Identification of novel markers of water pollution based on semi-targeted high-performance liquid chromatography coupled with high-resolution mass spectrometry

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2 Abstract

This research project aims to identify a set of markers to be used as a group in order to evaluate wastewater pollution worldwide. Markers are powerful tools used to reveal anthropological contamination. They can be any type of substance used by humanity, such as pharmaceuticals, artificial sweeteners, personal care products, herbicides and bisphenols. The analysis was performed using semi-targeted HPLC-MS using samples from a wastewater treatment plant (WWTP) in Northern Poland for a year, from September 2018 to July 2019. The samples were collected before and after the WWTP, which helped with the study of the efficiency of removal of the plant. Seasonality of the markers is studied, not finding it in the available dataset. It is analysed using statistical tools, such as principal component analysis. The monitoring of these markers will be useful to analyse the habits of the population.

3 List of Acronyms and abbreviations

t_R	Retention time
WWTP	Wastewater treatment plant
WBE	Wastewater based epidemiology
Inf	Influent
Eff	Effluent
Dis	Disposal
WHO	World Health Organisation
UN	United Nations
SPE	Solid phase extraction
FA	Formic acid (CH_2O_2)
ACTN	Acetone (C_3H_6O)
EtOAc	Ethyl acetate ($C_4H_8O_2$)
MeOH	Methanol (CH_3OH)
ACE	Acesulfame ($C_4H_4KNO_4S$)
AMP	Amphetamine ($C_9H_{13}N$)
ATZ	Atrazine ($C_8H_{14}ClN_5$)
BP	Bisphenol ($C_{12}H_{112}O_2$)
CAF	Caffeine ($C_8H_{10}N_4O_2$)
CBZ	Carbamazepine ($C_{15}H_{12}N_2O$)
DCF	Diclofenac ($C_{14}H_{11}Cl_2NO_2$
GB	Gabapentin ($C_9H_{17}NO_2$)
MET	Metformin ($C_4H_{11}N_5$
MP	Methyl-paraben ($C_8H_8O_3$)
MPD	Methylphenidate ($C_{14}N_{19}NO_2$)
MTO	Metoprolol ($C_{15}H_{25}NO_3$)
NC	Nicotine ($C_{10}H_{14}N_2$)
PARAC	Paracetamol ($C_8H_9NO_2$)
SUC	Sucralose ($C_{12}H_{19}Cl_3O_8$)

4 Introduction

Water is the most abundant liquid on Earth. However, only 1% of it is freshwater, the water with low percentage of salt, and can be used for human purposes, such as drinking water [1]. The freshwater cycle, which is the terrestrial part of the water cycle, enables humans to extract water sustainably, from streams, lakes, aquifers and human-made reservoirs [1]. The 99% of freshwater is groundwater, which is the freshwater present beneath Earth's surface, mainly in rock and soil pore spaces [1]. The other 1% is called surface water, which is the water present in streams, lakes and human-made reservoirs. Groundwater provides more than half of the volume of water extracted for domestic use [1]. In Europe, groundwater is mostly used for drinking and agricultural purposes and it usually contains nitrates and pesticides [1, 2].

Once water has been extracted and used by humans in domestic or industrial applications, it is labelled as wastewater. At this stage, it is a complex matrix, containing thousands of compounds, including metabolites, personal care products, household appliances, etc., and it reflects the consumption patterns of the population and socioeconomic information [3, 4].

Figure 1 shows a schematic representation of types of water and their origin. Starting from the left, types of pesticides, such as herbicides, insecticides, fungicides, etc., are applied to protect all type of crops. After rain or watering processes, this group of contaminants is carried by water to groundwater. Contaminants with anthropological origin are used in houses and hospital and after the consumption, they finish in the sewage system. This wastewater is sent to the wastewater treatment plant (WWTP), where it will go through cleaning processes. Industries use different chemical compounds that also finish in the water system. This water is also sent to the WWTP before being released to surface water. Surface waters could be filtrated into groundwaters. From these surface or groundwaters, water is extracted for drinking purposes. To make it drinkable, it is cleaned by drinking water treatment plants and brought to the consumers. After that, this water will be affected again by products consumed by humans.

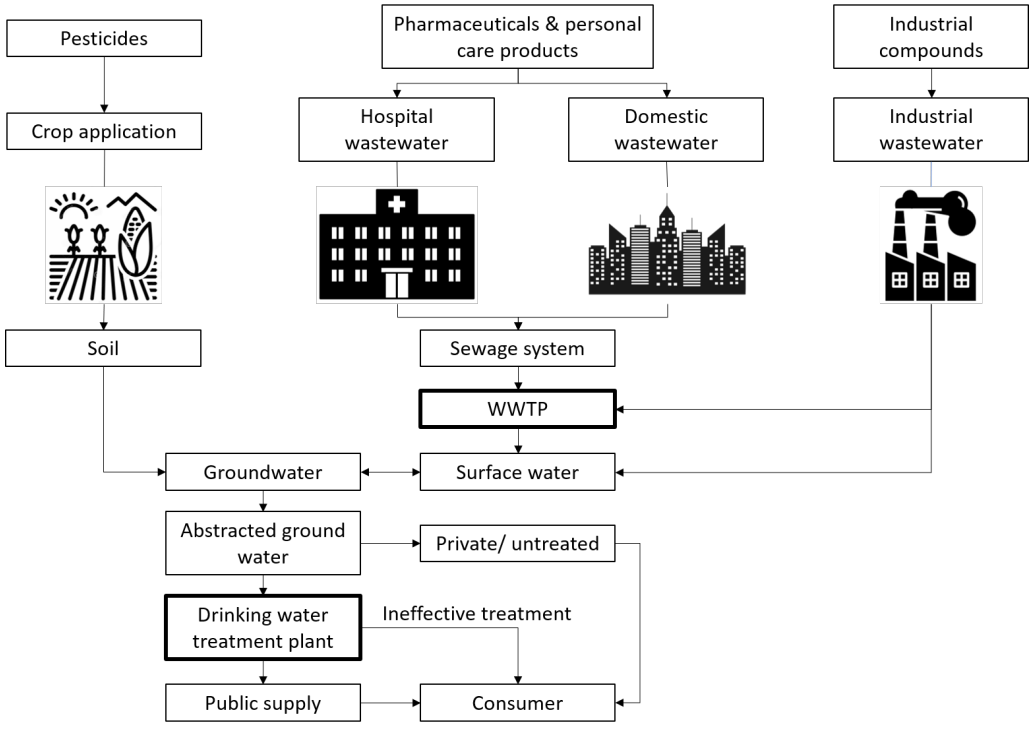


Figure 1: Schematic representation of the water cycle for human purposes [5].

The United Nations (UN) developed a list with 17 goals, shown in Figure 2, to ensure sustainable development addressing global challenges such as poverty, inequality, climate change, environmental degradation, peace and justice [6]. In this list, there is a goal related with water safety, goal number 6, which is to ensure access to water and sanitation for all human population. To accomplish this goal, water analysis has to be performed all around the world. At the moment, at least 3 billion of people do not know the quality of their water due to a lack of monitoring and resources [6]. The COVID-19 pandemic provided for an example of the importance of clean water: the disease was spread faster where there was no access to clean water, making more impact and increasing the difficulty of contention [6]. Cholera infections are another typical health issues that can be transmitted when wastewater is not managed properly [7]. Chemical contaminants are also present in freshwater from direct or indirect discharge of treated effluents of WWTP, inefficient pipe connections and broken pipes of untreated sewage [7].

Figure 2: Schematic representation of the 17 sustainable development goals [6].

Another challenge in achieving clean water is contamination due to human activity. Between 1930 and 2000, the production of chemical compounds has increased massively, from 1 million of tons/year to 400 million tones/year [5]. Most of these chemicals finish in the aquatic environment after the human use and they do not disintegrate easily and become persistent. During the last century, different types of persistent chemicals were investigated and monitored to see their effect on the environment. These compound were called persistent organic pollutants (POPs), because of their resistance to degradation. On 2001, a global convention was held in Stockholm with the aim of eliminating and restricting the use and production of these POPs [8]. Some of these compounds were Aldrin and DDT (Dichlorodiphenyltrichloroethane), in the pesticides category, and polychlorinated biphenyls, in the industry category [8]. However, due to the increase of chemicals produced, many more new contaminants have been detected. For this reason, the EU created a list of priority pollutants in 2008 and has updated it with new compounds since then. In this list, there are a broad range of inorganic and organic compounds, which are mostly used in industry, agriculture and households [9]. These compounds are called emerging contaminants (EC) and include, for example, pharmaceuticals, personal care products and endocrine disruptive compounds [10]. The goal of the list is to identify these EC present in wastewater and storm run-off water to analyse if they have an impact or a threat in aquatic ecosystems [11]. Pharmaceuticals, which are designed to perform a biological effect, in water could cause an increase of bacteria resistant to antibiotics if present in water, or affect negatively part of the ecosystem [10, 12]. They deserve special attention due to their continuous introduction to the environment in large quantities [12]. While the detection of chemicals in the environment does not directly imply adverse consequences to the different ecosystems [11, 13], it has been proven that more than 70% of

these chemicals have a negative impact on the environment [5]. More research has to be performed to study their effect. One way of analysing the effect is by using EC as chemical markers, because of the origin of these ECs in mainly human and industry sewage [13].

To identify whether a compound has the risk of becoming a dangerous EC, it lipophilicity is a key property. It helps to know the fate of a particular contaminant. A popular way to measure lipophilicity is with the partition coefficient n-octanol/water, also called log P [14], which describes the ratio of chemical's concentration in the equilibrium of an organic phase, using octanol, and aqueous phase [15]. The concept of log P was introduced in 1899 by Charles Ernest Overton and Hans Horst Meyer, to determine the ability of certain compounds to enter the different lipophilic compartments of cells [16]. They were using olive oil and water mixtures to calculate it. Later on, Corwin Hansch suggested the use of n-octanol as an inexpensive organic phase [16]. Since then, it has been used as a parameter to determine lipophilicity. In pharmaceutical studies, log P is used to study the how easily a drug can reach its target inside of the body. For environmental sciences, it is used to indicate how easily a compound can pollute different waters, such as ground and surface water, and the toxicity that compound has for aquatic ecosystems.

Wastewater is brought to a WWTP, where it is going to be cleaned and, then, disposed in a freshwater source, lake or stream. The percentage of the wastewater that is treated depend on the income level of the country. High-income countries treat above 70% of the wastewater but this drops to only 8% for countries with low income [17].

In Figure 3 a simplified schematic representation of how a WWTP works is shown. This is an example of a WWTP from a high income country. The raw wastewater arrives to the WWTP and is subjected to preliminary treatment, which include filtration of bigger items, such as flushable wipes, branches, sand and rocks, using bar screens and grit chambers. The goal of the pretreatment is to make the water more homogenous to avoid breakdowns of the machinery used on later steps. Then, the wastewater enters the primary treatment, where solid particles precipitate using the primary clarifiers. Clarifiers are big water pools with a slow mixing velocity to help the precipitation of solid particles, called sludge. Usually, the sludge undergoes a process that thickens it to remove the access of water, during approximately 10 h, and the excess water will be put into the primary clarifiers again. After the primary treatment, the wastewater should only contain organic matter and should be free of solid particles larger than 10 μm. Next, the secondary treatment, where the biological organisms will be degraded, using aeration basins. It is based on the addition of oxygen bubbles and microorganisms in the water to facilitate the aerobic digestion. After this step, the wastewater enters a secondary clarifier, with the goal to make the final removal of solid particles and biological matter. Then, the water is filtered using a sand filter to remove organic matter. The final step is the water is moved to the disinfection process. At this point, 85% of the organic matter has been removed. The disinfection is typically done using chlorine, ozone or UV-light. In the disinfection section is where some of the pharmaceuticals and other anthropogenic chemicals are partially removed. Finally, the water is now discharged into a nearby stream, river or lake or it could go to a water treatment plant, where the water will be treated for drinking purposes. Generally, the wastewater treatment would take between 11 to 20 h (from Zurich's WWTP website [1] and [18]). Most of the municipal WWTPs are not equipped to deal with removal of pharmaceuticals and other complex organic compounds [19]. They were built with the aim to remove N, P, C and microbiological compounds with concentrations from mg/L.

More generally, conventional WWTP present four mechanisms of removal of pollutants: (1) sorption, (2) biological transformation, (3) volatilisation and (4) abiotic degradation [21]. A schematic representation of the four mechanisms is shown in Figure 4. Sorption is based on adsorption of the hydrophobic

[1] *https://www.stadt-zuerich.ch/ted/de/index/entsorgung_recycling/publikationen_broschueren/werdhoelzli.html*

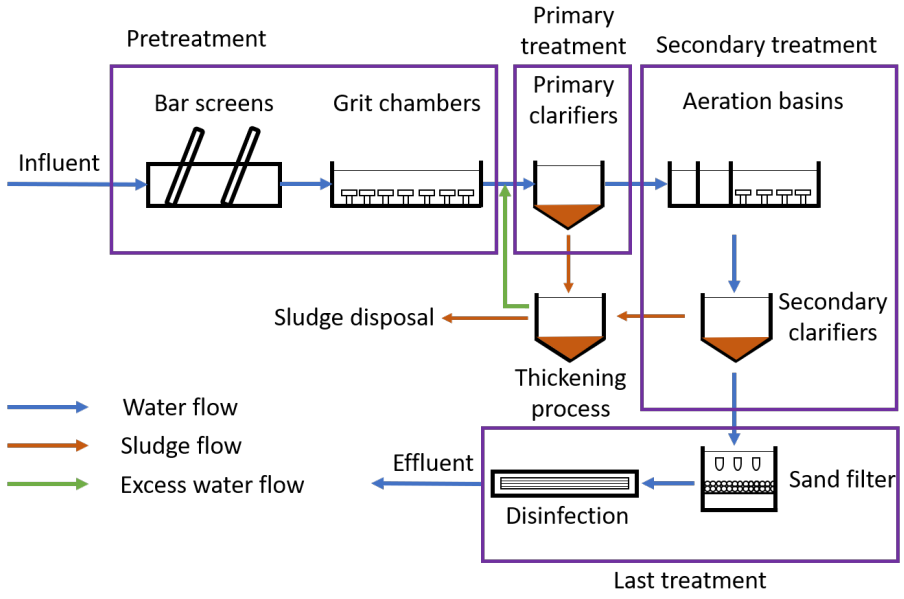


Figure 3: Simplified schematic representation of a WWTP [20].

or positively charged molecules into sludge, which is later incinerated or disposed on fields. Biological transformation can be separated in two processes, metabolic reactions, where the pollutant is used by the microorganism as a growth substrate, and co-metabolic reaction, where the pollutants are metabolised by the microorganisms by side reaction. The efficiency of the biological transformation depends on the type of microorganisms used, the quantity and the biodegradability of the pollutant. Volatilisation only occurs when the pollutant is volatile enough. The last mechanism is abiotic degradation, which is not a significant removal mechanism, and could be by photolysis or hydrolysis. Depending on the type of the pollutant and their characteristics, one mechanism will work better to remove and their fate will be different.

