2.4	0.8
Perfluorodecanoic acid (PFDeA)	3.6	7.7	7.5	82	75	96	0.5	3.1	0.9
Perfluorododecanoic acid (PFDoA)	7.0	12	14	65	84	103	1.0	4.4	1.3
Perfluorooctanoic acid (PFOA)	4.4	13	16	111	53	117	1.3	4.7	1.5
Perfluorooctanesulfonamide (PFOSA)	6.0	14	13	54	82	81	0.6	3.3	1.0
Perfluorotetradecanoic acid (PFTeDA)	5.4	8.4	8.2	71	76	69	1.0	2.4	0.6
Perfluoroundecanoic acid (PFUnA)	1.1	10	9.1	71	84	119	1.2	3.9	1.5
Perfluorobutanesulfonic acid (PFBuS)	2.1	9.2	8.5	84	63	105	1.1	3.8	1.4
Perfluoroheptanoic acid (PFHpA)	7.4	13	13	74	71	65	1.3	5.5	2.0
Perfluorohexanoic acid (PFHxA)	7.3	11	11	86	63	74	1.1	4.8	1.7
Perfluorononanoic acid (PFNA)	5.2	7.7	11	94	64	84	1.3	5.4	1.5
Perfluorooctanesulfonic acid (PFOS)	4.3	8.2	12	53	70	89	1.2	2.0	1.3
Perfluoropentanoic acid (PFPeA)	10	11	11	67	71	68	2.5	1.8	1.5
Perfluoroheptanesulfonic acid (PFHpS)	2.1	9.0	14	75	121	119	1.8	4.1	1.5

Compound	Instrumental %RSD	% Repeatability	% Reproducibility	% R (25 µg/L)	% R (70 µg/L)	% R (150 µg/L)	% FME (25 µg/L)	% FME (70 µg/L)	% FME (150 µg/L)
Perfluorohexanesulfonic acid (PFHxS)	2.4	12	14	73	84	61	2.2	5.1	1.9
Levamisol	7.3	6.0	13	70	71	70	3.0	18.0	4.6
Propranolol	5.9	11	11	64	84	94	1.0	4.3	1.2
Ketoprofen	9.4	13	11	69	72	74	1.1	3.0	0.9
Metformin	18	16	13	68	52	77	0.3	0.3	0.1
Oxprenolol	5.4	10	10	72	84	115	0.8	4.2	1.0
Tramadol	4.5	6.4	6.9	105	92	125	2.4	8.0	1.6
Trimethoprim	2.5	2.0	9.1	64	50	90	0.3	1.2	0.4
Flufenamic acid	7.0	6.5	6.3	65	71	56	1.0	10	0.9
Meloxicam	5.2	10	12	70	84	85	0.3	1.5	0.4
Citalopram	1.7	15	13	119	90	85	0.7	2.3	0.8
Clomipramine	2.1	12	15	77	94	71	0.6	3.2	1.0
Fluoxetine	7.0	14	12	102	65	74	0.9	3.9	1.1
Fosinopril	4.3	13	18	75	106	92	0.8	3.4	2.7
Quetiapine	2.7	10	8	92	86	71	0.5	1.2	0.9
Timolol	3.3	11	12	93	86	120	1.8	2.2	1.1
Mepivacaine	9.1	7	7.9	84	113	125	1.3	2.6	1.9
Meptazinol	6.4	6.1	6.1	93	114	123	1.2	2.4	1.8
4-Acetamido-Antipyrine	1.6	3.4	8.9	82	98	67	3.8	4.8	1.5
O-Desmethyl-Tramadol	10	18	21	82	78	100	0.6	2.9	0.8
4-Formylamino-Antipyrine	16	11	11	66	84	97	0.4	1.5	0.6
N-bisdesmethyl-Tramadol	3	9.5	11	81	72	76	1.9	2.3	1.0
DEET (Diethyltoluamide)	6.2	17	19	67	72	91	0.9	3.3	1.2
Spiroxamine	9.1	16	15	82	84	69	0.7	4.1	1.2
Bromoxynil	5.3	4.4	7.1	95	82	102	0.7	3.8	1.0
Dinoterb	7.4	20	17	64	72	63	2.6	8.2	3.9
Methiocarb-sulfoxide	4.8	18	20	61	75	68	0.9	1.1	0.9
Desethyl-Atrazine	9.1	11	13	74	67	81	2.5	1.8	1.6
3-Hydroxy-Carbofuran	13	18	16	76	81	68	1.7	2.4	3.2
Ethiofencarb-sulfoxide	3.4	13	15	65	67	63	0.0	1.1	0.9

Compound	Instrumental %RSD	% Repeatability	% Reproducibility	% R (25 μg/L)	% R (70 μg/L)	% R (150 μg/L)	% FME (25 μg/L)	% FME (70 μg/L)	% FME (150 μg/L)
Metamitron-desamino	5.0	8.3	7.3	101	84	128	0.6	1.0	0.8
Cotinine	11	8.3	13	100	68	87	1.5	3.6	1.4
Nor-Nicotine	12	13	11	72	84	65	2.3	1.5	0.6

Table 12:Validation results in muscles - Repeatability, Reproducibility, Recoveries, Factors of Matrix Effect.

Compound	Instrumental %RSD	% Repeatability	% Reproducibility	% R (25 μg/L)	% R (70 μg/L)	% R (150 μg/L)	% FME (25 μg/L)	% FME (70 μg/L)	% FME (150 μg/L)
Methamphetamine	1.2	1.2	57	64	57	63	1.7	2.7	0.8
Cathine	6.2	8.9	34	94	106	89	1.4	2.4	1.0
3,4-Methylenedioxyamphetamine (MDA)	4.0	3.9	26	65	106	65	2.8	3.3	3.7
3,4-Methylenedioxymethamphetamine (MDMA)	3.8	8.0	23	69	115	96	1.3	1.6	1.4
Codeine	3.2	7.9	24	75	81	72	2.3	4.1	1.7
Lysergic acid diethylamide (LSD)	2.1	9.1	24	54	64	53	1.4	2.1	1.7
3,4-Methylenedioxy-N-ethylamphetamine (MDEA)	5.4	13	19	102	96	89	1.1	1.7	1.5
Dihydro-Codeine	2.3	2.3	29	77	92	85	4.1	5.9	3.4
Nor-Fentanyl	5.0	8.9	41	65	110	115	0.9	0.9	0.7
D L-N O-Didesmethyl-Venlafaxine	7.0	9.4	17	54	85	84	1.2	1.5	1.1
2-Amino-Benzothiazole	4.7	8.6	24	87	92	103	1.0	1.2	0.8
2-OH-Benzothiazole	7.9	15	23	72	96	66	1.3	1.3	1.3
4-Me-Benzotriazole	2.3	8.3	24	74	62	94	3.7	4.2	2.2
2-4-Dinitrophenol (DNP)	3.3	14	27	61	84	119	0.7	1.3	1.0
Perfluorodecanoic acid (PFDeA)	5.0	9.8	14	74	89	113	1.6	1.8	1.9
Perfluorododecanoic acid (PFDoA)	5.2	5.7	25	73	95	82	1.4	1.6	1.7
Perfluorooctanoic acid (PFOA)	6.3	11	24	75	88	82	2.2	2.4	2.0
Perfluorooctanesulfonamide (PFOSA)	2.2	3.1	17	63	71	65	0.9	1.6	1.4
Perfluorotetradecanoic acid (PFTeDA)	4.6	12	32	59	68	55	0.9	0.8	0.5

Compound	Instrumental %RSD	% Repeatability	% Reproducibility	% R (25 μg/L)	% R (70 μg/L)	% R (150 μg/L)	% FME (25 μg/L)	% FME (70 μg/L)	% FME (150 μg/L)
Perfluoroundecanoic acid (PFUnA)	2.2	12	18	84	82	117	1.7	2.1	2.3
Perfluorobutanesulfonic acid (PFBuS)	1.6	11	21	83	65	123	1.4	1.7	1.9
Perfluoroheptanoic acid (PFHpA)	3.5	10	16	64	75	72	2.0	2.6	2.7
Perfluorohexanoic acid (PFHxA)	4.7	8.8	19	68	63	57	1.8	1.7	1.8
Perfluorononanoic acid (PFNA)	4.7	3.8	19	70	72	64	0.8	1.6	2.5
Perfluorooctanesulfonic acid (PFOS)	2.3	6.0	59	71	63	95	1.8	1.4	1.4
Perfluoropentanoic acid (PFPeA)	3.4	7.6	23	64	90	133	2.0	2.6	2.4
Perfluoroheptanesulfonic acid (PFHpS)	18	6.9	11	58	63	59	2.1	1.8	0.7
Perfluorohexanesulfonic acid (PFHxS)	18	6.6	12	55	71	64	2.4	0.8	4.2
Levamisol	7.0	7.4	26	54	69	56	4.2	3.2	5.6
Propranolol	4.9	9.5	37	129	92	86	1.1	1.5	1.2
Ketoprofen	1.6	12	19	64	67	62	1.3	0.9	1.0
Metformin	11	16	53	64	52	75	0.4	0.6	0.1
Oxprenolol	3.6	8.7	31	96	98	109	1.0	1.4	1.1
Tramadol	5.1	9.1	18	85	96	110	3.1	3.2	1.8
Trimethoprim	5.0	3.9	30	70	84	115	0.5	0.9	0.5
Flufenamic acid	8.1	9.0	23	70	64	75	1.1	1.6	1.3
Meloxicam	4.0	13	26	67	75	113	0.6	0.9	0.7
Citalopram	4.4	8.2	16	80	79	59	0.6	1.2	0.8
Clomipramine	3.1	7.5	22	82	74	64	0.7	1.3	1.0
Fluoxetine	9.0	11	16	65	79	110	0.9	0.7	1.1
Fosinopril	11	12	33	111	91	85	2.2	4.1	3.8
Quetiapine	7.6	7.7	24	98	87	72	0.6	1.1	0.9
Timolol	1.5	12	31	105	89	94	1.2	2.7	1.2
Mepivacaine	2.4	12	25	69	98	96	1.7	2.4	1.7
Meptazinol	7.0	15	27	71	96	87	1.5	2.2	1.7
4-Acetamido-Antipyrine	6.5	11	32	82	94	73	3.9	1.4	1.3
O-Desmethyl-Tramadol	6.9	16	31	119	102	98	0.9	1.6	1.0

Compound	Instrumental %RSD	% Repeatability	% Reproducibility	% R (25 μg/L)	% R (70 μg/L)	% R (150 μg/L)	% FME (25 μg/L)	% FME (70 μg/L)	% FME (150 μg/L)
4-Formylamino-Antipyrine	4.9	5.9	26	74	132	123	0.8	1.7	0.7
N-bisdesmethyl-Tramadol	2.5	2.2	2	92	72	81	1.8	3.5	1.4
DEET (Diethyltoluamide)	5.7	9.1	27	125	90	97	1.1	2.5	1.2
Spiroxamine	8.2	8.5	41	67	71	64	1.7	1.3	1.9
Bromoxynil	1.0	3.1	19	61	81	125	0.8	2.1	1.0
Dinoterb	11	11	18	70	67	81	5.3	0.6	4.7
Methiocarb-sulfoxide	12	10	12	68	50	70	0.2	0.6	1.0
Desethyl-Atrazine	9.4	8.6	37	71	67	112	1.1	1.3	1.2
3-Hydroxy-Carbofuran	15	11	30	65	73	68	1.8	1.4	1.6
Ethiofencarb-sulfoxide	3.7	12.0	28	68	49	57	2.1	1.6	1.0
Metamitron-desamino	4.0	7.1	22	90	61	115	0.6	0.9	0.8
Cotinine	4.1	9.9	32	81	74	130	1.9	1.4	1.5
Nor-Nicotine	10	14	32	91	119	62	7.8	2.6	0.8

Recovery experiments were performed at 3 different levels (instrumental concentrations: 25, 70 and 150 μg/L). The majority of the analytes had satisfactory recoveries between 60-120%. Method repeatability in terms of %RSD in 6 spiked samples at 150 ng/L was below 25% for all analytes and the % Reproducibility was between 20 and 50% in the majority of analytes.

The validation results for %Repeatability, %Reproducibility, %Recoveries, %Factors of Matrix Effect are presented in the tables 11 and 12 for livers and muscles respectively.

5.3 Target Screening Results

As mentioned in the chapter 4, 20 samples of top consumers (predators) and their prey (5 Buzzards, 5 Otters, 5 Harbour Seals And 5 Roaches) samples were gathered consecutively from different locations across the United Kingdom from the Center of Ecology and Hydrology (CEH). 165 polar and semi-polar Emerging Contaminants were detected in the samples. The biggest percentage of the detected ECs were pharmaceuticals (21%), followed by plant protection products (16%), surfactants (14%) and per- & polyfluoroalkyl substances (PFAS) (10%). The classes of the detected emerging contaminants are presented in the figure 30, as well as in the table 13.

Table 13: Classes of detected ECs.

Classes of ECs	***# detected ECs***	***% detected ECs***
Antidepressants	4	2
Illicit Drugs	11	7
Illicit Drugs TPs	11	7
Industrial Chemicals	10	6
Per- and Polyfluoroalkyl Substances (PFAS)	17	10
Pharmaceuticals	35	21
Pharmaceuticals TPs	8	5
Plant Protection Products	27	16
Plant Protection Products TPs	4	2
Stimulants	5	3
Stimulants TPs	5	3
Surfactants	23	14
Sweeteners	5	3
Total	***165***	***100***

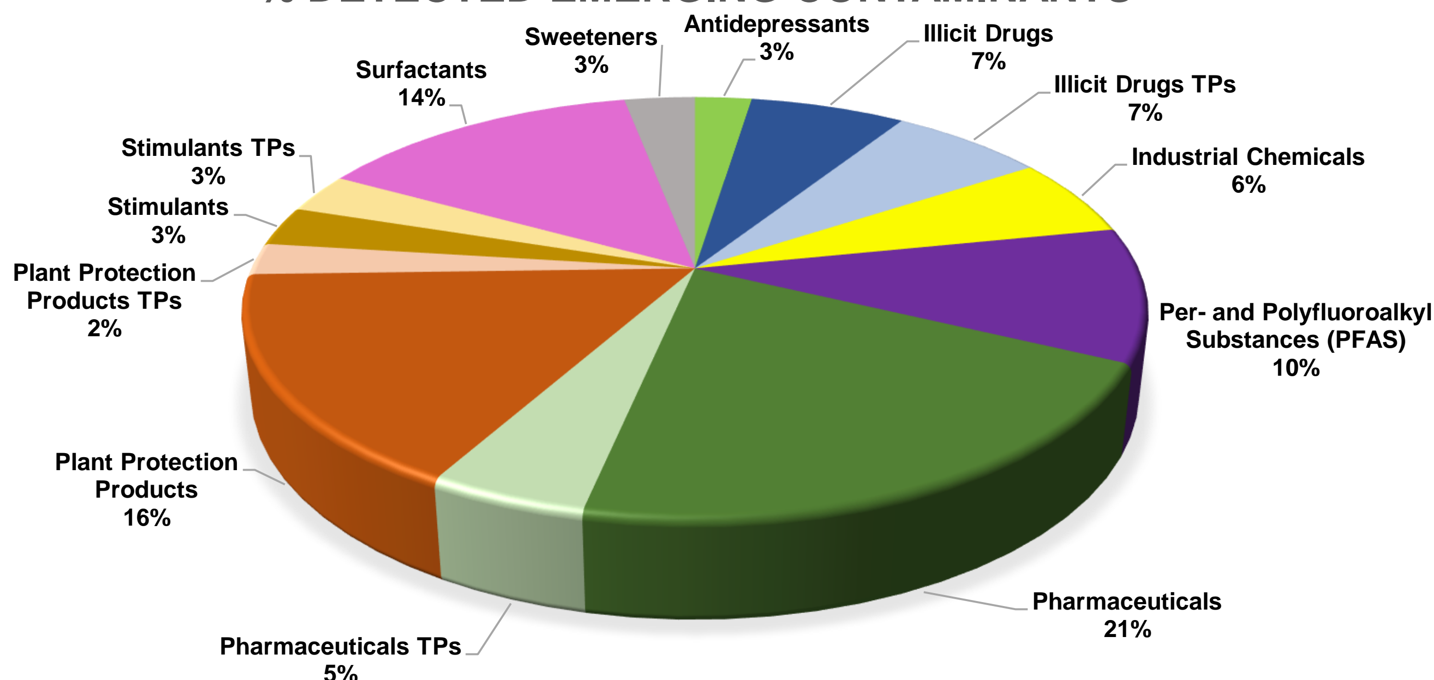


Figure 30: % detected ECs of different classes.

The detected emerging contaminants, as well as the number of the samples (in all different matrices), in which they were detected is presented in the table 14.

Table 14: Detected Emerging Contaminants in top predators' and their preys' samples which were gathered across the United Kingdom.

Compounds	*Classification*	*Roach*	*Buzzard*	*Otter*	*Harbour Porpoise*
Total number of samples		**5**	**5**	**5**	**5**
Fluoxetine	Antidepressants	1	0	0	0
Sertraline		4	0	0	0
Venlafaxine		2	2	3	0
Citalopram		3	0	2	1
2 C-D	Illicit Drugs	0	0	0	3
2C-T-4		2	0	0	0
Amphetamine		2	2	2	1
Methamphetamine		0	2	2	1
Benzylpiperazine		0	0	0	5
Cathine		2	0	0	5
Cathinone		0	2	3	0
Codeine		1	0	0	0

Compounds		Roach	Buzzard	Otter	Harbour Porpoise
Desomorphine		2	0	0	0
Ethylphenidate		0	4	1	0
Methedrone		0	5	3	2
N-Propyl-Amphetamine	Illicit Drugs TPs	0	0	0	1
P-Hydroxy-Methyl-Amphetamine		1	0	0	0
Dehydrometh-Amphetamine (DHMEPH)		0	2	0	0
Dihydro-Codeine		1	0	0	0
3,4-Dimethoxyphenethylamine (DMPEA)		0	0	0	2
3-Methylnor-Fentanyl		3	1	2	5
Nor-Fentanyl		0	0	0	3
MeOT (5-)		0	4	4	0
Methcathinone		0	0	0	2
D L-N O-Didesmethyl-Venlafaxine		2	0	0	0
O-Desmethyl-Venlafaxine		2	0	0	0
Benzenesulfonamide	Industrial Chemicals	1	0	0	1
2-4-Dinitrophenol (DNP)		1	0	0	0
Nonylphenol (4-NP)		4	0	0	0
2-Amino-Benzothiazole		5	0	0	0
2-Hydroxy-Benzothiazole		1	0	0	0
Benzotriazole (BTR)		4	0	0	0
4-Methyl-Benzotriazole		5	0	0	0
Diethyl-Phthalate		2	0	0	0
Dimethyl-Phthalate		3	0	1	1
Di-n-Butyl-Phthalate		0	0	0	1
Perfluorobutanesulfonic acid (PFBuS)	Per- and Polyfluoroalkyl Substances (PFAS)	1	0	3	0
Perfluorodecanoic acid (PFDeA)		5	1	5	5
Perfluorodecanesulfonic acid (PFDeS)		0	0	0	1
Perfluorododecanoic acid (PFDoA)		4	0	4	2
Perfluoroheptanoic acid (PFHpA)		0	0	1	0
Perfluoroheptanesulfonic acid (PFHpS)		0	1	5	3
Perfluorohexanoic acid (PFHxA)		4	2	2	2
Perfluorohexanesulfonic acid (PFHxS)		1	4	5	4
Perfluorononanoic acid (PFNA)		3	1	5	4
Perfluorooctanoic acid (PFOA)		2	0	5	0
Perfluorooctanesulfonic acid (PFOS)		5	5	5	5
Perfluorooctanesulfonamide (PFOSA)		4	0	3	5
Perfluorotridecanoic acid (PFTrDA)		0	0	0	1
Perfluoroundecanoic acid (PFUnA)		3	0	5	5
Perfluorohexane sulfonic acid (PFHxS) monosubstituted isomer		0	0	1	0
Perfluorooctane sulfonic acid (PFOS) monosubstituted isomers		4	5	5	5
Perfluoropentane sulfonic acid (PFPeS)		0	0	2	0
Galaxolide	Pharmaceuticals	1	5	4	5
Galaxolidone		3	5	4	5
Octocrylene		0	0	0	1