The main pollutants found in municipal wastewater, their removal mechanism and percentage of removal are presented in Table 1. These pollutants are classified in nine different categories: (1) surfactants, (2) pharmaceuticals, (3) illicit drugs, (4) personal care products, (5) artificial sweeteners, (6) plasticisers and plastic additives, (7) pesticides, (8) persistent organic pollutants and (9) heavy metals. Surfactants, used in households for detergents and cleaners, are one of the contaminants found in raw wastewater in higher concentration (>40 mg/L). Most surfactants are easily biodegraded and, hence, well removed in the WWTP [21]. Pharmaceuticals are highly ingested, with more than 300 mg/inhabitant/day in western Europe [22], and excreted via urine and faeces, so these pharmaceuticals and their metabolites are sent to the wastewater system. They are typically present in the range of μg/L - ng/L [19, 10]. The most abundant pharmaceuticals found in wastewater are anti-inflammatories, analgesics, antibiotics, antidiabetics, antihypertensives/diuretics, β-blockers, lipid regulators, psychiatric drugs and antihistamines [21]. Only a few pharmaceuticals are removed by biodegradation and the majority are removed partially, less than 90% [21]. If the drugs are hydrophobic or positively charged, they can be partially removed by sorption. Most of the metabolites are more hydrophilic and polar than their parent compound, hence, they are not well removed by biological treatments or sorption [21]. Different pharmaceuticals are introduced in aquatic environments because of the difficulty of removal in the WWTP. Illicit drugs, such as amphetamine and cocaine, are detected in raw wastewater from 100-2000 ng/L. Fortunately, they are easily removed, from 79 to 98%, in conventional WWTP by biodegradation mechanism [21]. Personal care products (PCP) are another big group of water

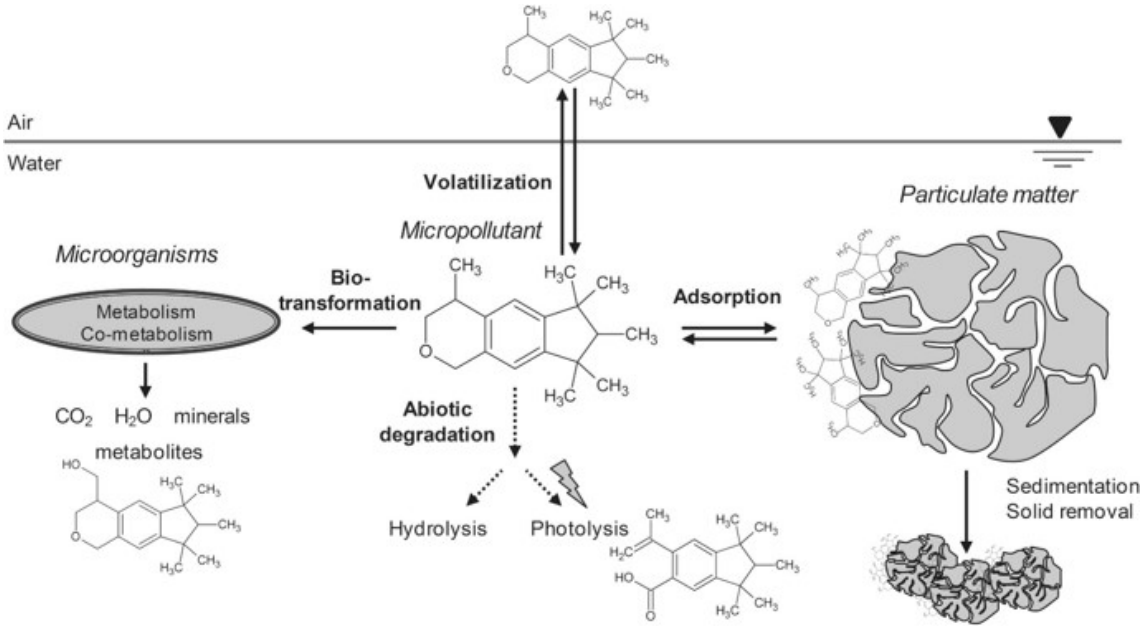
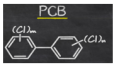
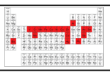


Figure 4: Schematic representation of the mechanisms of removal in a conventional WWTP. Reprinted with permission from [21].

pollutants. They include chemical compounds present in shampoos, washing lotions, skin and dental care, sunscreen agents, etc. They mainly enter the wastewater system via wash-off during showers. Parabens are broadly used as antimicrobials in different products. Fortunately, they are well removed (>95%) in the WWTP by biodegradation [21]. Artificial sweeteners, such as acesulfame, aspartame, cyclamate, saccharine and sucralose, are widely used as a substitute of sugar. They are design to not being metabolised, so they are not a source of energy for the human body. Aspartame, cyclamate and saccharine are well eliminated in the WWTP, by biodegradation. However, acesulfame and sucralose are not removed by any treatment and become very persistent [21]. Plasticisers and plastic additives are another group of main contaminants in wastewater. Plasticisers, such as phthalates, are used to improve flexibility in plastics. In WWTP, they are partially removed (>60%) by biodegradation and sorption [21]. Bisphenols are one of the most used plastic additives, used widely in all types of products, such as food containers, bottles, cans and toys. The removal in WWTP depend on their particular properties. For example, bisphenol A is well removed (>80%) by biodegradation [21]. Pesticides are design and synthesised to control and destroy growth of certain plants, insects or fungi. They are present in wastewater due to rain run-off. Generally, they are not properly removed (<50%) in WWTP [21]. Persistent organic pollutants, such as polychlorinated biphenyls (PCB), were the first pollutants monitored. PCBs were mainly used as heat exchange fluid, among other uses, before being banned between 1970 and 1990. Despite the ban, they are still detected in wastewater. The removal in WWTP is partial, with a removal percentage of 75%, mostly by sorption due to their high hydrophobicity [21]. Heavy metals traditionally were associated with industrial emissions, but after new regulations on recent years, the main source of them in wastewater is domestic, such as corrosion of old pipes and paint. Generally, heavy metals are adsorbed in solids, hence, the main removal mechanism is the pretreatment part, using bar screens and grit chambers, with an efficiency higher than 75% [21].

All the main types of pollutants presented in the last paragraph are not efficiently eliminated in a typical WWTP. Hence, the elimination mechanisms presented in these plants need to be improved and optimised to ensure that all the water released into the natural environment will not have any human-made chemical

Table 1: Table with the fate and removal of groups of pollutants in WWTP.

Types of pollutants		Main mechanism of removal	Removal in WWTP
Surfactants		Biodegradation	>95%
Pharmaceuticals		Biodegradation and sorption	<90%
Illicit drugs		Biodegradation	79-98%
Personal care products		Biodegradation	>95%
Artificial sweeteners		Biodegradation	0-100%
Plasticisers and plastic additives		Biodegradation	>80%
Pesticides		Sorption	<50%
Persistent organic pollutants		Sorption	~75%
Heavy metals		Suspended solids	>75%

and is safe for the ecosystem without causing any harm. Optimising the mechanisms that are currently used would not add a big extra expense to that facility and would make a difference.

Further demands on improving WWTP also come from the increasing challenges on how to manage drinking water sustainably posed by the rapid increase in population [7]. Also, over the years more areas have became water sensitive, such as southern California, Israel, South Africa, Australia and Spain [23]. Studies performed by the EU show that at least 11% of Europeans suffer from water scarcity [24]. One way of solving these problems of lack of water is recycling, re-using the treated wastewater as a drinking water, also called potable, source [24]. There are two main ways of re-using treated wastewater, the indirect and the direct way, shown in Figure 5. The indirect way is the traditional one, where treated wastewater is disposed into surface water, where the aquatic ecosystem environment acts as a buffer, and, later on, the water is collected to treat for drinking water purposes [25]. The direct way avoids the step of disposing the treated wastewater and it is directly sent to the drinking water treatment plant [25]. The advanced water, treatment shown in Figure 5, refers to some extra cleaning steps, such as reverse osmosis and micro-filtration, present in some cities around the world depending on the local regulations. Typically, the water cleaned in the WWTP was mainly used for agricultural purposes. In 2015, the European Commission published a Communication "Closing the loop - An EU action plan for the Circular Economy" which states "in addition to water-efficiency measures, the reuse of treated wastewater in safe and cost-effective conditions is a valuable but under-used means of increasing water supply and alleviating pressure on over-exploited water resources in the EU" [26]. After this, numerous initiatives aim at recirculation of water, with at least 1 billion of treated wastewater being re-used [24]. For example, Cyprus re-uses more than 90% of sewage water, Malta 60% and Spain, Italy and Greece, between 5 and 12% [24].

In order to ensure a sufficiently good quality of the cleaned water, it is fundamental that all wastewater is collected and appropriately treated in well-functioning WWTP. To ensure that, routine analysis must be performed to control water quality both at the exit of WWTP as well as in the natural environment

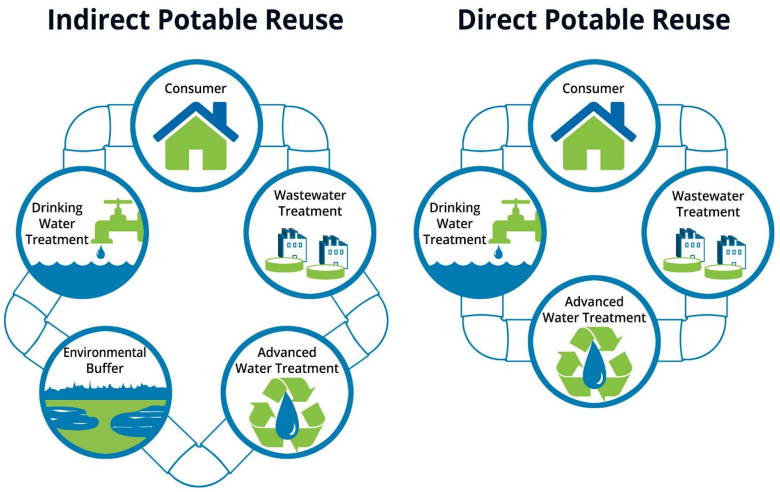


Figure 5: Representation of the reuse of wastewater for drinking purposes. Extracted from [25].

and collection points. Besides quality control, water analysis can also be used to gather real time social information and temporal trends for a specific region in an approach called Wastewater based epidemiology (WBE) [27]. It is based on the analysis of wastewater to determine analytes to collect information about general well-being and habits of the population [28]. This approach has been mainly used to monitor the consumption of illicit drugs, alcohol, nicotine, pharmaceuticals and diseases [3, 28, 29]. Sometimes, it relies on the analysis of specific human metabolites [28]. WBE has been successfully used for the detection of smoking habits, SARS-CoV-2 and polio. In summer of 2014, Mackul'ak et al., studied smoking habits in 80,000 music festivals in Czech Republic and Slovakia. They analysed wastewater aiming for the detection of cotinine, the main metabolite of nicotine [30]. WBE has been used as one of the sources used to asses infection spread, for diseases such as SARS-CoV-2 [29]. In Israel, sewage surveillance, established in 1986, allowed them to track polio in wastewater and react quickly in 2013, when a breakout happened [29]. The advantages of WBE are numerous, such as early and reliable detection of infections and help control the spread, unbiased results and the ability to monitor habits of the population [29, 30, 28]. However, one disadvantage is that it cannot help in finding specific infected individual in a community [29].

Despite the importance of water analysis, it would not be feasible to monitor the bast set of all possible contaminants. Instead, a selected molecule or a group of them must be chosen that allow detecting and controlling anthropogenic contamination in wastewater. These molecules, which must be representative of the different sources of contamination, are called markers [7]. According to their origin, they can be distributed in several categories: those produced by humans, such as faeces and metabolites, those that go through human bodies without being metabolised, such as PPCPs and artificial sweeteners, those associated with sewage contaminated waste system, such as detergents, and bacteria, such as Escherichia coli (E.coli) [7]. Traditionally, bacterial markers have been used to trace domestic wastewater [2]. Makers have become a technique to identify sources of water contamination [7]. For this reason, an ideal marker should be source specific, released to the environment in sufficient quantities, reflect contamination in a quantitative sense and should be amenable to rapid and sensitive analysis [2]. Chemical markers present more advantages than microbial markers because they are direct proof of human contamination and they are easier to detect, with rapid and reliable techniques, such as HPLC [7]. The main disadvantage for chemical markers is that there is not a single chemical that could serve as a universal marker for wastewater contamination [7].

Bacterial markers main disadvantages are that the presence of those microorganisms not always related with wastewater contamination, the analysis are more time consuming and the short life expectancy of the bacteria [7]. A good understanding of the land and types and levels of human contamination is crucial to select suitable markers for a specific area and previous analysis should be performed to prove that the analyte is not detected in waters that are not contaminated by wastewater [7]. Recent studies have shown the effectivity of chemical markers to assess water pollution, using pharmaceuticals, personal care products and artificial sweeteners [7].

In this project, the goal is to find a combination of markers that could be used worldwide to detect wastewater contamination and malfunctioning of WWTP. The markers selected were specific for polish urban wastewater, but they are chemical compounds used around the world in a similar way. Nineteen possible markers were analysed using HPLC-HRMS (High pressure liquid chromatography coupled to high resolution mass spectrometry), which is the technique most commonly used for the detection of emerging contaminants [3]. It has high selectivity and sensitivity, flexibility, robustness, which makes it optimal for the detection of polar organic compounds, high availability and low cost [28, 12]. A critical part is the wide range of the analytes to be detected simultaneously. Principal component analysis (PCA) is a statistical tool typically used to evaluate the spatial and temporal variations of the markers [7]. Because of improvements of analytical chemistry methods, the detection and determination of trace substances in urban wastewaters is possible. It has helped to gain more and more information about life-habits, public health and general well-being of the population [28].

5 Experimental part

5.1 Reagents

The following reagents were used during the experimental part:

- Acetone of LC-MS purity - Sigma Aldrich (Germany)
- Ammonia (NH_3)(pure solution) - Chempur (Poland)
- Formic acid (FA)(>98%) - Merck (Germany)
- Methanol of HPLC purity - Merck (Germany)
- Methanol of LC-MS purity - VWR Chemicals (USA)
- Ethyl acetate of LC-MS purity - Sigma Aldrich (Germany)
- Demineralized water obtained using the Hydrolab system (Poland)

5.2 Standards

The following standards were used:

- Acesulfame-K (ACE) - Nutrinova company (Germany)
- Diclofenac (DCF) - Sigma Aldrich (Germany)
- Carbamazepine (CBZ) - Sigma-Aldrich (Germany)
- Caffeine (CAF) - Sigma Aldrich (Germany)
- Methyl paraben (MP) - Fluka company (Belgium)
- Paracetamol (PARAC) - Sigma-Aldrich (Germany)
- Sucralose (SUC) - Nestlè company (Switzerland)

5.3 Sampling and sample treatment

The wastewater samples were collected from a WWTP situated in Northern Poland. The wastewater comes from four cities and the towns nearby, with less than 500,000 inhabitants in total and food industry with 12% of the contribution of wastewater. The wastewater, after the cleaning treatment, is discharged into the Baltic sea. The sampling site is not identified for confidentiality purposes.

The samples were collected monthly from September 2018 to July 2019, on three different positions presented in Figure 6. The influent is the sampling point just before the wastewater enters the facilities of the WWTP, the effluent is the sampling point just after and the disposal is the sampling point where the water is released to the sea, 10 km away from the WWTP.

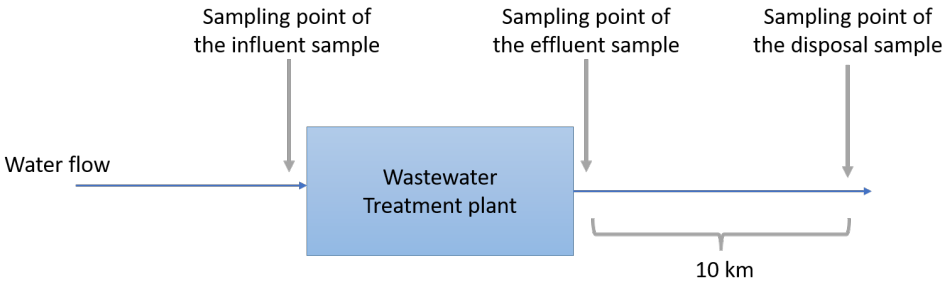


Figure 6: Scheme of the sampling sites located in the WWTP.