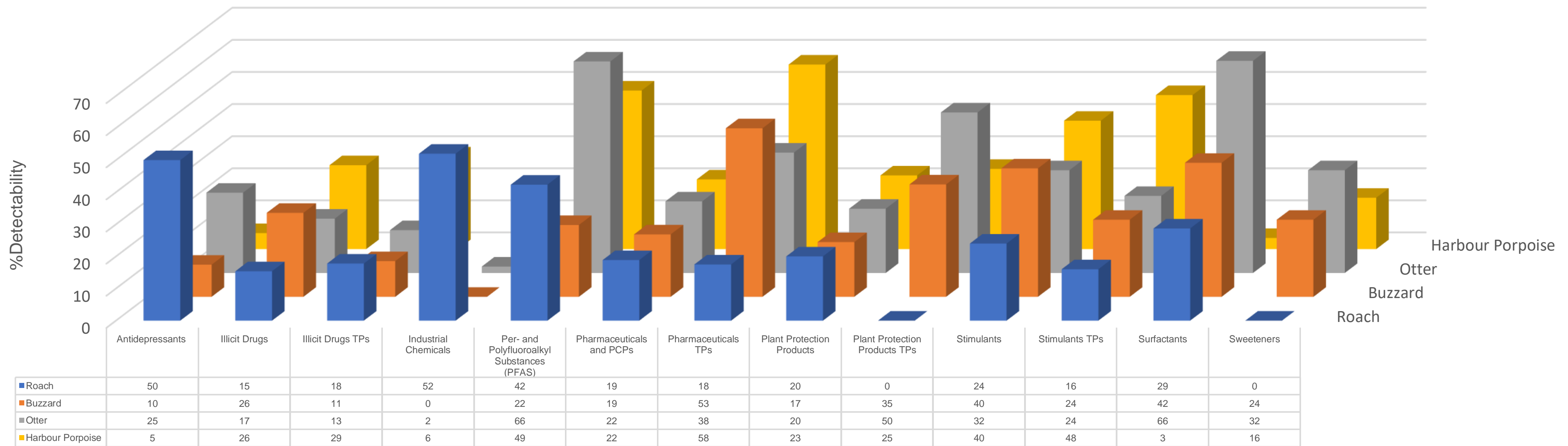
Compounds		***Roach***	***Buzzard***	***Otter***	***Harbour Porpoise***
Brinzolamide		1	0	2	2
Ibuprofen		1	0	0	5
Ketoprofen		1	0	1	0
Salicylic acid		0	1	5	0
Acebutolol		1	0	0	0
Alprenolol		0	0	1	0
Benperidol		2	0	0	0
Captopril		1	0	0	0
Carazolol		0	0	1	0
Cimetidine		0	0	2	1
Colchicine		1	0	0	0
Crotamiton		1	1	1	1
Cytarabin		2	0	0	0
Deprenyl		0	4	0	1
Levamisol		1	0	0	0
Levetiracetam		0	0	0	1
Lidocaine		3	0	0	1
Meptazinol		0	5	4	1
Metformin		2	0	0	1
Nalidixic acid		0	2	1	0
Nigericin		2	3	3	2
Phendimetrazine		0	0	0	2
Phentermine		0	0	0	2
Proguanil		0	0	0	1
Quetiapine		1	0	0	0
Salicylamide		0	0	0	1
Terbinafine		2	0	0	0
Timolol		2	0	0	0
Tolycaine		0	3	5	1
Tramadol		3	0	0	0
Trimethoprim		1	0	0	0
Vigabatrin		1	5	5	4
4-Acetamido-Antipyrine	Pharmaceuticals TPs	3	5	5	5
4-Formylamino-Antipyrine		0	2	1	0
Nor-Lidocaine		3	4	4	4
N-oxide-Lidocaine		0	0	0	1
N-bisdesmethyl-Tramadol		0	1	0	3
Nor-Tramadol		1	0	1	2
O-Desmethyldinor-Tramadol		0	4	1	4
O-Desmethylnor-Tramadol		0	5	3	4
Bromoxynil	Plant Protection Products	0	1	0	0
Dinoterb		5	0	0	1
Imazamox		0	0	5	0
Imazapyr		1	3	5	4
Atrazine		3	2	2	4

Compounds		***Roach***	***Buzzard***	***Otter***	***Harbour Porpoise***
Butachlor		1	0	0	0
Carboxin		3	5	5	4
Chlordimeform		1	0	0	0
Coumaphos		0	0	1	0
Cyprodinil		2	0	0	1
Diethyltoluamide (DEET)		1	0	0	0
Dichlorobenzamide		0	1	0	0
Dikegulac		0	1	1	0
Ethoxyquin		2	0	0	0
Etofenprox		0	1	0	0
Flutolanil		1	0	0	0
Isoprocarb		0	0	0	5
Mepronil		0	1	2	0
Metamitron		0	0	0	1
Methoprene		2	4	4	0
Monolinuron		1	0	0	0
Myclobutanil		0	0	0	1
Promecarb		1	0	0	0
Propoxur		0	4	2	0
Pymetrozine		2	0	0	4
Spiroxamine		1	0	0	5
Trimethacarb (2.3.5-)		0	0	0	1
Desisopropyl-Atrazine	Plant Protection Products TPs	0	0	1	0
Metolachlor-ESA		0	0	1	0
Propachlor-OXA		0	5	5	4
Amfepramone	Stimulants	0	5	3	4
Caffeine		5	0	1	0
Ephedrine		0	0	0	1
Harman		0	1	1	0
Nicotine		1	4	3	5
Cotinine	Stimulants TPs	0	1	3	3
Hydroxy-Cotinine		0	0	0	1
Nor-Ephedrine		3	0	0	3
Nor-Nicotine		1	5	3	4
Theophylline		0	0	0	1
AES-C12, n=0	Surfactants	2	5	5	1
AES-C12, n=1		3	5	5	1
AES-C12, n=2		4	4	5	0
AES-C12, n=3		4	4	4	0
AES-C12, n=4		4	4	4	0
AES-C12, n=5		3	4	4	0
AES-C12, n=6		3	4	4	0
AES-C12, n=7		3	3	4	0
AES-C12, n=8		3	2	4	0
AES-C12, n=9		2	2	4	0

Compounds		Roach	Buzzard	Otter	Harbour Porpoise
AES-C14, n=0		1	2	4	1
AES-C14, n=1		0	0	4	0
AES-C14, n=2		0	1	3	0
AES-C14, n=3		0	0	3	0
AES-C14, n=4		0	0	3	0
AES-C14, n=5		0	0	2	0
AES-C14, n=6		0	0	3	0
AES-C14, n=7		0	0	3	0
AES-C14, n=8		1	0	3	0
AES-C14, n=9		0	0	3	0
C11-LAS		0	3	1	0
C12-LAS		0	3	0	1
C13-LAS		0	2	1	0
Acesulfame	Sweeteners	0	0	1	1
Cyclamic acid		0	1	1	0
Saccharine		0	0	2	0
Aspartame		0	4	4	3
Cyclamic acid		0	1	0	0

In the figure 31 the %detectability of every class of compounds in every matrix is presented. In the most categories, the %detectability in top predators is higher in contrast with their prey. Especially, the (bio)transformation products were detected with higher detection frequency in the top consumers.

In the figures 32 and 33 (Box-Plot), the number of detected emerging contaminants in the different matrices is presented, as well as their normalized concentration. The highest abundance of emerging contaminants was found in the otter samples. In general, although the variation of the number of the detected ECs in top predators and in their prey doesn't indicate important differentiation, the total normalized concentration of detected emerging contaminants was higher in the top predators' samples in contrast with their prey samples.



	Antidepressants	Illicit Drugs	Illicit Drugs TPs	Industrial Chemicals	Per- and Polyfluoroalkyl Substances (PFAS)	Pharmaceuticals and PCPs	Pharmaceuticals TPs	Plant Protection Products	Plant Protection Products TPs	Stimulants	Stimulants TPs	Surfactants	Sweeteners
Roach	50	15	18	52	42	19	18	20	0	24	16	29	0
Buzzard	10	26	11	0	22	19	53	17	35	40	24	42	24
Otter	25	17	13	2	66	22	38	20	50	32	24	66	32
Harbour Porpoise	5	26	29	6	49	22	58	23	25	40	48	3	16

Figure 31: %Detectability in UK's samples.

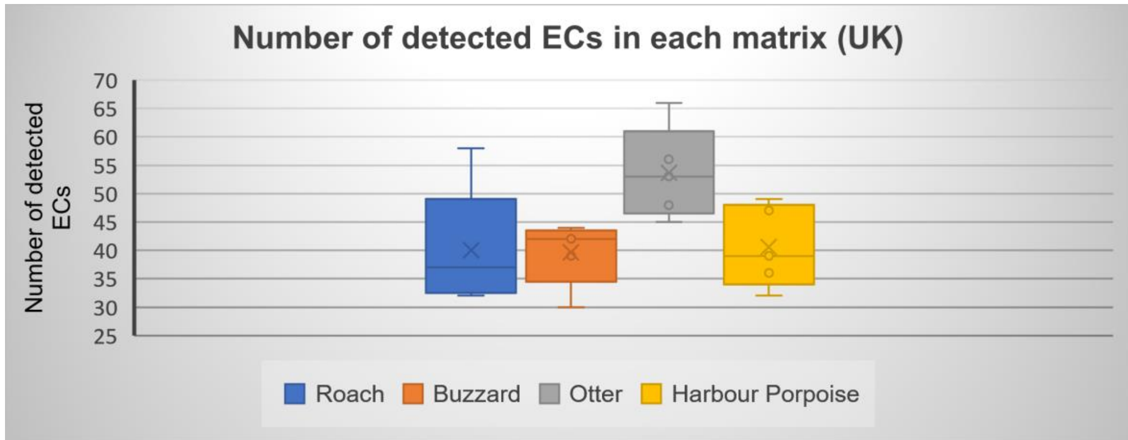


Figure 32: Number of detected ECs in each matrix.

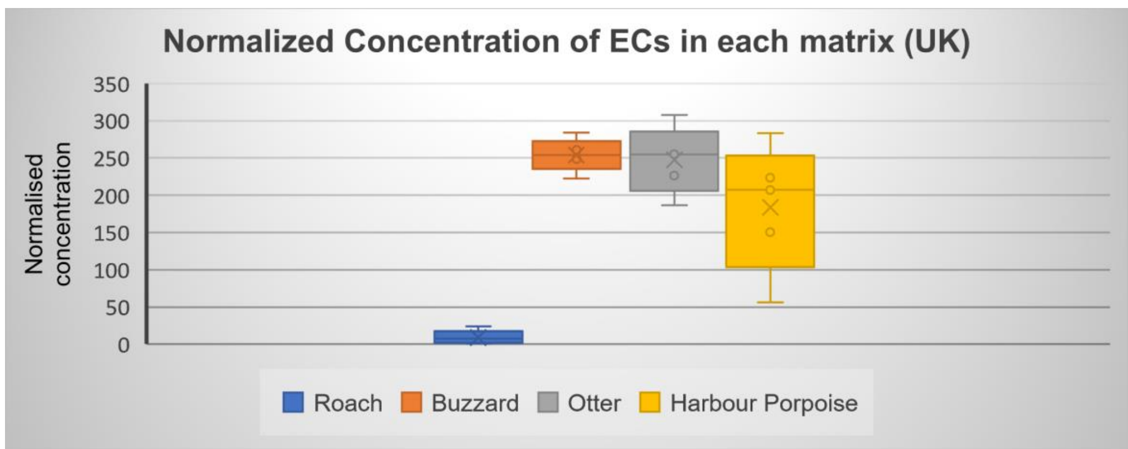


Figure 33: Normalized Concentrations of ECs in each matrix.