After the sample was collected, in 1 L amber glass bottles, using the under cap method, it was transported to the laboratory under refrigerator conditions (4 ^{o}C) and then, it was store from 24 up to 48 h maximum in a fridge. A sample of 50 mL of wastewater was taken from the refrigerator. The pH was measured to be around 8. Occasionally the sample was acidified in order to avoid the degradation of the analytes caused by microorganisms and to check if the sensitivity will improve. For the sample pre-treatment, 10 μL of internal standards (IS) were added, which are paracetamol-methyl-d_3 and N-methylcyclohexylsulfamic acid. The sample was concentrated from 50 mL to 1 mL using SPE cartridges (Strata-X Polymeric RP 200 mg/3 mL). SPE helps reducing the matrix effect, making the matrix more simple, and it enhances and isolates the signal obtained on the instrument. The SPE cartridges were conditioned with 5 mL of MeOH and 5 mL of H_2O. Influent wastewater samples were filtered with glass beads to remove solid particles, the effluent and disposal samples were not because the WWTP already removed them. Fifty mL of sample was added and the solvent was dried for 20 min. The elution was performed with 6 mL of MeOH; 3 mL MeOH, acetone (ACTN) and ethyl acetate (EtOAc) solution (2:2:1); 3 mL of 5% NH_3 in MeOH solution (50 mL MeOH + 2.5 mL NH_3). The extract was evaporated to dryness in a gentle stream of N_2 and the remaining solid was frozen. Before performing the analysis, the extract was reconstructed with 1 mL H_2O:MeOH mixture. A schematic representation of the procedure is presented in Figure 7.

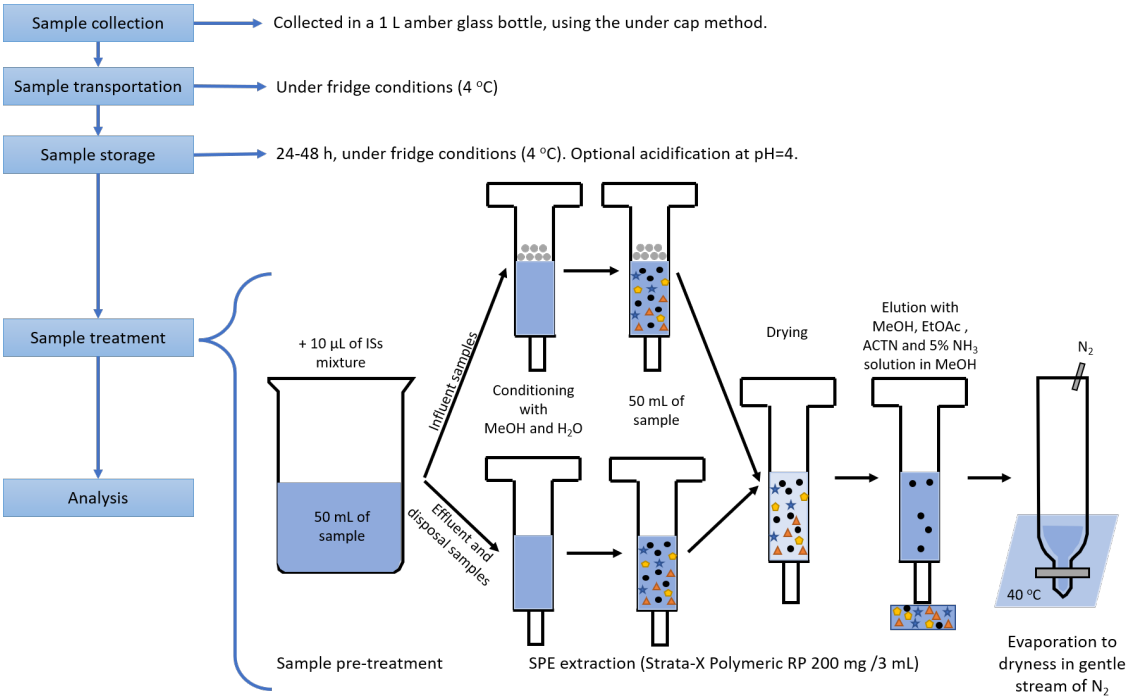


Figure 7: Scheme of the sample's path from the collection to the analysis. The blue stars, the yellow pentagons and the orange triangles represent the analytes, the black circles represent the matrix and the grey circles represent the glass particles used for filtration.

6 Instruments

The following instruments and equipment were used during the research:

- Liquid chromatograph coupled with Q-TOF, time-of-flight analyser, model 6540 by Agilent Technologies (USA) equipped with a degassing device 1290 Infinity Samples (Model G4227A), 1290 Infinity Pump (Model G4220A), 1290 Infinity autosampler (model G4226A), column thermostat 1290 Infinity (model G1316C)
- LiChrospher 100 RP-18e chromatography column (250 x 4.6 mm, 5 μm) Merck (Germany)
- Vacuum chamber for performing extractions using the SPE technique by Grace (USA)
- SPE Strata-X 33 μm Polymeric RP 3 mL/200 mg columns from Phenomenex (USA)
- Three-piece syringes for infusion pumps, including a luer-lock tip, about vol. 50(60) mL by Margomed (Poland)
- Luer plugs by J.T. Baker (US)
- HLP 5 water deionizer by Hydrolab (Poland)
- XP50H analytical balance by Mettler-Toledo (Switzerland)
- Automatic pipettes by Eppendorf (Germany) with a capacity of 10, 100, 1000, 2500 μL
- Device for evaporation of samples in a stream of nitrogen, equipped with baths water TurboVap LV by Capiler Life Science (USA)
- 1.5 mL autosampler vials with sealing membranes made of polytetrafluoroethylene (PTFE) and plastic caps by Anchem (Poland)
- Measuring cylinders with a volume of 50 and 250 mL by LABART Sp. z o. o. (Poland)
- Glass bottles of 1000 mL by Schott Glass (Germany)

The semi-targeted analysis were performed using HPLC-HRMS. The parameters of the chromatographic system are presented in Table 2

The chromatographic conditions for targeted analysis are presented in Table 3. For the quantitative analysis, Multiple reaction monitoring (MRM) was used. It is a highly sensitive method of targeted mass spectrometry used to detect and quantify compounds within complex mixtures [31]. The parameters are presented in Table 4.

Table 2: Operating conditions of the HPLC-QToF-MS system.

<table>
<tr><td colspan="3">HPLC</td><td colspan="2">Q-ToF-MS</td></tr>
<tr><td>Chromatographic column</td><td colspan="2">LiChrospher 100 RP-18e chromatography column (250 x 4.6 mm, 5 μm)</td><td>Ionisation</td><td>ESI (+)
ESI (-)</td></tr>
<tr><td>Composition of the mobile phase</td><td colspan="2">A: H_2O + 0.1% FA (v/v)
B: MeOH</td><td>Flow rate of drying gas</td><td>8 L/min</td></tr>
<tr><td>Elution type</td><td colspan="2">Gradient</td><td>Temperature of the drying gas</td><td>300 ^oC</td></tr>
<tr><td rowspan="5">Elution program</td><td>t (min)</td><td>%B</td><td rowspan="2">Nebuliser gas pressure</td><td rowspan="2">35 psig</td></tr>
<tr><td>0.0</td><td>5.0</td></tr>
<tr><td>20.0</td><td>100.0</td><td rowspan="2">Capillary voltage</td><td rowspan="2">3500 V</td></tr>
<tr><td>25.0</td><td>100.0</td></tr>
<tr><td>Mobile phase flow rate</td><td colspan="2">0.7 mL/min</td><td>Fragmentor voltage</td><td>100 V</td></tr>
<tr><td>Injection volume</td><td colspan="2">3 μL</td><td>Scanning mode range</td><td>100-1700 m/z</td></tr>
<tr><td>Thermostat temperature</td><td colspan="2">35 ^oC</td><td>Collection data speed</td><td>1.5 spectra/s</td></tr>
</table>

Table 3: Operating conditions of the UHPLC-MS/MS system.

<table>
<tr><td colspan="3">UHPLC</td><td colspan="2">MS/MS</td></tr>
<tr><td>Chromatographic column</td><td colspan="2">Kinetex C_{18} UHPLC column (150 x 2.1 mm, 1.6 μ)</td><td>Ionisation</td><td>ESI (+)
ESI (-)</td></tr>
<tr><td>Composition of the mobile phase</td><td colspan="2">A: H_2O + 0.1% FA (v/v)
B: ACN</td><td>Flow rate of drying gas</td><td>10 mL/min</td></tr>
<tr><td>Elution type</td><td colspan="2">Gradient</td><td>Temperature of dessolvation gas</td><td>250 ^oC</td></tr>
<tr><td rowspan="5">Elution program</td><td>t (min)</td><td>%B</td><td rowspan="2">Nebuliser gas flow rate</td><td rowspan="2">3 mL/min</td></tr>
<tr><td>0.0</td><td>5.0</td></tr>
<tr><td>10.0</td><td>60.0</td><td rowspan="2">Capillary voltage</td><td rowspan="2">4000 V</td></tr>
<tr><td>13.0</td><td>90.0</td></tr>
<tr><td>Mobile phase flow rate</td><td colspan="2">0.6 mL/min</td><td>Heat block temperature</td><td>400 ^oC</td></tr>
<tr><td>Injection volume</td><td colspan="2">2 μL</td><td>Interface temperature</td><td>300 ^oC</td></tr>
<tr><td>Thermostat temperature</td><td colspan="2">35 ^oC</td><td>Heating gas flow rate</td><td>10 mL/min</td></tr>
</table>

Table 4: MRM parameters used for targeted analysis with HPLC-MS/MS.

Analyte	Ionisation mode	Precursor ion [m/z]	Product ions [m/z]	Collision energy (V)	Q1 Prerod (V)	Q3 Prerod (V)
Acesulfame	Negative $[M\text{-}H]^-$	161.80	82.00	16.0	18.0	30.0
			78.00	32.0	17.0	29.0
Amphetamine	Negative $[M\text{-}H]^-$	136.10	119.10	-14.0	-12.0	-11.0
			91.10	-20.0	-11.0	-16.0
Bisphenol A	Negative $[M\text{-}H]^-$	226.95	211.30	29.0	18.0	20.0
			133.10	26.0	12.0	11.0
			93.00	43.0	17.0	17.0
Caffeine	Positive $[M+H]^+$	194.90	138.00	-23.0	-20.0	-29.0
			83.00	-12.0	-20.0	-23.0
Carbamazepine	Positive $[M+H]^+$	236.50	194.00	-20.0	-25.0	-21.0
			192.05	-22.0	-25.0	-21.0
Diclofenac	Negative $[M+H]^+$	293.80	250.05	13.0	11.0	24.0
			214.05	21.0	11.0	21.0
Metformin	Positive $[M+H]^+$	129.60	71.05	-23.0	-30.0	-29.0
			60.05	-14.0	-30.0	-25.0
Methyl paraben	Negative $[M\text{-}H]^-$	151.10	136.10	19.0	11.0	20.0
			92.00	23.0	11.0	13.0
Paracetamol	Positive $[M+H]^+$	152.00	110.10	-17.0	-30.0	-19.0
			93.10	-23.0	-10.0	-20.0
Sucralose	Negative $[M\text{-}H+HCO_2{}^-]^-$	440.90	395.00	13.0	16.0	17.0
			359.10	17.0	16.0	15.0

7 Method

7.1 Experimental method

There are mainly three different approaches while using HPLC-HRMS, depending on its purpose. Non-targeted analysis, also called unknown-unknown screening and fingerprinting, is used when there is no information of the available compounds present in the sample. It is a very challenging approach and aims to detect and identify all compounds in a sample without the use of standards [32, 33, 34]. Targeted analysis, also called known-known screening, is a comparative analysis which allows the rapid detection of compounds present in a sample with the use of standards [32, 34]. The last approach is semi-targeted analysis, also called known-unknown or suspected screening. It is based on a mixture of the previous two approaches.The determination of compounds that might be present in the sample is performed without the use of standards, only using the monoisotopic mass [3]. Figure 8 shows a schematic version of how to do the procedure for the three different approaches. In Table 5 the advantages and disadvantages of the approaches are presented.

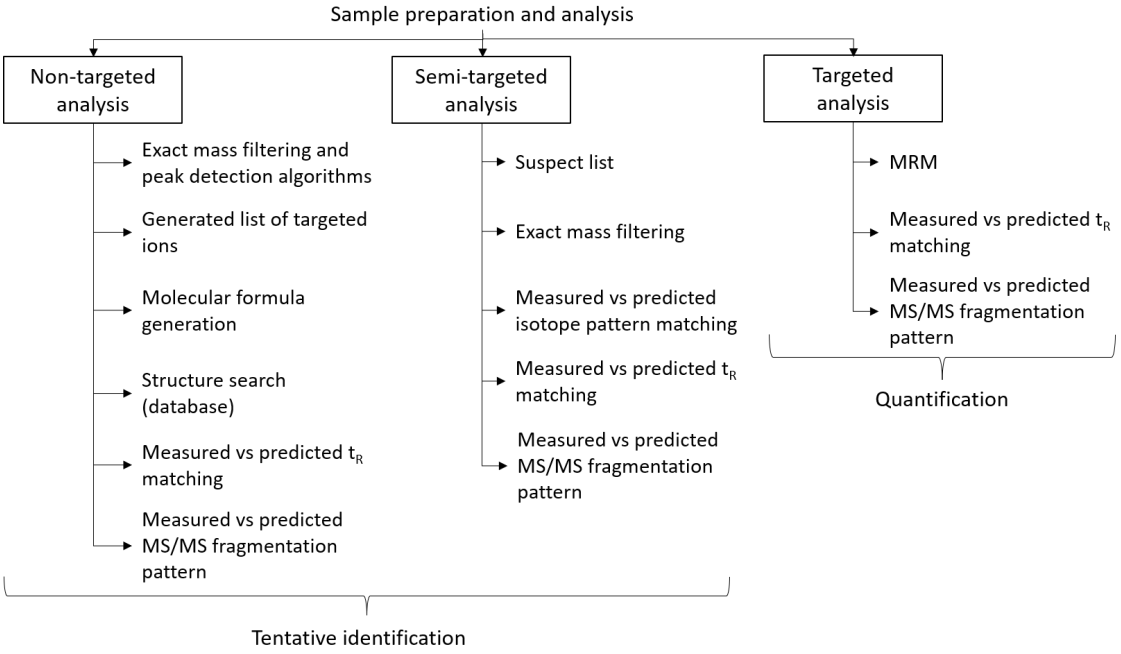


Figure 8: Schematic description of non-targeted, semi-targeted and targeted analysis using HPLC-HRMS [3].

Table 5: Advantages and disadvantages of targeted, semi-targeted and non-targeted analysis.

	Non-targeted analysis	Semi-targeted analysis	Targeted analysis
Short description	Obtention of a list of possible compounds present in a sample.	Identification of analytes present in a sample without using standards.	Quantification of specific analytes present in a sample.
Advantages	Qualitative analysis ppb level	Qualitative analysis ppb level No need of standards Based on literature review	Quantitative analysis ppt level High sensitivity High selectivity
Disadvantages	Time consuming Risk of false + results Highly trained analyst	Risk of false + results Time consuming	Expensive standards Calibration curve

7.2 Properties of a marker

For selecting markers, the environment of your sample must be taken into consideration. They should be different if the wastewater comes from an industrial zone or from a residential area [7]. The properties that an ideal marker should have are: (i) the compounds should only be present in sewage, (ii) the marker should be present with higher concentration than the detection limits, (iii) the compound should be not present in waters without anthropogenic contamination, and (iv) the potential marker should not go through degradation processes such as biodegradation, photo-degradation and/or absorption [35, 36]. The selection also needs to be based also on sales of each compound from each country according to each health system, and the compounds' pharmacokinetics, the percentage of excretion of the analyte non-metabolised [37].

7.3 Selected markers

Nineteen possible markers for monitoring wastewater were selected following the criteria presented on the previous section. The selection was established based on literature search and scientific discussion with specialists in environmental pollution and protection. Although all these compounds have an anthropogenic origin have different functions. Figure 9 presents a schematic representation of the markers selected, based on their origin. Eight of the markers are related to the pharmaceutical industry, three are related with food consumption, two are related with stimulant drugs, one is related with personal care, one is a herbicide and five are bisphenols, which are present in plastics.

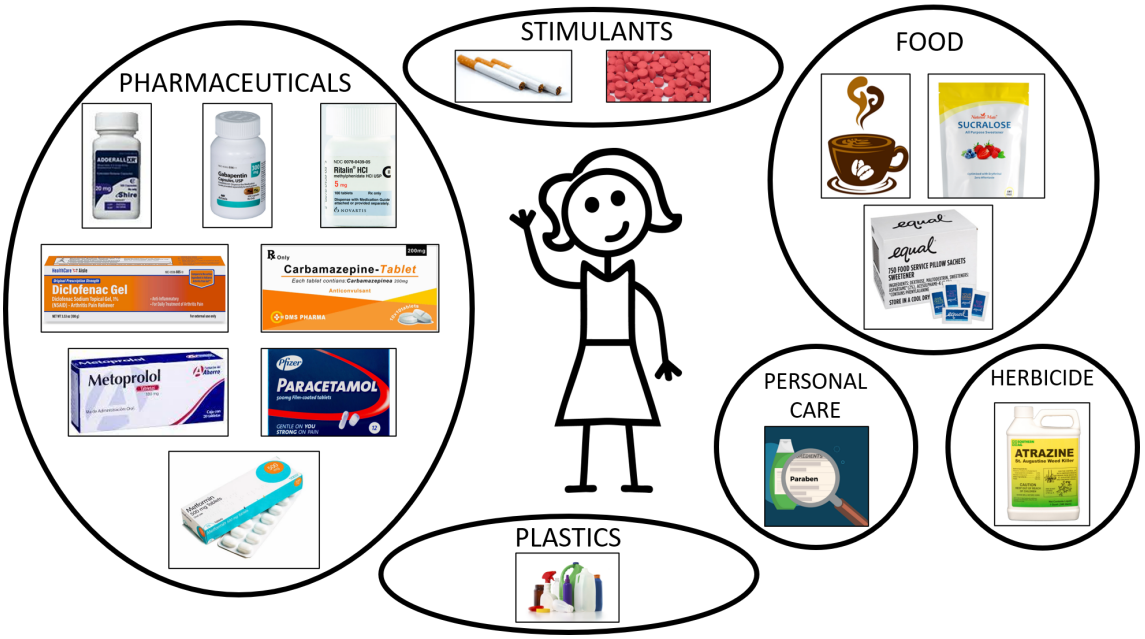


Figure 9: Representation of the population habits.

Table 6 presents the most relevant properties of each compound, lipophylicity, half-life, elimination in the WWTP, principal metabolites and its % of excretion. In the following, more detailed comments on these properties are given for each marker.

The markers related with pharmaceutical products are: carbamazepine and gabapentin, as anti-epileptic drugs; diclofenac, as an anti-inflammatory; metformin, used to treat diabetes; methylphenidate, as a stimulant; metoprolol, as a beta-blocker drug; and paracetamol, a universal analgesic and antipyretic.

Carbamazepine, whose chemical structure is shown in Figure 10, is an anti-epileptic drug, used since the 1970s. Nowadays, it is also used to treat trigeminal neuralgia and manic depressive illness. Although newer drugs for treating epilepsy have been synthesised, carbamazepine is one of the most used. For instance, it ranks number 12 the prescribing frequency list for long term illnesses in Ireland [62]. It is almost completely metabolised in the liver into 10,11-dihydroxy carbamazepine, which has a 33% of excretion rate, and dihydro-10,11-epoxycarbamazepine, with 2% of excretion rate [4]. Only 2-13% is excreted in the unchanged form in urine [63, 4]. Carbamazepine is known to be a persistent drug in the environment [10]. It has been detected all around the world, including Poland [10], Spain [64, 65], Ireland [62], Austria [63, 66], Germany [67, 68], France [69], Ukraine [69], Finland [70], Singapore [71], Japan[35, 72], China [4], Australia [73], Canada [74, 75, 76] and Brazil [77]. Carbamazepine has a half life between 119 and 328 days [38] and is considered moderately hydrophilic, with a log P =2.47 [41]. These two properties makes the compound difficult to remove from the WWTP and makes it persistent in the environment. Indeed, it has been demonstrated that it is extremely resistant to removal in the WWTP, it is neither degraded nor retained [73, 78, 45, 66] and only 7-19% is removed [41]. As a consequence of the low removal, carbamazepine is present in effluent samples, sometimes even with higher concentration, due to the conversion of the metabolites to their parent compound [75]. Because of its specific human source and it high persistence carbamazepine [74, 66],is one of the most widely used markers to evaluate the wastewater contamination in the published literature [7].

Table 6: Summary of the markers and their main properties.

Marker	logP [2]	Half-life	Eliminated in WWTP	Principal metabolites	% excretion
Carbamazepine	2.47	119-328 days [38]	No [10]	M1: 10,11-dihydroxy carbamazepine [4] M2: dihydro-10,11-epoxycarbamazepine [4]	M1: 33 [4] M2:2 [4] Unchanged: 2-13 [4]
Gabapentin	-1.10	20-100 h [39]	Yes [40]	Not substantially metabolised (CITA)	
Diclofenac	4.51	>1000 days [40]	No, <21% [41]	M1: 4-hydroxydiclofenac [42] M2: 5-hydroxydiclofenac [42] M3: 1-O-acyl glucuronide [42]	Unchanged: 15 [40]
Metformin	-2.6	>1000 days [40]	Yes, 94% [10]	Not substantially metabolised(CITA)	
Methylphenidate	0.2	40 h [43]	No [44]	M1: Ritalinic acid [44]	M1: 80 [44]
Metoprolol	1.88	N/A	N/A	M1: Metoprolol acid [4]	Unchanged: 5 [4]
Paracetamol	0.46	3-7 days [38]	Yes [45]	M1: Paracetamol glucuronide [46] M2: Paracetamol sulphate [46]	M1: 47-62 [46] M2: 25-36 [46] Unchanged: 4 [40]
Amphetamine	1.76	N/A	No [47]	M1: 4-hydroxyamphetamine [48] M2: phenyl-2-propanone [48]	M1: 15 [48] M2: 7 [48] Unchanged: 70 [49]
Nicotine	1.17	3 days [50]	Yes [51]	M1: Cotinine [30]	80 (CITA)
Acesulfame	-1.33	N/A	No, <40% [2]	Not metabolised [2]	
Caffeine	-0.07	10 years [52]	Yes [45]	To 20 different dimethylxanthines [52]	Unchanged: 0.5- 10 [52]
Sucralose	-1.00	Several years [2]	No, <20% [2]	Not metabolised [53]	Unchanged: 90 [53]
Methyl-paraben	1.96	<6 h [54]	Yes, 90% [55]	M1: Parahydroxybenzoic acid [56]	N/A
Atrazine	2.61	168 days [57]	No [58]	M1: Deethyl-atrazine [59] M2: Deisopropyl-atrazine [59] M3: Diaminochlorotriazine [59]	Metabolites: 93% [59] Unchanged: 7% [59]
BPA	3.32	15 days [60]	Yes, 60-90% [60]	M1: Glucuronidate-BPA [61]	N/A
BPAF	4.47	N/A	No [60]	N/A	N/A
BPE	3.60	N/A	No [60]	N/A	N/A
BPF	2.91	N/A	No [60]	N/A	N/A
BPS	1.65	37 days [60]	Yes [60]	M1: Glucuronidate- BPS [61]	N/A

[2] Values extracted from PubChem:https://pubchem.ncbi.nlm.nih.gov/

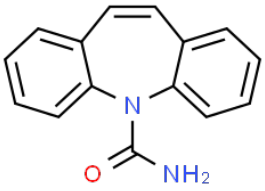


Figure 10: Chemical structure of carbamazepine.

Gabapentin, whose chemical structure is shown in Figure 11, is a non-opioids and non-steroidal anti-inflammatory medicine, used as an antiepileptic and neuropathic pain drug [79]. It is also used to treat panic and anxiety disorders, dementia, bipolar disorders and schizophrenia [80]. Gabapentin is widely prescribed in many countries. For instance, in Czech Republic, 15.9 tons of gabapentin was delivered to pharmacies and medical facilities in 2020 [80]. Gabapentin is not substantially metabolised and it is excreted via urine unchanged [80, 81, 40]. Even though the half-life of the compound is between 20 and 100 h [39], it is efficiently removed in the WWTP, with 80-100% [40], probably due to the compound hydrophilicity (log P = -1.1) . The compound has been detected in the rivers Rhine [82] and Elbe [80], Germany [39], Sweden [40], Norway [83], Latvia [28] and Israel [84]. Due to its high frequency of detection, it has been used previously as a marker for anthropogenic contamination of water [83].

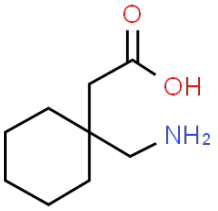


Figure 11: Chemical structure of gabapentin.

Diclofenac, whose chemical structure is shown in Figure 12, is a non-steroidal anti-inflammatory drug with analgesic and antipyretic effects, which is used mainly for arthritis, rheumatoid diseases and to relief moderate pain [85]. It is widely consumed around the world, examples of the consumption rate are: Spain is 2124 μg/capita/day [86], 2613 μg/capita/day for Germany [87] and 2658 μg/capita/day for Turkey [88]. It presents hepatotoxicity in humans [85]. Diclofenac is metabolised in the liver to mostly 4'-hydroxydiclofenac, to 5-hydroxydiclofenac and to 1-O-acyl glucuronide [42] and 15% is excreted unchanged [40]. Due to the big consumption of this medicament, the European Commission has included diclofenac on the list of substances to monitoring for the purpose of facilitating the determination of appropriate measures to address its risk (Directive 2013/39/EU of the European Parliament). It has been detected in Poland [10], Spain [65], Sweden [40], Latvia [28], Finland [70], France [69], Ukraine [69], Singapore [71], China [89], Brazil [77] and Canada [76]. Diclofenac is a persistent compound in water with a half-life higher than 1000 days [40]. While there is not an agreed value in the literature for the removal efficiency, it is generally consider low. Some sources give a range between 10 and 58% [40], while others find it to be less than 21% [41]. Such variability is due to the dependency of the percentage of removal on the temperature and the exposure to natural sunlight [88]. For instance, the exposure to natural sunlight has been shown to increase the degradation of the compound [85, 90]. The resistance of removal is related with

high lipophilicity (log P= 4.51) and the low biodegradability [41]. Diclofenac has been described as one of the most frequently detected drug in water [91] and it has been verified that diclofenac can be used as a chemical indicator of water pollution [77, 7].

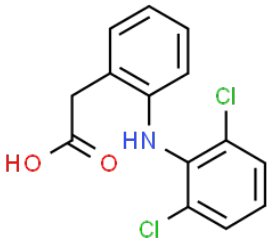


Figure 12: Chemical structure of diclofenac.

Metformin, whose chemical structure is shown in Figure 13, is a drug generally used to treat diabetes type 2, polycystic ovary syndrome, certain cancers and for weight loss [92, 93]. It regulates the glucose levels by activating adenosine monophosphate kinase, impacting cellular energy balance [94]. Around 59 million people are living with diabetes in Europe, which is approximately 8% of the population [95]. Most of the sources [92, 96, 97] indicate that the marker is not metabolised and is excreted in its original form. It must be noted, however, that some studies [40] indicate that only 41% is excreted unchanged, the rest being metabolised. Metformin has been detected in different types of water in Europe, including the river Rhine [82], Poland[10], Latvia [28] and Sweden [40]. It is efficiently removed in the WWTP, with a removal percentage of 94% [10, 45, 40]. According to some studies, it is one of the medicaments most found in water with high concentration [92, 10]. Hence, it is a good option for a marker of contamination and to monitor whether the WWTP works adequately.

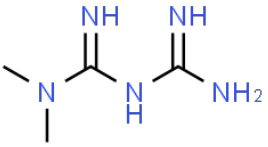


Figure 13: Chemical structure of metformin.

Methylphenidate, whose chemical is shown structure in Figure 14, is a drug used as a mild central nervous system stimulant. It is sold as Concerto® in Europe and Ritalin® in the USA and is mainly used to help with attention-deficit hyperactivity disorder (ADHD) of children and young people [44, 98]. It also helps to increase behavioural alertness, agitation or excitation. ADHD is a neurobehavioural disorder most commonly diagnosed in children in school-age, affecting between 3% and 7% of them [99]. Methylphenidate blocks the uptake of dopamine, an important neurologic messenger substance [44]. It is also consumed illicitly by students, especially common in the USA, where 7% of the students use it regularly to reach higher work productivity [44, 100]. Ritalinic acid is the main metabolite of methylphenidate, with 80% of the substance being excreted in this form in urine [44]. Despite the low percentage excretion of methylphenidate, it has been detected and monitored in waters of Germany [44, 101], USA and Spain [101]. It has poor

biodegradation, a half-life of around 40 h [43] and log P= 0.2. These properties and the stability of the metabolite in the environment, make the compound hard to eliminate in WWTP [44]. The compound is so hard to eliminate that even drinking water plants are unable to remove it from drinking water [44]. Due to the high consumption of this pharmaceutical and its difficult removal, it could be interesting to use it as a anthropological marker.

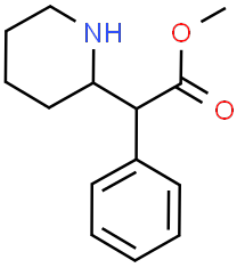


Figure 14: Chemical structure of methylphenidate.

Metoprolol, whose chemical structure is shown in Figure 15, is a beta-blocker medicine used to treat patients with high blood pressure, chest pain and heart failure [102]. It is one of the cheapest drug of this kind on the market, so low income communities have higher consumptions rates [102]. It is metabolised as metoprolol acid and only 5% is excreted in the unchanged form [4]. Not many studies on the removal of the compound in the WWTP. However, low removal efficiency was hypothesised due to the large detection in effluent waters and its high lipophilicity (logP= 1.88), which makes the removal more difficult [103]. It has been detected in Australia [102], Latvia [28], China [4, 89, 104] and Finland [70]. Metoprolol could be used as a marker to monitor its removal rates in WWTP.

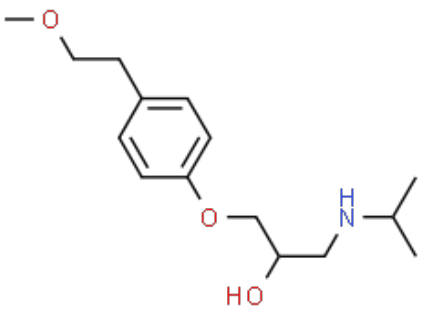


Figure 15: Chemical structure of metoprolol.

Paracetamol, whose chemical structure is shown in Figure 16, also called acetaminophen, is a universally used antipyretic and analgesic medicine [79]. It is one of the most produced and consumed drug worldwide [38]. It is used to reduce fever, relieve coughing, colds, and pain including muscular aches, chronic pain, migraine headache, backache, and toothache. Paracetamol is mainly metabolised in the liver into paracetamol glucuronide (47-62%) and paracetamol sulphate (25-36%) [46] and only 4% is excreted unchanged [40]. Paracetamol has a half-life between 3 and 7 days [38] and it is highly removed in WWTP, with up to almost 100% of removal [45, 19, 105, 106, 40]. Even with the good removal rates, the small

half-life and the low percentage of excretion, Paracetamol has been detected in different waters from many countries, including Poland [10], Latvia [28], Ireland [62], Spain [65], Sweden [40], Greece [107], Singapore [71] and China [89]. It has been successfully used as a wastewater marker and to monitor WWTP malfunctions in various situations [108, 105, 74].

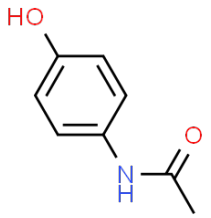


Figure 16: Chemical structure of paracetamol.