In the figure 34 the concentrations of atrazine and it's (bio)transformation product desisopropyl-Atrazine are presented. The concentrations in top predators are significantly higher in contrast with the concentration in their prey. The frequency of appearance is 60% in the roach muscles and 53% in the predator's livers.

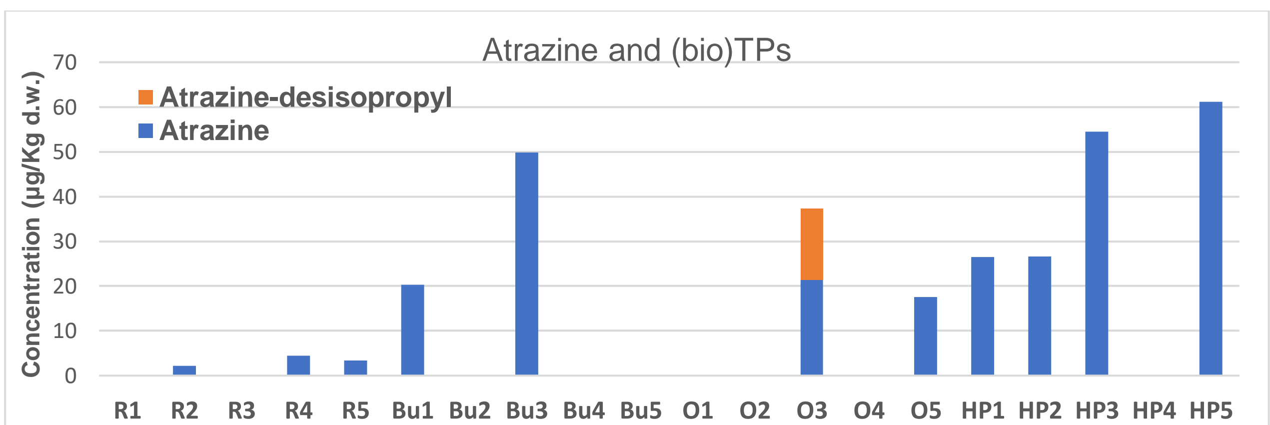


Figure 34: Atrazine and it's (bio)TPs

Nicotine and it’s (bio)transformation products, Nor-Nicotine, Cotinine and Hydroxy-Cotinine, were detected in extremely higher concentrations in the top predators’ samples in contrast with their prey. The Nor-Nicotine was the (bio)transformation product with the higher detection frequency and the highest concentrations in the samples.

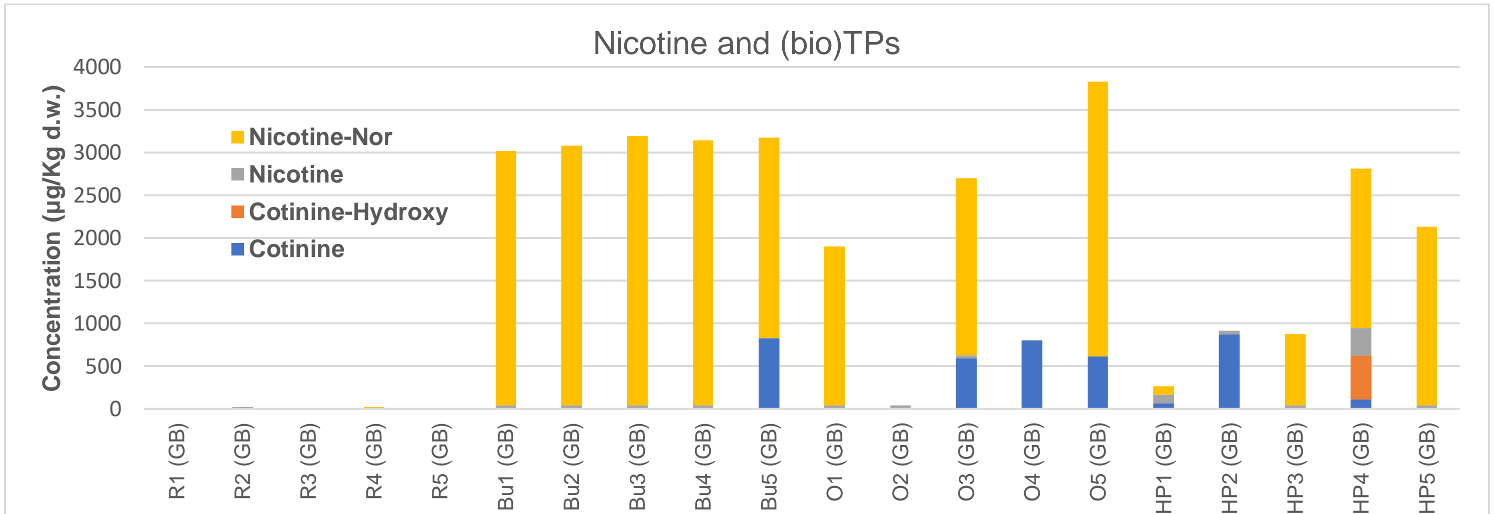


Figure 35: Concentration of Nicotine and its' (bio)TPs.

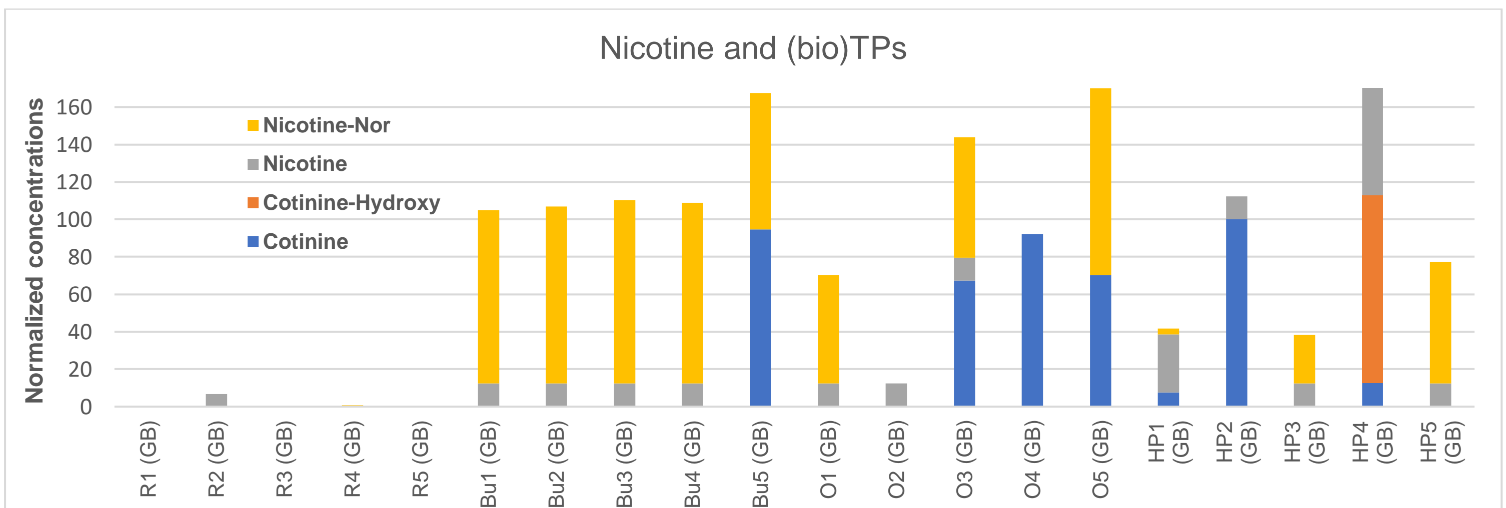


Figure 36: Normalized Concentrations of Nicotine and its' (bio)TPs.

Tramadol and it’s (bio)transformation products were detected mainly in top predators’ livers. In the figure 39 an extracted ion chromatogram is presented, which indicates the presence of numerous pharmaceuticals (Tramadol, Citalopram) and pharmaceuticals (bio)transformation products (Nor-Tramadol, 4-Acetamido-Antipyrine), as well as antidepressant drugs (Quetiapine, Sertraline) in roach samples which were gathered in the River Lee.

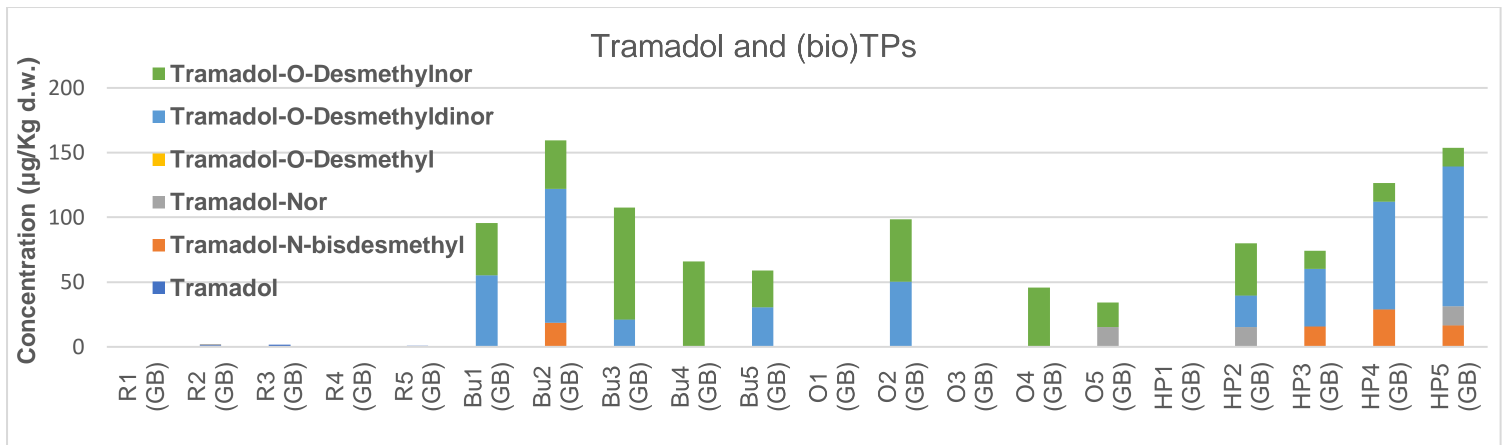


Figure 37: Concentration of Tramadol and its' (bio)TPs.

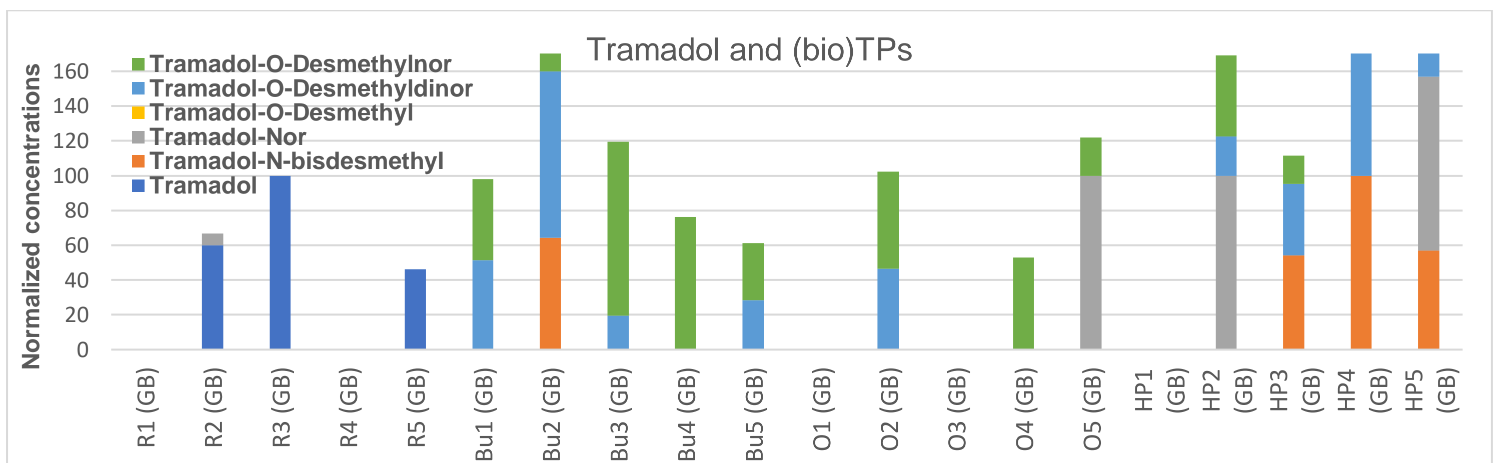


Figure 38: Normalized Concentrations of Tramadol and its' (bio)TPs.

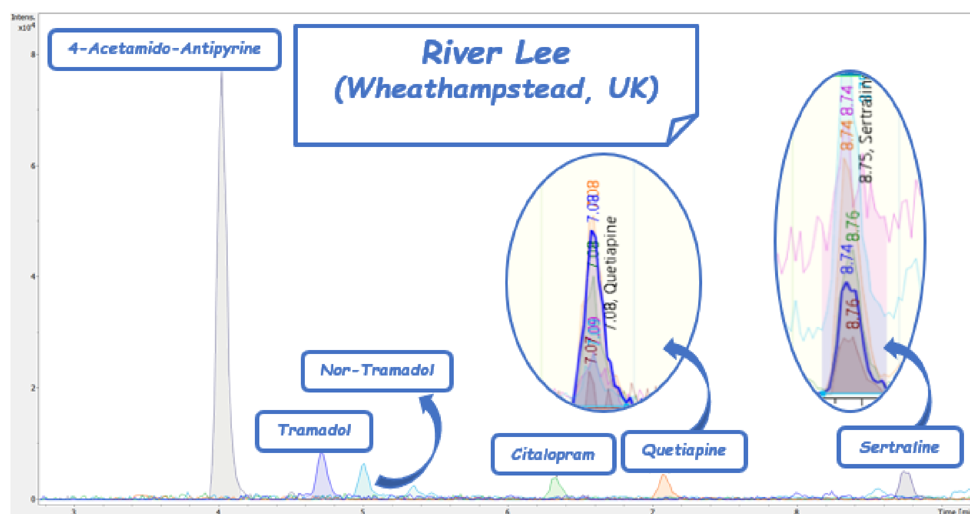


Figure 39: Existance of several pharmaceuticals, (bio)TPs and antidepressant drugs in Roach samples which were gathered in river Lee.

17 PFAS were detected in all the samples from United Kingdom. In the predators' samples the concentration of total PFAS was extremely higher in contrast with their prey. The most abundant PFAS was the Perfluorooctanesulfonic acid (PFOS) and it was detected in high concentrations in otter samples. In an otter sample which was gathered in Hertfordshire were detected 15 PFAS and in the highest concentration among the samples.

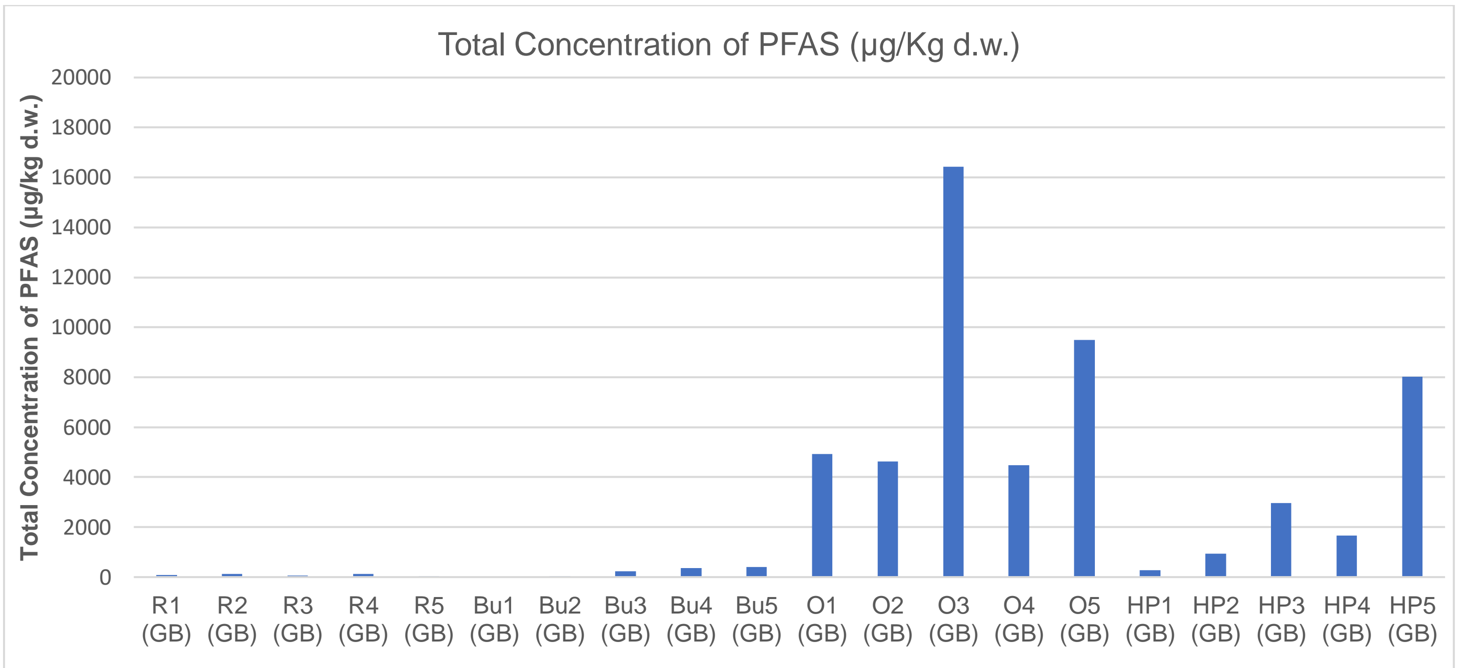


Figure 40: Total Concentration of PFAS.

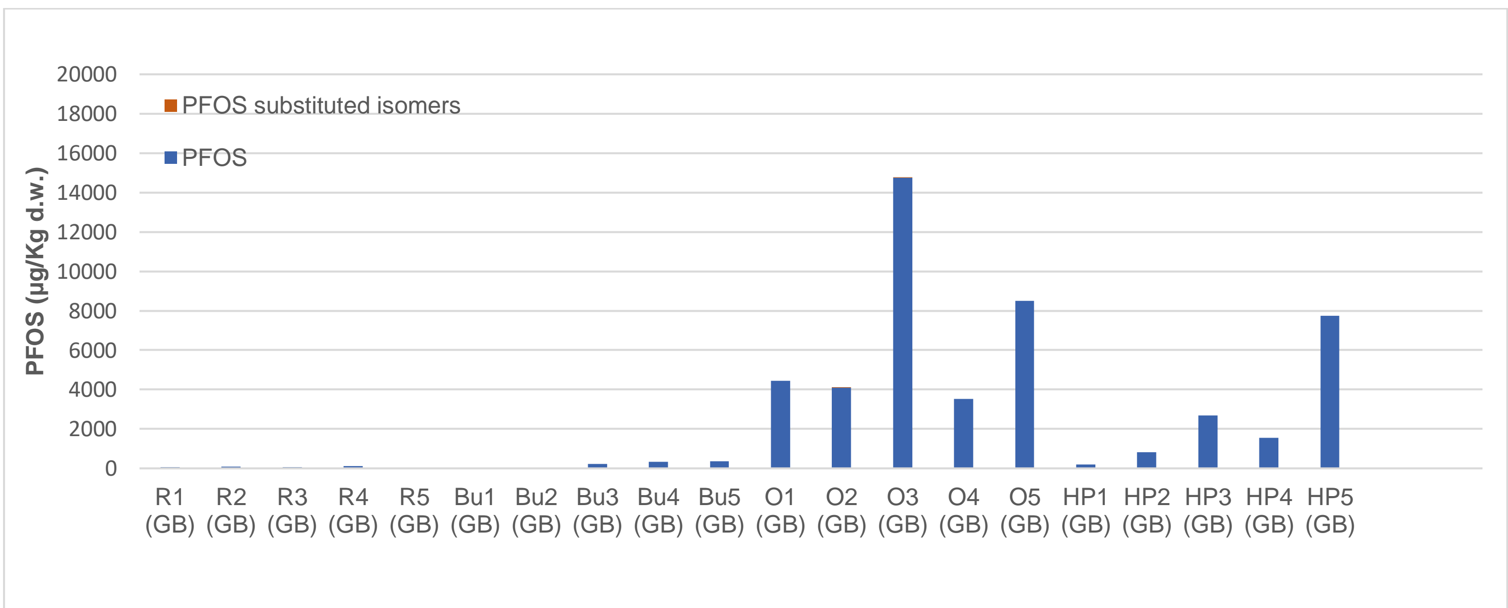


Figure 41: Concentration of PFOS.