The markers selected that are related with stimulants are: amphetamine and nicotine, with its metabolite continine.

Amphetamine, whose chemical structure is shown in Figure 17, also called α-methylbenzeneethanamine, is a strong central nervous system stimulant. In Europe, it is the most commonly used illicit associated with nightlife [109, 110]. Its illegal manufacturing takes place mainly in the Netherlands and Belgium [111]. It is also used legally as a medicine for ADHD. In this context, it helps improve cognitive control, and narcolepsy [99], a profound chronic neurologic sleep disorder associated with excessive daytime sleepiness that affects approximately 0.03% to 0.05% of the general population [112]. Amphetamine presents two main metabolites, 4-hydroxyamphetamine and phenyl-2-propanone, which are excreted in proportions of 15% and 7%, respectivelly [48, 49]. The majority of the compound, around 70%, is excreted in the unchanged form [49]. Amphetamine has been detected in aquatic environments in different countries around the world, such as the Netherlands[110], Spain [113], Malaysia [114] and the USA[115, 47]. The high frequency of detection is caused by a low removal of the analyte in the WWTP [47, 113]. The high consumption rate, legal and illegally, and the inefficiency of the removal, makes this compound a good candidate for a marker.

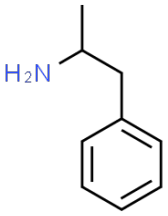
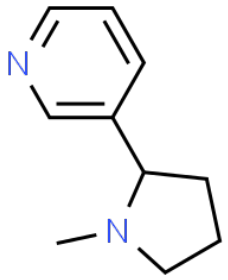


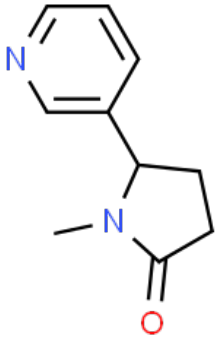
Figure 17: Chemical structure of amphetamine.

Nicotine, whose chemical structure is shown in Figure 18a, is a naturally produced alkaloid in the nightshade family of plants, mostly predominant in tobacco plants, and is widely used recreationally as a stimulant and anxiolytic. Nowadays, more than 1 billion people worldwide smoke cigarettes that contain nicotine [116], including 26% of EU citizens in 2017 [117]. The amount of people consuming nicotine regularly is seven times greater than all the illicit drugs combined, making tobacco one of the most important factors affecting population mortality worldwide [116], not only from direct consumption but also from passive smoking [51]. According to the WHO, in 2030, 8 million of deaths are expected from the tobacco consumption [118]. Seventy to eighty % of the nicotine ingested is metabolised to continine , whose chemical structure is shown in Figure 18b. Continine is later further sub-metabolised into other products. At the

end, only around 10-15% of the product is excreted as continine [30]. This metabolite is much more stable in the water environments than nicotine [30]. It has been studied and detected in countries such as Czech republic [30], Slovakia [30], Portugal [119], Italy[120], Switzerland [51] and Australia [121, 122]. Both nicotine and cotinine have a high elimination rate in WWTP, around 98% [51]. Even though the efficiency of removal is high, it is still detected some countries, as shown before, this implies that the consumption must be very high. Since Poland is the 6th country of the EU in terms of frequency of smoking [117], nicotine and its metabolite could be an optimal option as a marker [123, 124, 120].



(a) Chemical structure of nicotine.



(b) Chemical structure of cotinine.

The selected markers related with food products are: acesulfame, caffeine and sucralose.

Acesulfame, whose chemical structure is shown in Figure 19, is an artificial sweetener, considered to be 200 times sweeter than sugar while only providing a small amount calories [2]. It is used for a considerable variety of products, such as in beverages, pharmaceuticals, mouthwashes and toothpastes [108]. It is also used as a food preservative, with the code E950. Acesulfame consumption ranges from 4.9 to 17.6m g/d/capita, for different European countries [125]. It is not metabolised by the human body and it is therefore excreted via urine and faeces in its original form, from where it reaches the wastewaters [2, 126]. Acesulfame has a log P= -1.33. It is hence quite soluble in water. and it is known to persist in water environments [2, 127], less than 40% is removed [127]. The presence of the analyte in effluent samples proves its low removal [128] and high persistence in the environment [7, 129]. As a result, its concentration is often more than ten times higher than other sweeteners, like sucralose, in wastewater effluents [2, 130]. For these reasons, acesulfame has been suggested as one of the most suitable chemical markers [2, 130]. Indeed, it has been detected in numerous countries, such as Singapore [71], China [131], Germany [129, 127, 68], along the river Rhine [82], Greece (in the island of Lesbos) [132], Finland [133], Spain [128], Switzerland [2], Canada [134] and Sri Lanka [135].

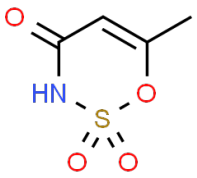


Figure 19: Chemical structure of acesulfame.

Caffeine, also called 1,3,7-trimethylxanthine, has a chemical structure as shown in Figure 20. It is an alkaloid present in numerous plant species, most famously in the seeds of coffee and cacao and in the leaves

of the tea tree. This compound used for different purposes, mainly in beverages (coffee, tea, some soft drinks and chocolate), but also in pharmaceutical products, where it is used as a diuretic and for cardiac, cerebral and respiratory stimulants [52]. It is the most consumed psychoactive in the world [136], with an average global consumption of is 70 mg/person/day. The consumption, however, it varies significantly on the country. For instance, the consumption in the UK is 440 mg/person/day, while it is 210 mg/person/day in the U.S.A, 300 mg/person/day in Switzerland and 140 mg/person/day in Poland [52, 137]. The main source of caffeine intake also depends on the country. In average, 71% of the intake for adults comes from coffee, 16% from soft drinks and 12% from tea, for adults [138]. In the case of children, the main intake comes from carbonated soft drinks [138]. Caffeine is transformed in the liver into more than 20 metabolites, primarily dimethylxanthines (paraxanthine, theobromine, theophylline line), dimethyl and monomethyl uric acids. Between 0.5% and 10% is excreted unmetabolised via urine [52, 136]. Its metabolites are not a good option for markers since most of them have natural sources other than the consumption of caffeine [138]. Despite the low percentage of excretion of un-metabolised caffeine, it is one of the compounds most often detected in high concentration in raw wastewater [75]. It has been detected all around the world, including countries such as Poland [10], Spain [65], Latvia [28], France [69], Ukraine [69], Ireland [62], China [89], Singapore [71], Canada [75, 76] and Brazil [77]. Caffeine has several characteristics important for a good marker of water contamination: it is highly soluble in water (log P= -0.07), it has low evaporation rate and is highly persistence in water, with a half-life of about 10 years [52, 136]. Despite this, it is rapidly degraded in the WWTP [45, 108]. Hence, its presence in effluent wastewaters could indicate a failure on the WWTP. The efficiency of caffeine as a marker for wastewater has been proven in the literature [52, 136, 139, 140, 141, 7, 108]. Despite that, some studies have argued that it is not as a good option [77], since its presence is not restricted to anthropogenic origins. It must be noted, however, that most studies ahve shown that the concentrations of caffeine originating from naturally occuring plant sourncs are usually neglibible [139].

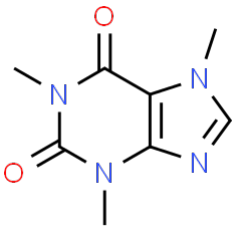


Figure 20: Chemical structure of caffeine.

Sucralose, whose chemical structure is shown in Figure 21, is another non-caloric artificial sweetener, considered to be 600 times sweeter than sucrose, a disaccharide of glucose and fructose [53]. It was discovered in 1976 by the company Tate & Lyle and it is commercially sold under the trade name Splenda® by McNeil Nutritionals since 1999 in the USA and Canada and since 2004 in Europe. Currently, Splenda® is sold in more than 30 countries around the world [3]. Sucralose is a polar, chlorinated sugar containing five hydroxyl groups. It is synthesised from sucrose by the selective replacement of three hydroxyl groups with chlorine atoms [53]. As acesulfame, it is used in food (with the code E955) and beverages. It has been observed that in acid solution, like in soft drinks, sucralose hydrolyses slowly. Therefore, it is necessary to assess its safety in drinks [142, 53]. In 1990 the Joint FAO/WHO Expert Group on Food Additives (JECFA) approved the daily intake of 15 mg/kg/bw (body weight) [143]. Doses up to 3% of the daily intake could

[3] https://www.splenda.com/

cause adverse effects such as pelvic mineralisation [144]. It does not metabolise in the human body and 90% is excreted unchanged via urine and faeces [53, 2, 126]. In 2008 the European Environment Agency expressed their concern about the presence of sucralose in aqueous environment. It came after Scientists from the Norwegian Institute for Air Research and the Swedish Environmental Research Institute found it omnipresent in their waters [53]. Later, a big study carried in 27 different European countries analysed of 120 river samples and it showed that sucralose, which is in use in Europe since beginning 2005, can be found in the aquatic environment, at concentrations up to 1 μg/L [53]. Several studies have demonstrated that sucralose does not undergo through degradation in WWTP: its elimination rate is 20%. Also, it is extremely persistent in the environment, with a half-life of several years depending on the pH and temperature [2]. In Europe, it has been detected in Spain [128, 53], Germany [127, 68], the UK [53], Belgium [53], the Netherlands[53], France[53], Switzerland[53], Italy[53], Norway[53], Sweden[53], Finland [133]. It has also been detected in Asia, including in China [131] and Singapore [71], as well as in America, including Canada[134], the USA [145, 146, 147, 144].Despite this wide detection, the effects of sucralose on the environment have not been deeply investigated yet. Early research shows that it could interfere with the sucrose production in cane sugar, that it could effect alga's photosynthesis and that it could change feeding behaviours of some organisms [148, 149]. Due to its high consumption and low elimination rate, it could be a good option of marker and interesting to monitor.

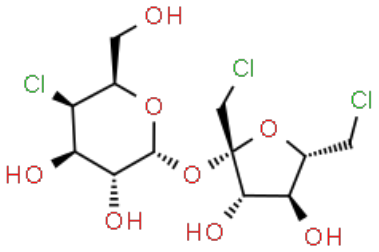


Figure 21: Chemical structure of sucralose.

As a personal care marker, methyl-paraben, whose structure is given in Figure 22, was selected. Methyl-paraben has been chosen as a representative of the paraben family, which is composed of are esters of the 4-hydroxybenzoic acid with an alkyl group. The most common used ones are methyl-paraben, ethyl-paraben, propyl-paraben, butyl-paraben, benzyl-paraben, isopropyl-paraben and isobutyl-paraben. They are present in a big variety of products, like pharmaceuticals, shaving gels, soaps, shampoos, lotions, canned foods and beverages [150]. Parabens have been used for almost 100 years due to their anti-fungal and anti-bacterial properties, affecting mainly gram-positive bacteria, moulds and yeast [151]. In particular, methyl-paraben is often used in a variety of cosmetics and personal-care products as well as in food as a preservative (E218). Studies have shown that these compounds have a potential carcinogenic and oestrogenic threats [152]. Despite that, the Scientific Committee on Consumer Products (SCCP) of the European Union assessed the risks of this family in 2005 and concluded that methyl and ethyl-parabens are safe when used in cosmetics with a concentration under 0.4% [56]. The European Union also authorised the use of parabens as a food additive by the Regulation (EC) No 1333/2008. In contrast, there are no regulations concerning their presence in the environment [151]. After consumption and dermal adsorption, parabens

are highly metabolised in the liver and intestine, mainly to parahydroxybenzoic acid, which is then excreted in urine and faeces [56]. Despite their high metabolisation, parabens have been detected in water in various countries, such as Spain [153, 154, 155], Portugal [150], Switzerland [156], Canada [157], the USA [158] and China [89]. At the same time, it has been proven that WWTP remove efficiently all parabens and exceeds more than 90% [55, 151, 156]. Hence, they may be a good option as a marker for anthropogenic contamination and to evaluate WWTP efficiency [151].

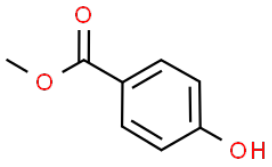


Figure 22: Chemical structure of methyl-paraben.

For a marker representing the agricultural life, the herbicide atrazine was selected.

Atrazine, whose chemical structure is shown in Figure 23, is a herbicide, which belongs to the s-triazines group. The s-triazines are a family of herbicides intensively used for agriculture worldwide since 1960 to control pre- and post-emergent weeds [76]. Its used was banned in the EU since 2004 [132], it is present on a list with other polar contaminants that pose a toxicological threat to aquatic ecosystems, which are of inte-rest to monitor [58, 132], (EC, 2015). In the USA, it is still in use with approximately $3.3x10^7$ kg/year [76], making this herbicide among the most frequently detected in water environments [76]. Globally, atrazine is probably the most used herbicide, used in around 80 countries [58]. Atrazine is extensively metabolised by the human body with more than 25 different metabolites identified [59]. The main metabolites, obtained through dealkylation processes, are deethyl-atrazine, deisopropyl-atrazine and diaminochlorotriazine. Together, these metabolites present 93% of the excretion of the analyte [59]. Studies have shown that atrazine presents moderate acute oral toxicity in rats, but it is not likely to posses carcinogenic risk to humans [59]. It is a very persistent compound, hydrophobic and with a half-life of 168 days in water exposed to sunlight [57]. Consequently, it is a difficult compound to remove in WWTP [58, 159]. Atrazine has been detected mostly on lakes and in inland seas, where disposals from agriculture are sent, in countries where it is legal and where is not [58]. Some of these places are Canada [75, 76, 76], in the USA [76], in the Black, the Aegean, the Mediterranean and the Baltic sea [58], in Germany [160, 159] and in Lesbos Island, Greece [132]. In Germany, atrazine was forbidden in 1992 and it is detected in the aquatic environments more than 20 years later [159], proving its persistence. Atrazine and its metabolites could be interesting options to use as a chemical marker to monitor aquatic contamination, due to agricultural activities, and its persistence [75, 132].

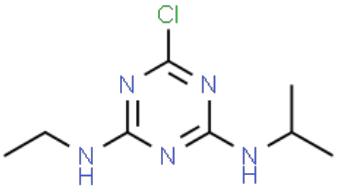


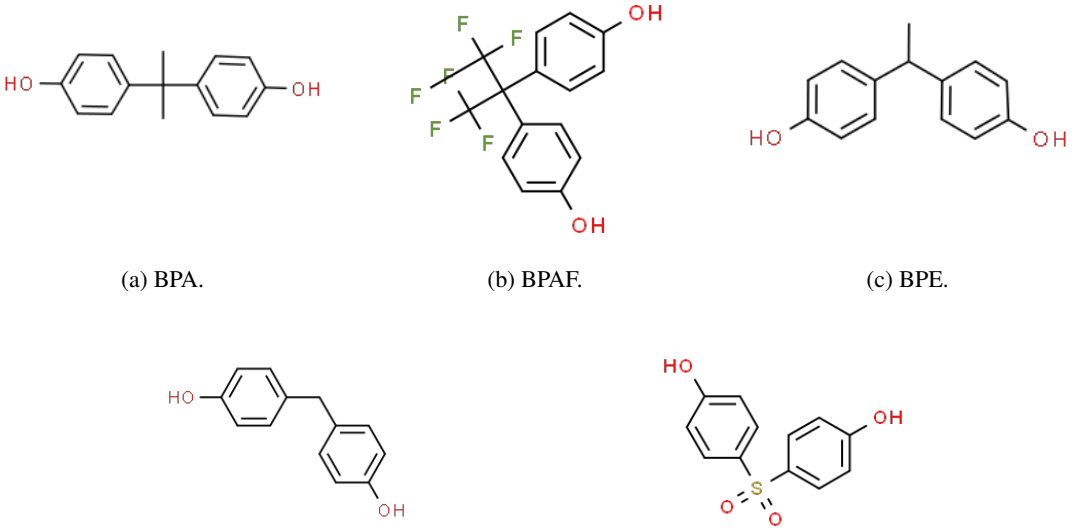
Figure 23: Chemical structure of atrazine.