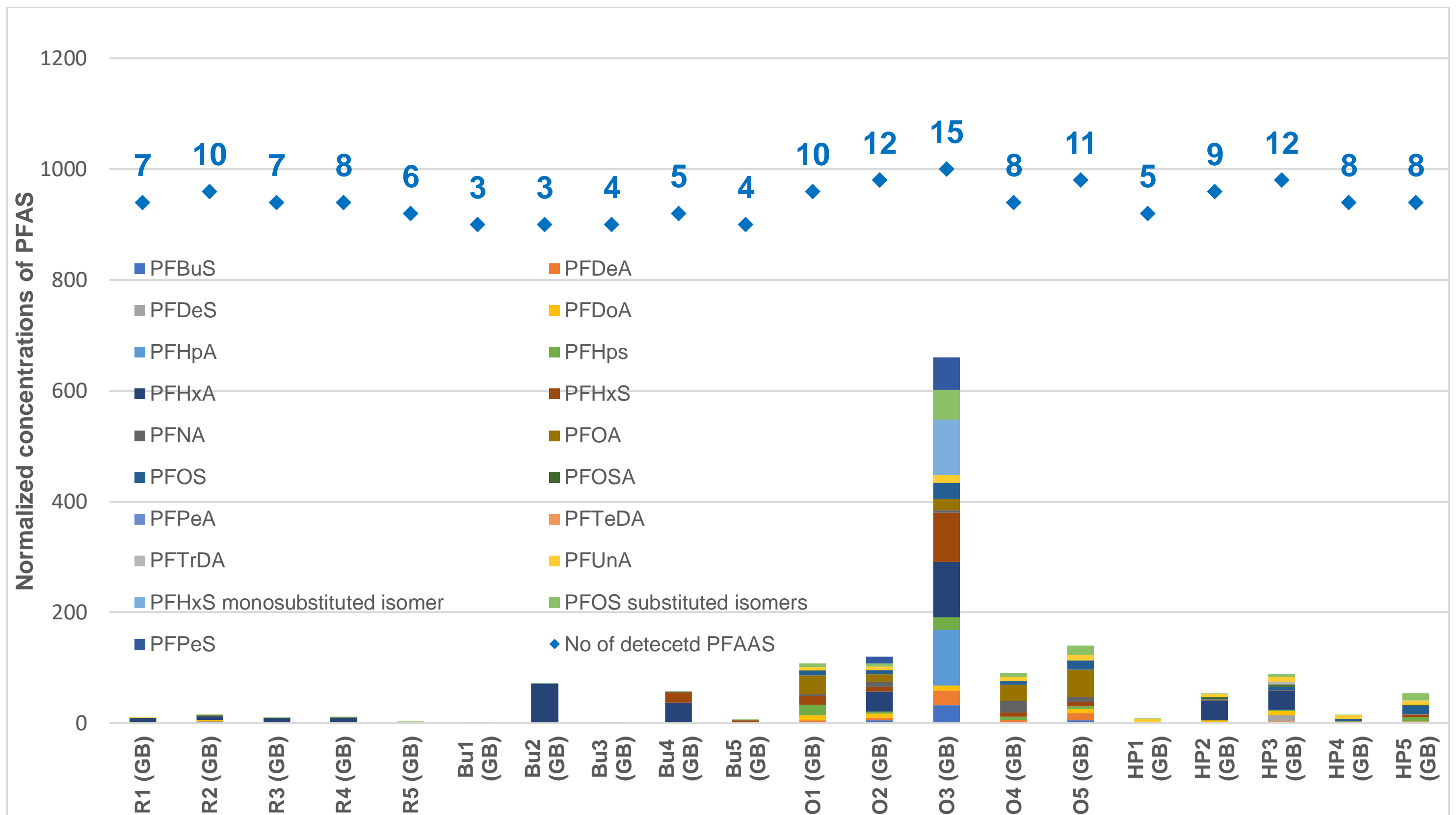


Figure 42: Normalized Concentration of PFAS.

5.3 Discussion

165 polar and semi polar organic emerging contaminants were detected in top predators and their prey samples across the United Kingdom. The detection of some emerging contaminants (e.g. Nicotine and it’s (bio)TPs, Quetiapine, Sertraline) reflexes the consumption levels of these compounds in the United Kingdom. In the top predators’ samples were detected numerous organic emerging contaminants and in extremely higher concentrations in contrast with their prey samples, something which indicates possible bioaccumulation through the food chain.

CHAPTER 6
CONCLUSIONS

Emerging contaminants and priority pollutants end up to the aquatic ecosystem through various ways and, then, they enter to the food webs, where they are bioaccumulated through the food chain. Top predators (consumers), due to their unique biology, habits and physiology, are highly sensitive indicators of local environmental quality, including the presence of emerging contaminants and priority pollutants. For this reason, the analysis of the top consumers (predators) is crucial.

The development of a novel, generic methodology for the detection of polar and semi-polar organic emerging contaminants and priority pollutants in biota muscles and livers was the first step in our study. The application of a generic sample treatment using ASE for the extraction of analytes and SPE with four different extraction sorbents, along with the data dependent and data independent acquisition mode by LC-HRMS allowed the wide-scope target screening for the detection of polar and semi-polar emerging contaminants in livers from top predators and muscles from their prey.

The validation of the developed protocol and the evaluation of the linearity, accuracy, precision, matrix effect and the detectability, using a representative validation dataset of 60 compounds with different physicochemical properties from a wide variety of classes of emerging contaminants was following step in our study.

Wide-scope target screening using a database with more than 2,100 emerging contaminants, priority pollutants, as well as their (bio)transformation products, was applied based on some performance criteria; mass accuracy, retention time, isotopic pattern and MS/MS information, were attributed in order to facilitate confidence.

The results of our research assured that dozens of polar and semi-polar organic emerging contaminants from wide variety of classes are existed in the top consumers (predators) and their prey. The frequency of appearance and the

concentrations of the detected emerging contaminants in livers of top consumers (predators) were higher in contrast with the detected compounds in muscles of their prey. These results indicate that the top consumers are ideal indicators of local environmental quality and they should be used as samples in the environmental monitoring studies.

To conclude, such a study can indicate the presence of polar and semi-polar organic emerging contaminants in the biota matrices (livers and muscles). The same extracts can be used for the wide-scope target screening, as well as in the suspect, non-target screening and retrospective analysis, due to the generic protocol, which was used in the sample preparation. The results are mainly qualitative and give information about the current state of the environmental pollution in the United Kingdom, with the presence of emerging contaminants in different locations across the country.

As LIFE APEX is an ongoing project there are existed, also, future perspectives. The quantification of all analytes in all samples will take place using standard addition curves, in order to identify possible bioaccumulation through the food chain. Furthermore, with statistical processing of the data there will be existed evidence in regards with the predator which is the most appropriate to be used in environmental monitoring studies. In the tier 2 of LIFE APEX, samples of top predators and their prey, which were gathered from a specific location from 1996-2018 and are stored in ESBs and NHMs, will be analyzed for the determination of emerging contaminants, in order to detect the variation of the top emerging contaminants through the years (time-trend analysis). Finally, during the 3rd tier of LIFE APEX, samples of top predators and their prey, which were gathered in different countries of Europe, will be analyzed for the determination of emerging contaminants in order to identify the possible dispersion of the top emerging contaminants across the Europe.

ABBREVIATIONS – ACRONYMS

Persistent Organic Pollutants	**POPs**
Polycyclic Aromatic Hydrocarbons	**PAHs**
Polychlorinated Biphenyls	**PCBs**
Emerging Contaminants	**ECs**
Emerging Pollutants	**EPs**
Persistent, Bioaccumulative, Toxic	**PBT**
Endocrine Disrupting	**ED**
Carcinogenic, Mutagenic, Toxic for Reproduction	**CMR**
Personal-Care Products	**PCPs**
Per- and Polyfluoroalkyl Substances	**PFAS**
(Bio)Transformation Products	**(bio)TPs**
Amphetamine Type Substances	**ATSs**
Sewage Treatment Plants	**STPs**
Linear Alkylbenzene Sulfonates	**LAS**
Nonylphenol Ethoxylates	**NPEOs**
Alcohol Ethoxy Sulphates	**AES**
Alkyl Ethoxylates	**AEOs**
Perfluoroalkyl Acids	**PFAAs**
Perfluorooctane Sulfonate	**PFOS**
Perfluorooctanoic Acid	**PFOA**
Perfluorohexane Sulfonate	**PFHxS**
Wastewater Treatment Plants	**WWTPs**
Apex Predators and their Prey	**AP&P**
Environmental Specimen Banks	**ESBs**
Natural History Museums	**NHMs**
Priority Pollutants	**PPs**
Liquid-Solid Extraction	**LSE**
Microwave-Assisted Extraction	**MAE**
Ultrasound Assisted Extraction	**UAE**
Matrix Solid-Phase Dispersion	**MSPD**
Liquid-Phase Micro Extraction	**LPME**
Supercritical Fluid Extraction	**SFE**
Pressurized Liquid Extraction	**PLE**
Accelerated Solvent Extraction	**ASE**
Solid Phase Extraction	**SPE**
Liquid Chromatography coupled to Mass Spectrometry	**LC-MS**
Electrospray Ionization	**ESI**
Reversed Phase Ultra High Performance Liquid Chromatography	**RP-UHPLC**

Reversed-Phase	**RP**
High-Resolution Mass Spectrometry	**HRMS**
Triple Quadrupole	**QqQ**
Atmospheric Pressure Chemical Ionization	**APCI**
Atmospheric Pressure Photoionization	**APPI**
Time-of-Flight	**TOF**
Full Width at Half Maximum	**FWHM**
Quadrupole-Time-of-Flight	**QTOF**
Data Dependent Acquisition	**DDA**
Data Independent Acquisition	**DIA**
Low Energy	**LE**
High Energy	**HE**
Single Reaction Monitoring	**SRM**
Identification Points	**IPs**
Gel Permeation Chromatography	**GPC**
Low Resolution Mass Spectrometry	**LRMS**
Quadrupole-Ion Trap	**QIT**
Benzotriazole	**BTR**
2-4-Dinitrophenol	**DNP**
Nonylphenol	**4-NP**
Perfluoro Butane Sulfonic Acid	**PFBuS**
Perfluoro Decanoic Acid	**PFDeA**
Perfluoro Dodecanoic Acid	**PFDoA**
Perfluoro Heptanoic Acid	**PFHpA**
Perfluoro Hexanoic Acid	**PFHxA**
Perfluoro Nonanoic Acid	**PFNA**
Perfluoro Undecanoic Acid	**PFUnA**
internal standards	**IS**
Center of Ecology and Hydrology	**CEH**
broad-band Collision Induced Dissociation	**bbCID**
instrumental limits of detection	**ILOD**
instrumental limits of quantification	**ILOQ**
Extracted Ion Chromatogram	**EIC**

REFERENCES

[1] N.S. Thomaids, A.G. Asimakopoulos, A.A. Bletsou, Emerging contaminants : A tutorial mini- review, Global NEST Journal, vol. 14, 2012, pp. 72–79.

[2] N. Mastroianni, M.L. De Alda, D. Barceló, Emerging organic contaminants in aquatic environments : state-of-the-art and recent scientific contributions, Contributions to Science, vol. 6, 2010, pp. 193–197.

[3] M. Gavrilescu, K. Demnerová, J. Aamand, S. Agathos, F. Fava, Emerging pollutants in the environment: Present and future challenges in biomonitoring, ecological risks and bioremediation, New Biotechnology, vol. 32, 2015, pp. 147–156.