The markers related with the plastic usage are five different bisphenols: BPA, BPAF, BPE, BPF and BPS.

Bisphenols (BP) are of organic compounds with two hydroxyphenyl functional groups [60]. There are different types of BP, used in a grand variety of products, mainly for plastics. The most commonly synthesised are bisphenol A (BPA), bisphenol AF (BPAF), bisphenol E (BPE), bisphenol F (BPF) and bisphenol S (BPS) [161]. BPA, whose chemical structure is shown in Figure 24a, chemically called 2,2-(4,4'-dihydroxydiphenyl) propane, has been in used since 1930 for the manufacture of polycarbonate plastics and epoxy resins with a wide range of daily life products, from electronics and toys to food protection [60, 162]. Its useful properties, including low volatility and moderate water solubility has made its use widespread. The global demand for BPA in 2006 was 3.9 million tones [162] and it kept increasing since then. Despite that it has been demonstrated that it presents endocrine disrupting activity [60, 163, 65]. Due to the potential hazard, the European Food Safety Authority has limit the intake to 0.04 ng kg weight^{-1} day^{-1} (EFSA, 2021). As a consequence of the low limits, companies have started using BPA analogues, with similar characteristics but with no demonstrated disrupting activity. BPAF, BPF and BPS (chemical structures in Figure 24b, 24d, 24e, respectively) are the main substitutes of BPA in the manufacturing of polycarbonate plastics and epoxy resins[60]. BPS is also used in the thermal paper [60]. Recent studies have shown that the analogues also present disrupting activity, but it has yet to be regulated [161, 61]. BPA and BPS are highly metabolised in the liver, mainly as glucuronidated metabolites and then are excreted in the urine [61]. These metabolites do not present oestrogenic disrupting activity [61]. These BPs are widely detected in wastewater samples. BPA has been detected in Spain [65], Germany[164], detected in UK [163], China [60] and Singapore [71, 36]. And BPAF, BPE, BPF and BPS have been detected in China [60]. The removal in WWTP depends on the compound and their lipophilicity (see Table 7), the higher the log P, the higher the lipophilicity and the higher difficulty of removal. BPS is easily removed during the treatment, BPA removal efficiencies vary from 60 to 90% and the rest are resistant to biodegradation [60]. Some studies have shown that some microorganisms, such as B. thuringiensis, S. meliloti and T. viride, could degrade BPA and other BP, helping reduce their environmental impact and toxicity [165]. In conclusion, BP are good candidates for chemical markers due to their high usage, easy detection and the difficulty of removal in ordinary WWTP.

Table 7: Lipophicility values for BPs analogues.

	BPA	BPAF	BPE	BPF	BPS
log P [4]	3.32	4.47	3.60	2.91	1.65



(a) BPA. (b) BPAF. (c) BPE.

(d) BPF. (e) BPS.

Figure 24: Chemical composition of bisphenols.

7.4 Semi-targeted analysis

It is not trivial to perform semi-targeted analysis, due to the high amount of compounds that could be detected in the sample and the lack of description of the process of analysis in the literature. Semi-targeted analysis has a similar approach, possible compounds are selected and search in the sample.

Semi-targeted analysis represents an intermediate analytical approach that relies on a MS full-scan screening to identify compounds known from literature without using standards.

The program used is for analysing the chromatograms is *MassHunter Workstation Software, qualitative analysis, Version B.04.00, build 4.0.479.5, Service Pack 1 with BioConfirm Software, Agilent Technologies, Inc. 2011.*

Here are detailed instructions based on personal experience on how to perform the analysis. In Figure 25, a flow chart with the procedure is presented.

[4]Values extracted from PubChem:https://pubchem.ncbi.nlm.nih.gov/

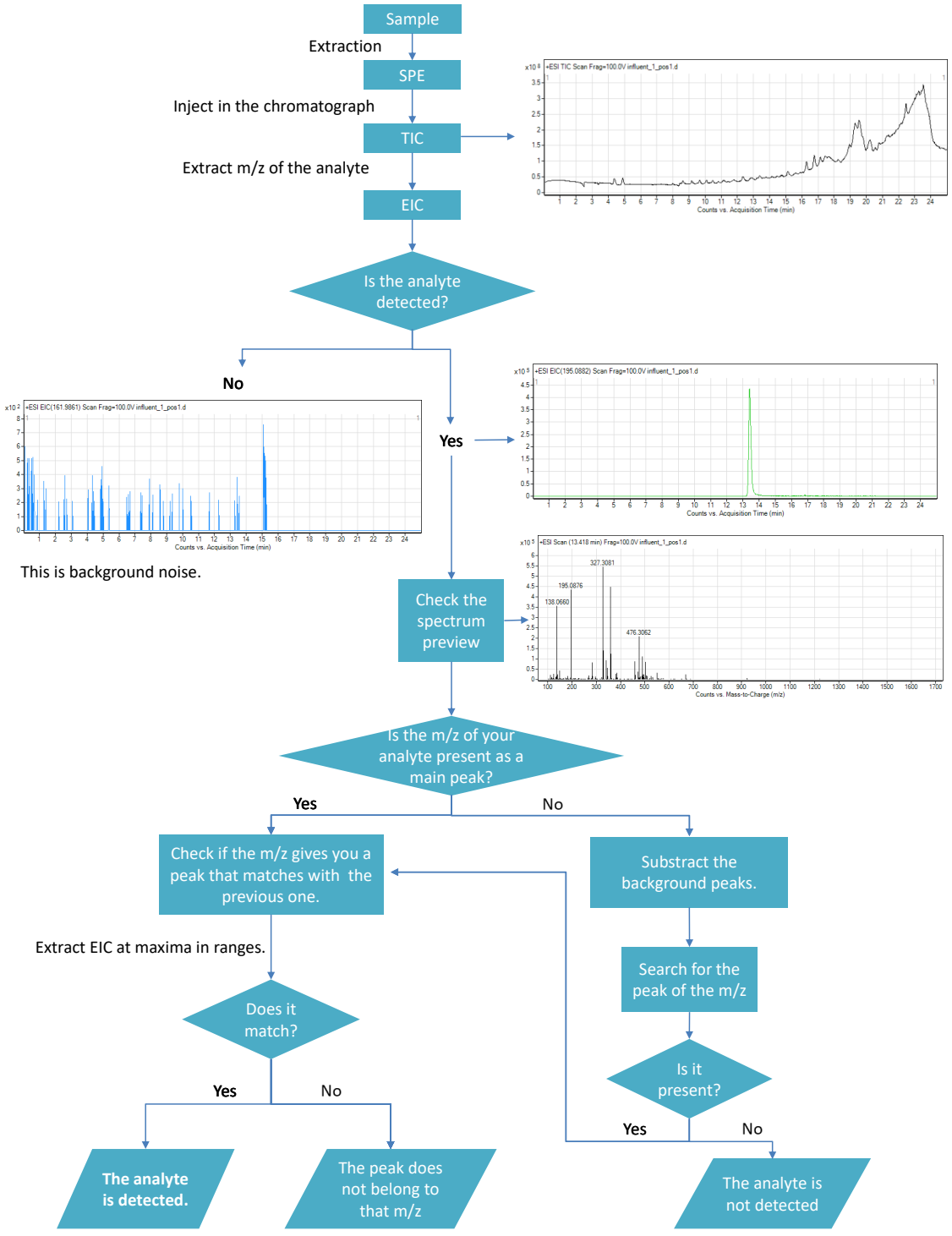


Figure 25: Flow chart of the semi-targeted analysis procedure.

Instructions for semi-targeted analysis:

1. Start for searching in the literature for a molecule that could be present in the sample.
2. Next, calculate for the monoisotopic mass of that analyte, for both ionisation modes, $[M+H]^+$ and $[M-H]^-$.
3. Inject the sample in the chromatograph.
4. Then, in the program, open a data file to see the total ion chromatogram (TIC).
5. Next, right click on the TIC representation, extract chromatogram to display the extract chromatograms dialogue box.
6. If there is more than one data file open, select from which data files you want to extract a chromatogram. To select more than one, press Ctrl key while selecting the files with the left mouse button.
7. After, select the Type of chromatogram to extract, EIC (extracted ion chromatogram) in this case. Extract the m/z value of the analyte of interest.
8. Next, the EIC is displayed in the Chromatogram Results window and to confirm the presence of the analyte, check if peak appears and it is more than 3 times bigger than the background noise. If it is, continue in point 9. If there is not a peak, the analyte is not detected in the sample.
9. To check if the peak obtained is your molecule or not, check the MS spectrum. It is present in the "spectrum preview" section. To check the MS spectrum of the peak, click on the maximum intensity of it. Is the m/z of the analyte present as one of the main peaks?

   In case of the answer being yes (the m/z of the analyte is presented as one of the main peaks):

   Check that the m/z gives a peak that matches with the previous one. Procedure to check if it matches is:

   (a) First, select the peak, left click and drag.

   (b) Next, right click on the mouse and extract EIC at maxima in ranges.

   (c) Then, select only the two chromatograms, the extracted at the beginning and the extracted from the MS spectrum. In Figure 26, a screen print of the data navigator section is presented.

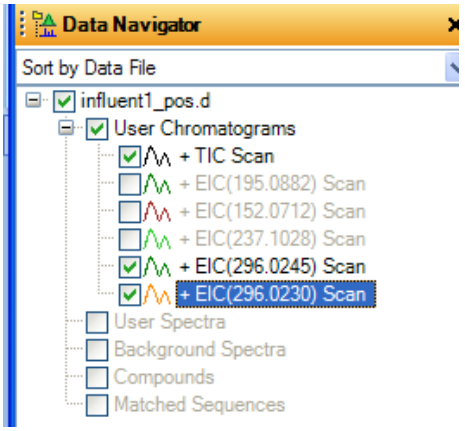


Figure 26: Printed screen of data navigator section.

(d) Click overlaid mode on the "chromatogram results" bar, on the chromatogram results section, which will overlay the chromatograms selected.

Repeat the same procedures with all the main peaks and check if they have the same retention time. An example is presented in Figure 27 and it is a chromatogram with four different m/z. In this case, the green line is the analyte of interest with m/z of 138.0660, the pink like has 476.3060 m/z, the black one 327.3079 m/z and the red one has 359.3341 m/z. It can be observed that the red and black line, clearly do not match with the green one.

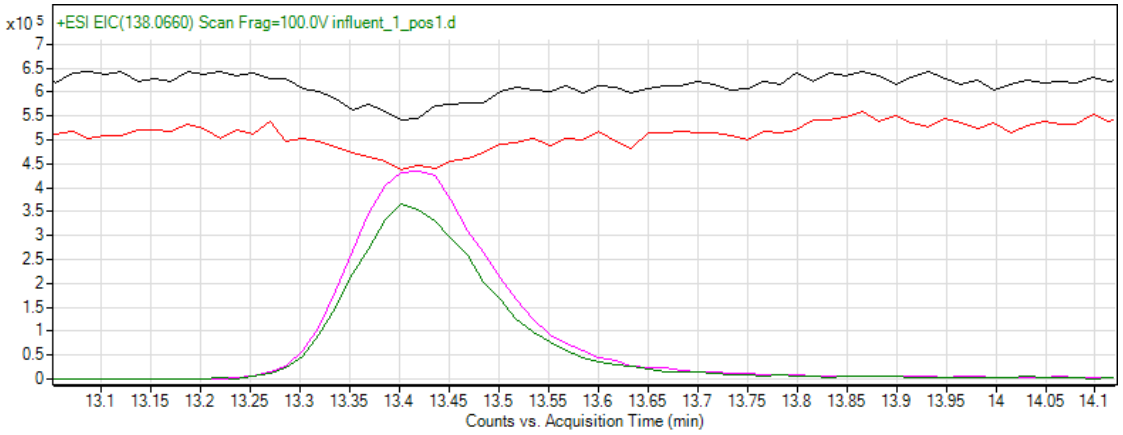


Figure 27: Chromatograms with peaks that do not match. (change)

In case of the answer being no (the m/z of the analyte is not presented as one of the main peaks):

Subtract the background MS peaks. How to do it:

(a) Select in the chromatogram, a point just before the peak. The zone is represented with a blue line in Figure 28.

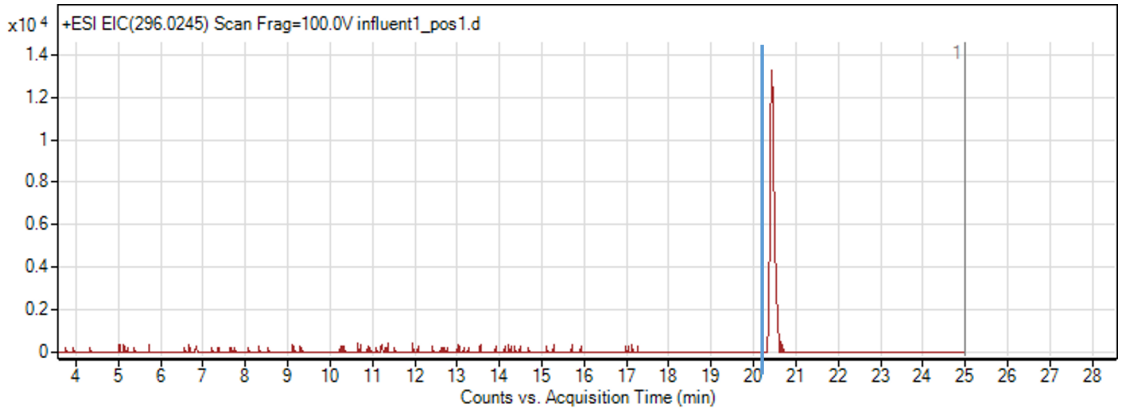


Figure 28: Chromatogram with a blue line marking where the peak begins.

(b) Next, go to the MS spectrum, right click and move to "Background spectrum". Under the section called "Background Spectra", the background spectrum will appear.

(c) After that, select in the chromatogram the maximum of the peak.The zone with the maximum of the peak is represented with a blue line in Figure 29.

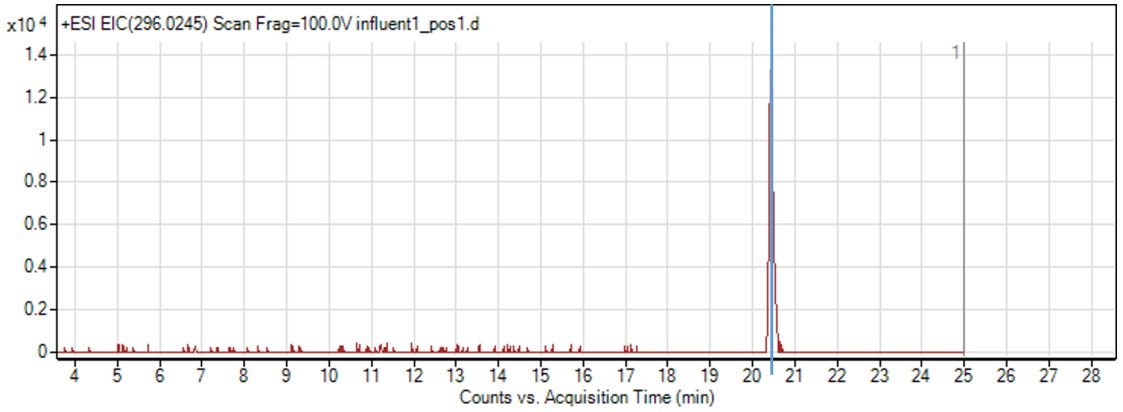


Figure 29: Chromatogram with a blue line marking where the maximum of the peak is.

(d) Go to the MS spectrum, right click and "Copy to user spectra". Under the section called "User spectra", the spectrum will appear with the retention time of the peak, right click and "Subtract background spectrum". The spectrum obtained has the background noise removed.