[4] Y. Picó, D. Barceló, Emerging contaminants in biota, Analytical and Bioanalytical Chemistry, vol. 404, 2012, pp. 2525–2526.

[5] A.H. M. Stuart, D. Lapworth, E. Crane, Science of the Total Environment Review of risk from potential emerging contaminants in UK groundwater, Science of the Total Environment, vol. 416, 2012, pp. 1–21.

[6] M. Farre, S. Perez, L. Kantiani, D. Barcelo, Fate and toxicity of emerging pollutants , their metabolites and transformation products in the aquatic environment, Trends in Analytical Chemistry, vol. 27, 2008, pp. 991–1007.

[7] M. Farré, L. Kantiani, M. Petrovic, S. Pérez, D. Barceló, Achievements and future trends in the analysis of emerging organic contaminants in environmental samples by mass spectrometry and bioanalytical techniques, Journal of Chromatography A, vol. 1259, 2012, pp. 86–99.

[8] J. Rivera-Utrilla, M. Sánchez-Polo, M.A. Ferro-García, G. Prados-Joya, R. Ocampo-Perez, Chemosphere Pharmaceuticals as emerging contaminants and their removal from water . A review, Chemosphere, vol. 93, 2013, pp. 1268–1287.

[9] P. Rzymski, A. Drewek, P. Klimaszyk, Pharmaceutical pollution of aquatic environment: an emerging and enormous challenge, vol. 17, 2020, pp. 97–107.

[10] S. Bruna, F. Costa, I. Neves, T. Tavares, Psychiatric Pharmaceuticals as Emerging Contaminants in Wastewater, Springer International Publishing, 2015.

[11] T. Kosjek, E. Heath, M. Petrovic, D. Barceló, Mass spectrometry for identifying pharmaceutical biotransformation products in the environment, Trends in Analytical Chemistryrends in Analytical Chemistry, vol. 26, 2007, pp. 1076–1085.

[12] D. Barceló, Pharmaceutical-residue analysis, Trends in Analytical Chemistry, vol. 26, 2007, pp. 454–455.

[13] P. Chinnaiyan, S. Thampi, M. Kumar, K. M. Mini, Pharmaceutical products as emerging contaminant in water: relevance for developing nations and identification of critical compounds for Indian environment, Environmental Monitoring and Assessment, vol. 190, 2018, pp. 287–300.

[14] R. Pal, M. Megharaj, K.P. Kirkbride, R. Naidu, Science of the Total Environment Illicit drugs and the environment — A review, Science of the Total Environment, vol. 463, 2013, pp. 1079–1092.

[15] S. Castiglioni, E. Zuccato, Illicit Drugs as Emerging Contaminants, 2010, pp. 119–136.

[16] D. Montes-Grajales, M. Fennix-Agudelo, W. Miranda-Castro, Science of the Total Environment Occurrence of personal care products as emerging chemicals of concern in water resources : A review, Science of the Total Environment, vol. 595, 2017, pp. 601–614.

[17] A. J.Ebele, M.A. Abdallah, S. Harrad, Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment, Emerging Contaminants, vol. 3, 2017, pp. 1–16.

[18] G.F. Birch, D.S. Drage, K. Thompson, G. Eaglesham, J.F. Mueller, Emerging contaminants (pharmaceuticals , personal care products , a food additive and pesticides) in waters of Sydney estuary, Australia, Marine Pollution Bulletin, vol. 97, 2015, pp. 56–66.

[19] T. Ivanković, J. Hrenović, Surfactants in the environment, Archives of Industrial Hygiene and Toxicology, vol. 61, 2010, pp. 95–110.

[20] IUPAC, Compendium of Chemical Terminology - Gold Book (Version 2.3.3), 2014.

[21] J.D. F. Calvo-Flores, J. Isac-García, Surfactants in the Environment, Emerging Pollutants: Origin, Structure and Properties, 2018, pp. 341–384.

[22] B.O. Clarke, S.R. Smith, Review of ‘ emerging ’ organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids, Environment International, vol. 37, 2011, pp. 226–247.

[23] J.S. Boone, C. Vigo, T. Boone, C. Byrne, J. Ferrario, R. Benson, J. Donohue, J. Ellen, D.W. Kolpin, E.T. Furlong, S.T. Glassmeyer, Science of the Total Environment Per- and poly fl uoroalkyl substances in source and treated drinking waters of the United States, Science of the Total Environment, vol. 653, 2019, pp. 359–369.

[24] B.C. T. Coggan, T. Anumol, J. Pyke, J. Shimeta, A single analytical method for the determination of 53 legacy and emerging per- and polyfluoroalkyl substances (PFAS) in aqueous matrices, Analytical and Bioanalytical Chemistry, vol. 411, 2019, pp. 3507–3520.

[25] C. Dassuncao, H. Pickard, M. Pfohl, A.K. Tokranov, M. Li, B. Mikkelsen, A. Slitt, E.M. Sunderland, Phospholipid Levels Predict the Tissue Distribution of Poly- and Per fl uoroalkyl Substances in a Marine Mammal, Environmental Science & Technology Letters, vol. 6, 2019, pp. 119–125.

[26] M. Kokotou, A. Asimakopoulos, N. Thomaidis, Artificial sweeteners as emerging pollutants in the environment: analytical methodologies and environmental impact, Analytical Methods View, vol. 4, 2012, pp. 3057–3070.

[27] F.T. Lange, M. Scheurer, H. Brauch, Artificial sweeteners — a recently recognized class of emerging environmental contaminants: a review, Analytical and Bioanalytical Chemistry, vol. 403, 2012, pp. 2503–2518.

[28] S. Praveena, M. Cheema, H.-R. Guo, Ecotoxicology and Environmental Safety Non-nutritive arti fi cial sweeteners as an emerging contaminant in environment : A global review and risks perspectives, Ecotoxicology and

Environmental Safety, vol. 170, 2019, pp. 699–707.

[29] S.D. Richardson, Environmental Mass Spectrometry: Emerging Contaminants and Current Issues, Analytical Chemistry, vol. 84, 2012, pp. 747–778.

[30] K. Knauer, Pesticides in surface waters : a comparison with regulatory acceptable concentrations (RACs) determined in the authorization process and consideration for regulation, Environmental Sciences Europe, vol. 28, 2016, pp. 1–7.

[31] M. Miszczyk, P. Marlena, T. Stobiecki, D. Kronenbach-Dylong, K. Waleczek, Official control of plant protection products in Poland: detection of illegal products, Environmental Science and Pollution Research, vol. 25, 2018, pp. 31906–31916.

[32] L. Ruiz-Gil, R. Romero-Gonzalez, A. Garrido Frenich, J. L. Martínez Vidal, Original Paper Determination of pesticides in water samples by solid phase extraction and gas chromatography tandem mass spectrometry, Journal of Separation Science, vol. 31, 2008, pp. 151–161.

[33] A. Boxall, The Handbook of Environmental Chemistry, Vol. 2: Transformation Products of Synthetic Chemicals in the Environment, Springer, 2009.

[34] I. Michael, M.I. Vasquez, E. Hapeshi, T. Haddad, E. Baginska, K. Kummerer, D. Fatta-Kassinow, Metabolites and Transformation Products of Pharmaceuticals in the Aquatic Environment as Contaminants of Emerging Concern, Wiley, 2014.

[35] Y. Picó, D. Barceló, Transformation products of emerging contaminants in the environment and high-resolution mass spectrometry : a new horizon, Analytical and Bioanalytical Chemistry, vol. 407, 2015, pp. 6257–6273.

[36] K. Nodler, M. Tsakiri, T. Licha, The Impact of Different Proportions of a Treated Effluent on the Biotransformation of Selected Micro-Contaminants in River Water Microcosms, International Journal of Environmental Research and Public Health, vol. 11, 2014, pp. 10390–

10405.

[37] T. Clarkson, Environmental contaminants in the food chain, The American Journal of Clinical Nutrition, vol. 61, 1995, pp. 682S-686S.

[38] P. Bienfang, H. Trapido-Rosenthal, E. Laws, Bioaccumulation/Biomagnification in Food Chains, in: Encycl. Sustain. Sci. Technol., 2012: pp. 822–845.

[39] P. Movalli, R. Dekker, J. Koschorreck, G. Treu, Bringing together raptor collections in Europe for contaminant research and monitoring in relation to chemicals regulations, Environmental Science and Pollution Research, vol. 24, 2017, pp. 24057–24060.

[40] A. Küster, P.R. Becker, J.R. Kucklick, R.S. Pugh, J. Koschorreck, The international environmental specimen banks — let ' s get visible, Environmental Science and Pollution Research, vol. 22, 2015, pp. 1559–1561.

[41] A. Koizumi, K. Harada, K. Inoue, T. Hitomi, H.-R. Yang, C.-S. Moon, P. Wang, N. Ngoc Hung, T. Watanabe, S. Shimbo, M. Ikeda, Past, present, and future of environmental specimen banks, Environmental Health and Preventive Medicine, vol. 14, 2009, pp. 307–318.

[42] H. Ruedel, Use of environmental specimen banks for investigations of emerging pollutants - recent case studies and outlook for future applications, Norman Bulletin, vol. 2, 2011, pp. 1–4.

[43] J. Koschorreck, C. Heiss, J. Wellmitz, A. Fliedner, H. Ruedel, The use of monitoring data in EU chemicals management — experiences and considerations from the German environmental specimen bank, Environmental Science and Pollution Research, vol. 22, 2015, pp. 1597–1611.

[44] A.M. Lister, Chimate Change Research Group, Natural history collections as sources of long-term datasets, Trends in Ecology and Evolution, vol. 26, 2011, pp. 153–154.

[45] H. Ziarrusta, M. Olivares, A. Delgado, O. Posada-Ureta, O. Zuloaga, N. Etxebarria, Multi screening determination of organic pollutants in molluscs

using matrix solid phase dispersion, Journal of Chromatography A, vol. 1391, 2015, pp. 18–30.

[46] G. Bajger, P. Konieczka, J. Namieśnik, Speciation of trace element compounds in samples of biota from marine ecosystems, Chemical Speciation and Bioavailability, vol. 23, 2011, pp. 125–142.

[47] N. Fidalgo-Used, E. Blanco-González, A. Sanz-Medel, Sample handling strategies for the determination of persistent trace organic contaminants from biota samples, Analytica Chimica Acta, vol. 590, 2007, pp. 1–16.

[48] A. Kettle, Use of Accelerated Solvent Extraction to Improve Laboratory Workflow, Dionex.Com, 2013, pp. 1–5.

[49] A. Nieto, F. Borrull, R.M. Marce, E. Pocurull, Pressurized liquid extraction of contaminants from environmental samples, Current Analytical Chemistry, vol. 4, 2008, pp. 157–167.

[50] B.E. Richter, B.A. Jones, J.L. Ezzell, N.L. Porter, N. Avdalovic, C. Pohl, Accelerated Solvent Extraction: A Technique for Sample Preparation, Analytical Chemistry, vol. 68, 1996, pp. 1033–1039.

[51] S. Kern, K. Fenner, H.P. Singer, R.P. Schwarzenbach, J. Hollender, Identification of Transformation Products of Organic Contaminants in Natural Waters by Computer-Aided Prediction and High-Resolution Mass Spectrometry, Environmental Science & Technology, vol. 43, 2009, pp. 7039–7046.

[52] K. Diamanti, R. Aalizadeh, N. Alygizakis, A. Galani, M. Mardal, N.S. Thomaidis, Wide-scope target and suspect screening methodologies to investigatethe occurrence of new psychoactive substances in influent wastewaterfrom Athens, Science of the Total Environment, vol. 685, 2019, pp. 1058–1065.

[53] J. Aceña, S. Stampachiacchiere, S. Pérez, D. Barceló, Advances in liquid chromatography–high-resolution mass spectrometry for quantitative and qualitative environmental analysis, Analytical and Bioanalytical Chemistry, vol. 407, 2015, pp. 6289–6299.

[54] M. Krauss, H. Singer, J. Hollender, LC–high resolution MS in

environmental analysis: from target screening to the identification of unknowns, Analytical and Bioanalytical Chemistry, vol. 397, 2010, pp. 943–951.

[55] M. Ibáñez, J. V. Sancho, D. McMillan, R. Rao, F. Hernández, Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-light mass spectrometry, Trends in Analytical Chemistry, vol. 27, 2008, pp. 481–489.

[56] S. D. Richardson, Water Analysis: Emerging contaminants and current issues, Analytical Chemistry, vol. 79, 2007, pp. 4295–4324.

[57] C. Postigo, M.J.L. de Alda, D. Barceló, Analysis of drugs of abuse and their human metabolites in water by LC-MS2: A non-intrusive tool for drug abuse estimation at the community level, Trends in Analytical Chemistry, vol. 27, 2008, pp. 1053–1069.

[58] R. Cariou, E. Omer, A. Leon, G. Dervilly-Pinel, B. Le Bizec, Screening halogenated environmental contaminants in biota based on isotopic pattern and mass defect provided by high resolution mass spectrometry profiling, Analytica Chimica Acta, vol. 936, 2016, pp. 130–138.

[59] B. Du, J.M. Lofton, K.T. Peter, A.D. Gipe, C.A. James, J.K. McIntyre, N.L. Scholz, J.E. Baker, E.P. Kolodziej, Development of suspect and non-target screening methods for detection of organic contaminants in highway runoff and fish tissue with high-resolution time-of-flight mass spectrometry, Environmental Science: Processes & Impacts, vol. 19, 2017, pp. 1185–1196.

[60] A.L. Heffernan, M.M. Gómez-Ramos, C. Gaus, S. Vijayasarathy, I. Bell, C. Hof, J.F. Mueller, M.J. Gómez-Ramos, Science of the Total Environment Non-targeted, high resolution mass spectrometry strategy for simultaneous monitoring of xenobiotics and endogenous compounds in green sea turtles on the Great Barrier Reef, Science of the Total Environment, vol. 599–600, 2017, pp. 1251–1262.

[61] J.A. Baz-Lomba, M.J. Reid, K. V. Thomas, Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF, Analytica Chimica Acta, vol. 914, 2016, pp. 81–90.

[62] A.A. Bletsou, D.E. Damalas, P.G. Ferrero, E.L. Schymanski, H.P. Singer, J. Hollender, N.S. Thomaidis, Wide-scope quantitative target screening of 2327 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS, in: Proc. 14th Int. Conf. Environ. Sci. Technol. Rhodes, Greece, 3-5 Sept. 2015, 2015.

[63] P. Gago-Ferrero, E.L. Schymanski, A.A. Bletsou, R. Aalizadeh, J. Hollender, N.S. Thomaidis, Extended suspect and non-target strategies to characterize emerging polar organic contaminants in raw wastewater with LC-HRMS/MS, Environmental Science and Technology, vol. 49, 2015, pp. 12333–12341.

[64] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Official Journal of the European Communities, 2002, pp. 1–29.

[65] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence, Environmental Science and Technology, vol. 48, 2014, pp. 2097–2098.

[66] E. L. Schymanski, H.P. Singer, J. Slobodnik, I.M. Ipolyi, P. Oswald, M. Krauss, T. Schulze, P. Haglund, T. Letzel, S. Grosse, N.S. Thomaidis, A. Bletsou, C. Zwiener, M. Ibáñez, T. Portolés, R. de Boer, M.J. Reid, M. Onghena, U. Kunkel, W. Schulz, A. Guillon, N. Noyon, G. Leroy, P. Bados, S. Bogialli, D. Stipaničev, P. Rostkowski, J. Hollender, Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis, Analytical and Bioanalytical Chemistry, vol. 407, 2015, pp. 6237–6255.

[67] H. Berrada, F. Borrull, G. Font, R.M. Marcé, Determination of macrolide antibiotics in meat and fish using pressurized liquid extraction and liquid chromatography-mass spectrometry, Journal of Chromatography A, vol. 1208, 2008, pp. 83–89.

[68] J.D. Blair, M.G. Ikonomou, B.C. Kelly, B. Surridge, F.A.P.C. Gobas, Ultra-trace determination of phthalate ester metabolites in seawater, sediments,

and biota from an urbanized marine inlet by LC/ESI-MS/MS, Environmental Science and Technology, vol. 43, 2009, pp. 6262–6268.

[69] B. Shao, H. Han, D. Li, Y. Ma, X. Tu, Y. Wu, Analysis of alkylphenol and bisphenol A in meat by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry, Food Chemistry, vol. 105, 2007, pp. 1236–1241.

[70] Y. Tao, D. Chen, X. Chao, H. Yu, P. Yuanhu, Z. Liu, L. Huang, Y. Wang, Z. Yuan, Simultaneous determination of malachite green, gentian violet and their leuco-metabolites in shrimp and salmon by liquid chromatography-tandem mass spectrometry with accelerated solvent extraction and auto solid-phase clean-up, Food Control, vol. 22, 2011, pp. 1246–1252.

[71] G. ten Dam, O. Pardo, W. Traag, M. van der Lee, R. Peters, Simultaneous extraction and determination of HBCD isomers and TBBPA by ASE and LC-MSMS in fish, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, vol. 898, 2012, pp. 101–110.

[72] G.T. Tomy, T. Halldorson, R. Danell, K. Law, G. Arsenault, M. Alaee, G. MacInnis, C.H. Marvin, Refinements to the diastereoisomer-specific method for the analysis of hexabromocyclododecane, Rapid Communications in Mass Spectrometry, vol. 19, 2005, pp. 2819–2826.

[73] J. Wang, P.R. Gardinali, Analysis of selected pharmaceuticals in fish and the fresh water bodies directly affected by reclaimed water using liquid chromatography-tandem mass spectrometry, Analytical and Bioanalytical Chemistry, vol. 404, 2012, pp. 2711–2720.

[74] S. Tavazzi, E. Benfenati, D. Barcelo, Accelerated Solvent Extraction then Liquid Chromatography Coupled with Mass Spectrometry for Determination of 4-t-Octylphenol , 4-Nonylphenols , and Bisphenol A in Fish Liver, Chromatographia, vol. 56, 2002, pp. 463–467.

[75] J.J. Wu, Y.L. Mak, M.B. Murphy, J.C.W. Lam, W.H. Chan, M. Wang, L.L. Chan, P.K.S. Lam, Validation of an accelerated solvent extraction liquid chromatography- tandem mass spectrometry method for Pacific ciguatoxin-1 in fish flesh and comparison with the mouse neuroblastoma

assay, Analytical and Bioanalytical Chemistry, vol. 400, 2011, pp. 3165–3175.

[76] Z. Xiao, X. Li, X. Wang, J. Shen, S. Ding, Determination of neonicotinoid insecticides residues in bovine tissues by pressurized solvent extraction and liquid chromatography-tandem mass spectrometry, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, vol. 879, 2011, pp. 117–122.

[77] V. Carretero, C. Blasco, Y. Picó, Multi-class determination of antimicrobials in meat by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry, Journal of Chromatography A, vol. 1209, 2008, pp. 162–173.

[78] D. Chen, Y. Tao, H. Zhang, Y. Pan, Z. Liu, L. Huang, Y. Wang, D. Peng, X. Wang, M. Dai, Z. Yuan, Development of a liquid chromatography-tandem mass spectrometry with pressurized liquid extraction method for the determination of benzimidazole residues in edible tissues, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, vol. 879, 2011, pp. 1659–1667.

[79] R. Draisci, C. Marchiafava, L. Palleschi, I. Purificato, E. Ferretti, Accelerated solvent extraction and liquid chromatography tandem mass spectrometry quantitation of corticosteroid residues on bovine liver, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, vol. 753, 2001, pp. 217–223.

[80] A. Gentili, D. Perret, S. Marchese, M. Sergi, C. Olmi, R. Curini, Accelerated solvent extraction and confirmatory analysis of sulfonamide residues in raw meat and infant foods by liquid chromatography electrospray tandem mass spectrometry, Journal of Agricultural and Food Chemistry, vol. 52, 2004, pp. 4614–4624.

[81] S. Herranz, M.C. Moreno-Bondi, M.D. Marazuela, Development of a new sample pretreatment procedure based on pressurized liquid extraction for the determination of fluoroquinolone residues in table eggs, Journal of Chromatography A, vol. 1140, 2007, pp. 63–70.

[82] H. Hooijerink, E.. van Bennekom, M.W.. Nielen, Screening for gestagens

in kidney fat using accelerated solvent extraction and liquid chromatography electrospray tandem mass spectrometry, Analytica Chimica Acta, vol. 483, 2003, pp. 51–59.

[83] N. Salgueiro-González, I. Turnes-Carou, S. Muniategui-Lorenzo, P. López-Mahía, D. Prada-Rodríguez, Fast and selective pressurized liquid extraction with simultaneous in cell clean up for the analysis of alkylphenols and bisphenol A in bivalve molluscs, Journal of Chromatography A, vol. 1270, 2012, pp. 80–87.

[84] I. Schmitz-Afonso, J.E. Loyo-Rosales, M. De La Paz Avilés, B.A. Rattner, C.P. Rice, Determination of alkylphenol and alkylphenolethoxylates in biota by liquid chromatography with detection by tandem mass spectrometry and fluorescence spectroscopy, Journal of Chromatography A, vol. 1010, 2003, pp. 25–35.

[85] M.E. Dasenaki, N.S. Thomaidis, Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography-tandem mass spectrometry, Analytica Chimica Acta, vol. 880, 2015, pp. 103–121.