(e) Then, search for your m/z and follow the same procedure as the point 9.a.

8 Results and Discussion

In the results and discussion section, different comparisons were performed for the data obtained from the semi-targeted analysis. There is a study about the seasonal effect of the markers detected, performed comparing the areas, a study of the shift of the retention time, a comparison of two different the pHs for the mobile phase, a comparison between the semi-targeted and the targeted analysis, a principal component analysis and a statistical evaluation of the performance of the WWTP.

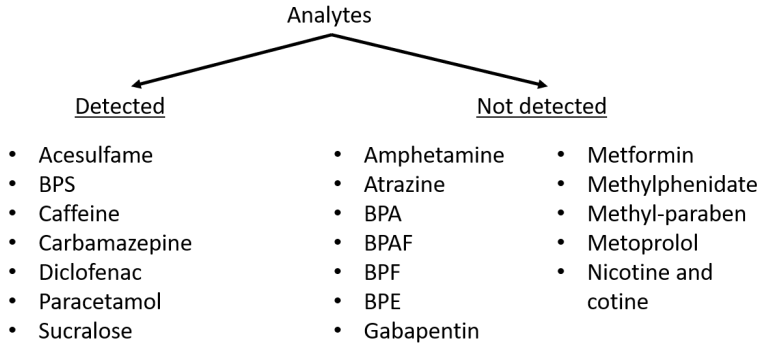


Figure 30: Schematic representation of the detected and not detected markers in the samples evaluated during this study.

The reason for not all the compounds search are present in this section is that some of them were not detected with the method used, most probably, they were under the limit of detection. Hence, they are not present in the graphical representation. These compounds are metformin, methylphenidate, metoprolol and gabapentin, from the health block; four of the bisphenols, BPA, BPE, BPF, BPAF; amphetamine, nicotine and cotine, its metabolite, from the stimulants section; atrazine from the herbicides; and methyl-paraben, from personal care. A schematic representation of them is shown in Figure 30.

8.1 Markers detected in the wastewater samples

Data in Table 8 shows the markers selected and found in semi-targeted analysis, the ionisation mode, their retention time, the theoretical mass/charge ratio and the experimental one and the frequency of detection of the markers found.

In mass spectrometry, the analyte needs to be ionised to be detected. In this case, it is ionised using electrospray. Compounds can be ionised in positive or negative mode, $[M+H]^+$ and $[M-H]^-$ respectively, depending on the affinity of the compound to electrons. The applied mode of ionisation is present in the second column in Table 8. The next column is the average retention time in minutes of the analyte for the different months detected during the study. The following two columns in Table 8 show the theoretical and experimental mass/charge ration (m/z). The theoretical m/z ratio was obtained using a monoisotopic calculator [5] and the experimental mass/charge ratio was obtained from the results of the analysis. The last column in Table 8 shows the detection frequency, calculated using the percentage of detection for the analyte.

The mass measurement error or accuracy, Δppm, was used to avoid false positives identification, which would mean that another compound was detected. Δppm was calculated using Equation 1 [166]. It is important to have the error as close to zero as possible, high value could mean a false positive identification.

[5] Scientific Instrument services: https://www.sisweb.com/referenc/tools/exactmass.htm

Table 8: Markers detected in the wastewater samples.

Analite	Type of water sample	Ionisation mode	t_R (min)	$m/z_{theoretical}$	$m/z_{experimental}$	Detection frequency (%)
Acesulfame	Influent	ESI -	5.7	161.9861	161.9857	82
Bisphenol S	Influent	ESI -	13.4	199.0759	199.0778	55
Caffeine	Influent	ESI +	12.2	195.0882	195.0874	100
Carbamazepine	Influent	ESI +	17.0	237.1028	237.1018	95
	Effluent	ESI +	16.6	237.1028	237.1018	88
	Disposal	ESI +	16.6	237.1028	237.1018	88
Diclofenac	Influent	ESI +	20.6	296.0245	296.0245	64
	Influent	ESI -	20.9	294.0089	294.0085	77
	Effluent	ESI +	20.3	296.0245	296.0237	85
	Effluent	ESI -	20.3	294.0089	294.0082	81
	Disposal	ESI +	20.3	296.0245	296.0233	88
	Disposal	ESI -	20.3	294.0089	294.0082	100
Paracetamol	Influent	ESI +	9.0	152.0710	152.0703	91
Sucralose	Influent	ESI -	12.1	395.0067	395.0060	100
	Effluent	ESI -	11.2	395.0067	395.0054	73
	Disposal	ESI -	11.3	395.0067	395.0045	88

No references in the literature were found on which value to use as a limit, however, based on experience of researchers presenting the data, we settled 10 as a maximum value for Δppm. All the results with Δppm>10 were eliminated.

$$\Delta ppm = \frac{m/z_{\text{theoretical}} - m/z_{\text{experimental}}}{m/z_{\text{theoretical}}} * 10^6 \tag{1}$$

Acesulfame, BPS, caffeine and paracetamol were only detected in the influent sample. Acesulfame with 82%, BPS with 55%, caffeine with 100% and paracetamol with 91%. Therefore, they were not detected for effluent and disposal samples. This implies that they were removed efficiently from the wastewater. Carbamazepine was detected in the three types of sample, in 95% of the influent samples and in effluent and disposal with 88% of detection frequency. This could mean that some of the analyte was removed during the cleaning processes on the WWTP. The same happened with sucralose, 100% of detection for influent, 73% for effluent and 88% for disposal. Diclofenac was detected in the three kinds of sample too and with both ionisation modes. For influent, the frequency of detection was lower than in the effluent and disposal.

In my opinion, there are three possibilities on why the percentage of detection increases between the influent, effluent and disposal.

The first hypothesis is related with the lipophilic character of the analyte. The lipophilic analytes are collected in sludge during the cleaning process, when the maximum concentration accepted in the sludge is reached, no more compounds can be adsorbed and they are not efficiently removed from the water.

The second option is related with the metabolites of the analytes, when the metabolite is converted to

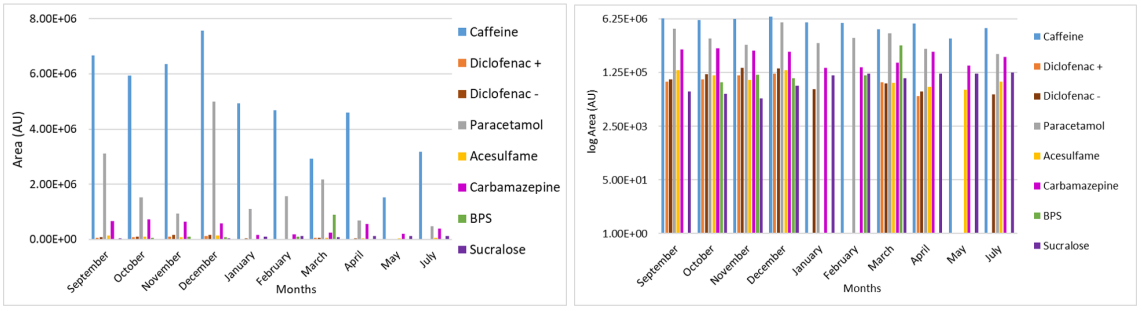
their parent compound after the treatment [107], hence the selected marker is detected.

The third hypothesis is that the three samples were collected at the same time, this means that the wastewater collected is not the same water after the cleaning process, therefore the detected concentration might be higher in some of the samples.

8.2 Seasonality in the marker's response

The goal of this section is to study whether there are seasonal effects in the detection of the markers. For that, the areas detected for each analyte were compared through the year, trying to identify any significant variation.

As shown in Figure 31a, the markers are present in the sample with very different concentrations. Hence, if they are plotted in the same graph the difference between each month and compound can only be appreciated for those compounds detected with higher concentrations. Putting the area in the logarithmic scale, as shown in Figure 31b, equalises the results and allows for a better comparison. Nonetheless, it also hides the differences that we want to observe. Therefore, the areas of each month for each marker are normalised by its annual mean. This results, which allow focusing on the variability, are shown in Figure 32.



(a) Linear scale.

(b) Logarithmic scale.

Figure 31: Representation of the areas of the detected markers in the influent sample.

In the influent sampling point, shown in Figure 32, seven of the selected markers were detected. Error bars show the standard deviation between the replicates. It is important to point that there was no sample for June and August to analyse. In Figure 33, a box plot is presented to help visualising whether there are any outliers among the months.

Generally, the standard deviation calculated with replicates, and presented in the error bars, was smaller than 10%, which is the expected range in these types of analysis due to the low concentration detected [3]. The deviation between replicated was higher than 10% with paracetamol in October, December and February and for acesulfame in October and December. This issues could be solved by performing more replicates and monitoring, but the original samples were not available any more.

The area of caffeine peaks, shown in light blue bars, is similar in all months. The normalized values fluctuate close to one, the mean, never surpassing 1.5 and only going below 0.5 on May. It is interesting to point that from September to December the values are above 1 and from march to July below, which could indicate a small seasonal effect, indicating more consumption of caffeine in the colder months. Observing the box plot, there are no outliers.

Diclofenac is shown in light (positive ionisation) and dark orange bar (negative ionisation). In its positive ionisation mode, the marker is not detected in January, February, May and July. This makes it difficult to use it as a marker, given that its concentration is often too low to be detected using these method. The

area in the detected months is more or less constant, staying between 0.5 and 1.5 times the mean value. The negative ionisation mode seems a better marker candidate, as it is detected in all months except for February and May. However, the variation of its concentration is larger, reaching twice the mean in November and December. Similarly, it is below 0.5 times the mean in several months. It is interesting that from September to December the values are above the mean and, from March to July, bellow. This could indicate a small seasonal effect. According to the calculation performed to build a box plot, there are not outliers in either of the cases.

Paracetamol, shown in grey bars, was detected in all months except for May. The concentration in December stands out as much higher than the others with a high dispersion. The box plot indicates that there is one outlier, which is one of the replicates from December. Perhaps this is due to a high cold season, more replicates and long-therm monitoring should be performed to confirm this hypothesis.

Acesulfame, Carbamazepine and Sucralose, shown in yellow, pink and purple bars respectively, are all detected in almost all months. Acesulfame is not detected in January and February. Both acesulfame and carbamazepine show fairly constant concentrations, although the area is seems to be higher between September and December. Sucralose, instead, shows higher areas between January and July. According to the box plot, there are no outliers for any of these markers.

BPS, shown in green, is only detected in some of the months with a small area, except on March, where the area is very big compared to the rest. In the box plot, it is shown that both of the replicates in March are outliers.

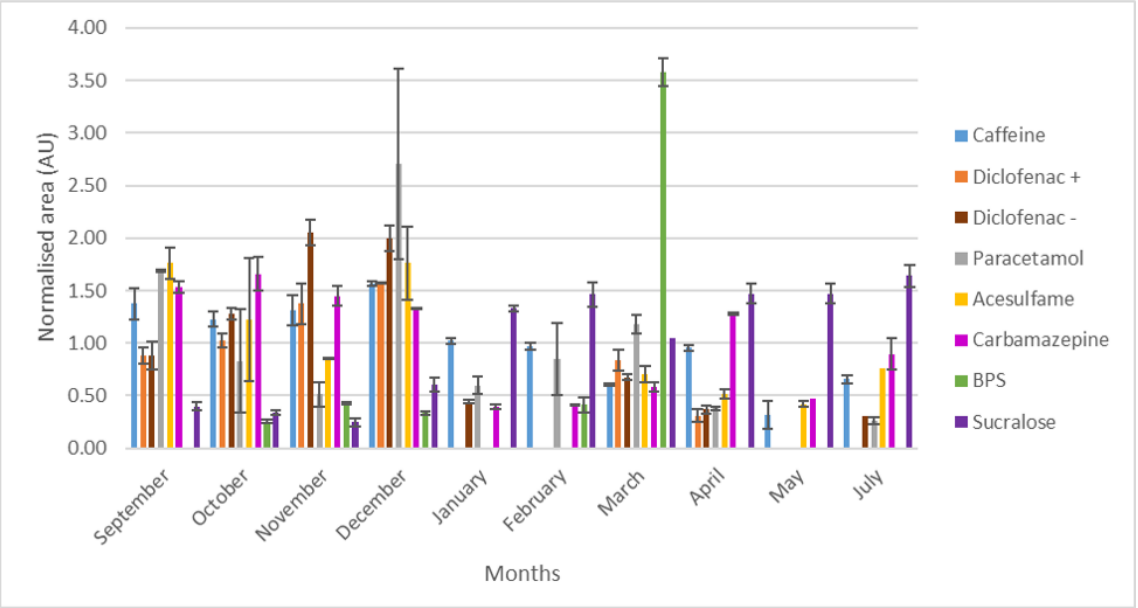


Figure 32: Normalised peak areas of the detected markers in the influent wastewater sample.

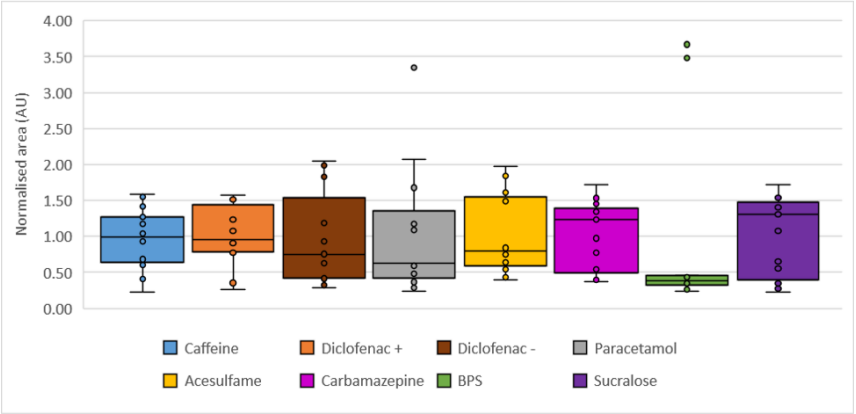


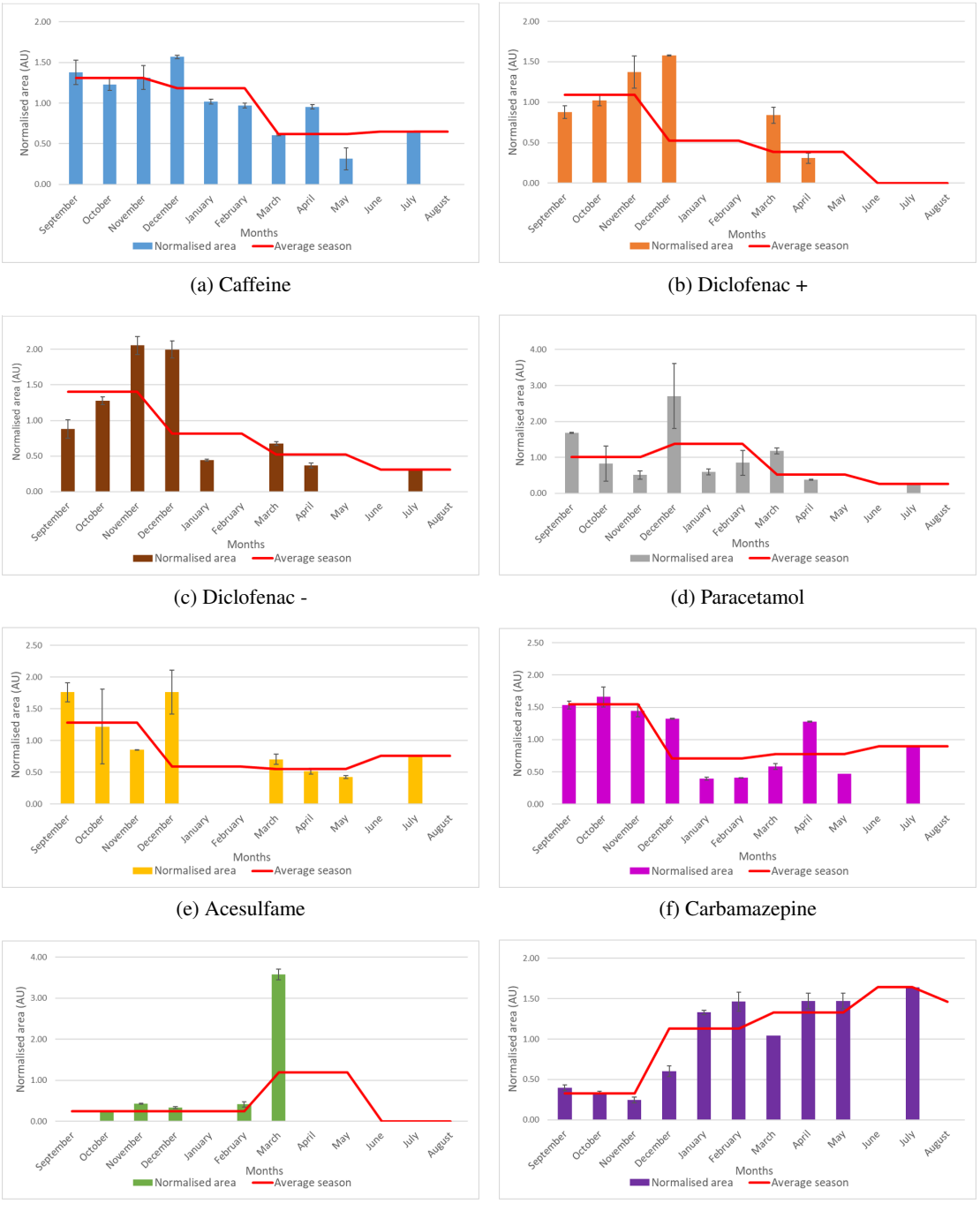
Figure 33: Box plot representation the detected markers detected in the influent wastewater.

To check if the differences observed during the different months are significantly different, Analysis of variance (ANOVA) was done. One-way ANOVAs were performed, comparing the samples of the different months for all the analytes using two replicates. In Table 9, the results of these ANOVAs are presented. It can be observed that there is a significant difference in every case for all the analytes, the calculated F is always bigger than the tabulated F value. However, are these differences seasonal? To check this point, Figure 34 shows the normalised areas of each analyte with their own error bars. The seasons considered are autumn (September-November), winter (December-February), Spring (March-May) and summer (June-August). The mean for summer must be looked at with care as it is not representative, it shows only the results from July (there were no samples from June and August). Nonetheless, it is added for completeness. The plots reveal that the variability is reduced when season averaged values are considered as compared to monthly values. However, the area for most of the analytes decreases during the warmer months. The only exception for this trend is sucralose. A hypothesis to explain this decrease during the summer is the photodegradation of the compounds due the increase of UV light, which could cause the fragmentation of double bonds. It should be noted that sucralose is the only marker without double bonds, and it is also the only one not showing a decrease in warmer months. Another option could be that more sucralose is consumed during the warmer months due to the increase of consumption of carbonated drinks.

Table 9: Analysis of variances for the markers in the influent sample between the different months.

Analyte	Caffeine	Diclofenac +	Diclofenac -	Acesulfame	Carbamazepine	Sucralose
F	42.79	36.93	131.85	8.85	71.94	116.80
F crit	3.02	4.39	3.87	3.87	3.23	3.23

Analyte	Paracetamol	Paracetamol no-outliers	BPS	BPS no-outliers
F	8.97	9.05	906.79	9.32
F crit	3.23	3.50	5.19	6.59



(a) Caffeine

(b) Diclofenac +

(c) Diclofenac -

(d) Paracetamol

(e) Acesulfame

(f) Carbamazepine

(g) Biphenol S

(h) Sucralose

Figure 34: Monthly (bars) and season average (red line) areas for each detected marker.

In general, all markers are detected in almost all the months, with the exception of diclofenac and BPS. The other compounds seem to be detected in a sufficiently consistent manner to act as markers. While the areas have shown to be statistically significantly different, the variation is not large enough to disregard the compounds as markers. Indeed, it is common in the literature to accept markers with this range of variability [167]. Moreover, the seasonality of the results seems to be small enough to allow the use of the compounds as markers. However, further studies on the reproducibility of the method should be conducted

before affirming with certainty that there is no seasonal variation. Hence, more samples, spanning the larger time period, would be needed.

In the effluent sampling point, three of the selected markers were detected. The same graphical representations as with the influent are presented. In this case, unlike for the influence samples, there is a sample from June.

Figure 35 shows the monthly detection areas for the effluent waters. In this case, only diclofenac (in both ionization modes), carbamazepine and sucralose were detected. Hence, only these markers are shown in the graphs. The error bars for the different months and markers are smaller than 10%, except for sucralose in March. A box plot to analyse the presence of outliers is shown in Figure 36.

Diclofenac in the positive and negative ionisation mode presented the same behaviour. Only diclofenac detected using the negative mode is not detected in October. It was detected in almost all the samples with normalised areas around the mean, with the exception of December, where the areas were higher, and March and April, where the areas were smaller than 0.5. As shown in the box plot, there are no outliers.

Carbamazepine and sucralose were detected in almost all the months, with normalised area around the mean. For carbamazepine the only exception is in September, where the area is bigger, and observing the box plot, it is not an outlier. Sucralose is not detected in October and November. For the other months, the concentration is around the mean, except for April, where the area is much higher. The box plot shows that these two replicates for April are outliers.

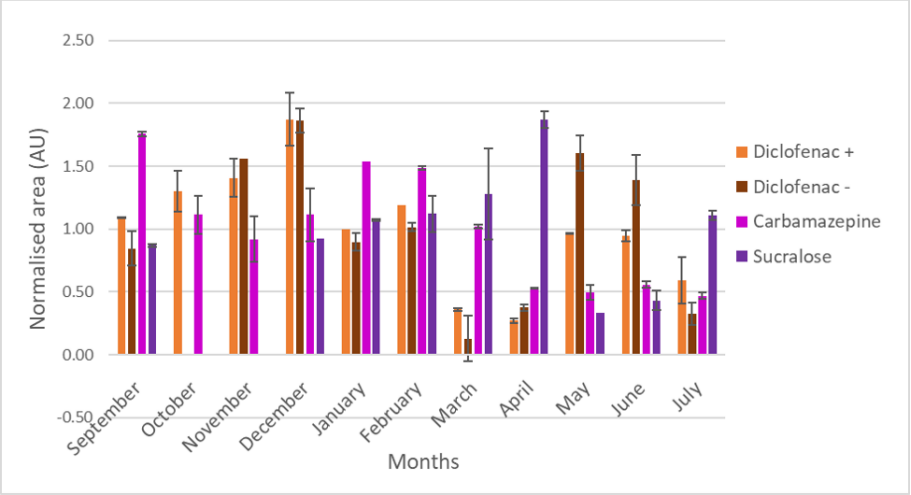


Figure 35: Normalised peak areas of the marker in the effluent wastewater sample.

Once again, to check if the differences between months are significantly different, one-way ANOVAs were performed. The results are presented in Table 10. It can be observed that for all the cases, the area between months is significantly different with 95% of confidence level. However, looking at the average per season presented in Figure 37, the difference between seasons does not seem to differ significantly enough to avoid considering them as markers.

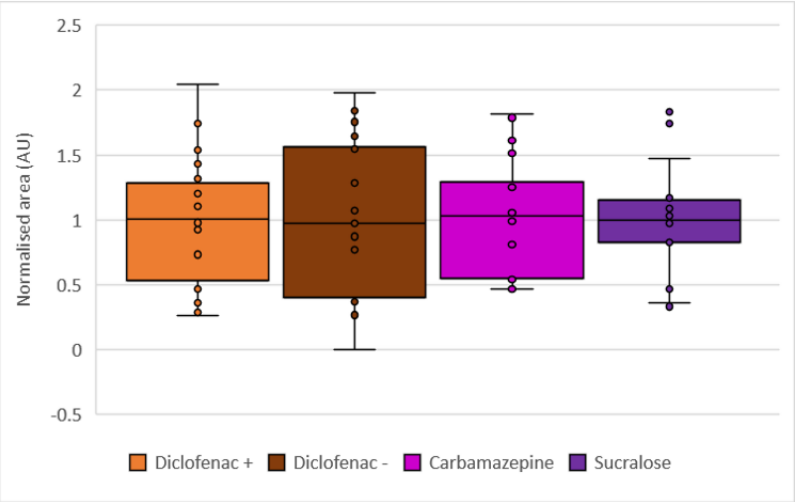
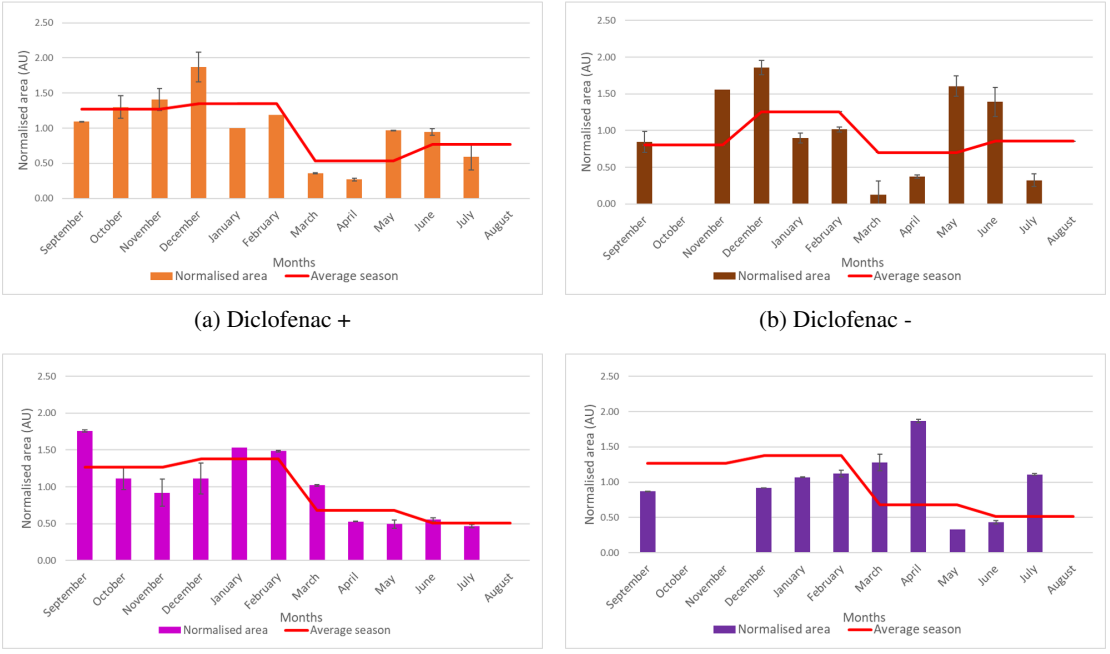


Figure 36: Box plot representation for the markers detected in the effluent wastewater.

Table 10: Analysis of variances for the markers in the effluent sample between the different months.

Analyte	Diclofenac +	Diclofenac -	Carbamazepine	Sucralose
F	36.32	47.71	36.73	16.53
F crit	3.23	3.23	3.02	4.39



(a) Diclofenac +

(b) Diclofenac -

(c) Carbamazepine

(d) Sucralose

Figure 37: Monthly (bars) and season average (red line) areas for each detected marker.

Summarising, the three compounds might be considered as markers for effluent samples. They present fluctuations between the different months, but using long-therm monitoring the spread will decrease and it could help when trying to improve the removal of these compounds in wastewater.

In the disposal sample, the same markers as in the effluent sample were detected. The sample was collected only on November, March, April, May, June and July. No study of the seasonal effect can be performed due to the lack of representativeness.

In Figure 38, the areas of the markers are presented. The markers were detected in almost all the months, except diclofenac - and sucralose in November, and the only one detected in April was diclofenac -.

Diclofenac with positive ionisation was detected with higher area in November and March and lower during May and July. The error bars are lower than 10% except in November, where the dispersion between the replicates is bigger. For diclofenac detected with the negative ionisation mode, the area is bigger during March and again lower for May and July. The dispersion of the replicates is below 10% except for the sample from April. No outliers were observed in the box plot. Carbamazepine and sucralose were detected with areas around the mean in all the months.

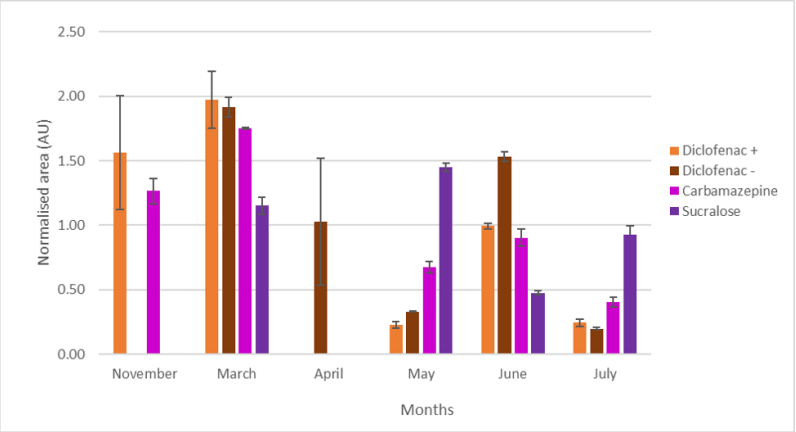


Figure 38: Normalised peak areas of the marker in the disposal wastewater sample.

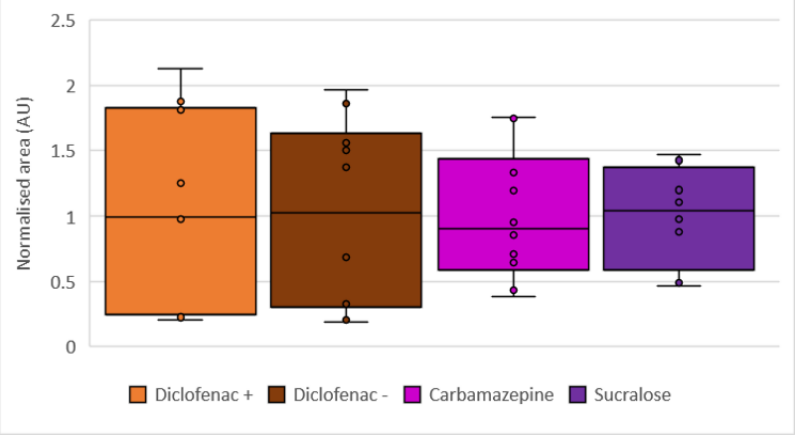


Figure 39: Box plot representation for the markers detected in the disposal wastewater.

The ANOVAs showed that the areas between the months are significantly different. As said before, the seasonality effect cannot be analysed for this sampling point due to the lack of samples and representativeness.

In conclusion, in this section, the area of the markers in every month was compared to see if there was a seasonal effect, meaning that the area changed depending on the consumption in each month. For

Table 11: Analysis of variances for the markers in the disposal sample between the different months.

Analyte	Diclofenac +	Diclofenac +	Carbamazepine	Sucralose
F	24.80	22.40	153.28	128.56
F crit	5.19	5.19	5.19	6.59

example, during winter season (December, January and February), the medicines used for colds and flu, such as Paracetamol, were expected to have higher concentration than the rest of months. Overall, monthly variations can be observed for all the markers in all three of the sampling points. However, the general view of the year showed that the fluctuations did not seem high. So the analytes detected in the majority of the samples are good options for markers. For the influent, caffeine, paracetamol, acesulfame, carbamazepine and sucralose are good options and for effluent and disposal, diclofenac, carbamazepine and sucralose. More samples would be needed to confirm the effectiveness of this markers.

8.3 Identification criteria

During the analysis of the water samples, a small shift of the retention time was observed, as shown in Figure 40. It was seen that this shift occur due to a failure of the HPLC-MS system, which was caused by the high pressure pump, which stopped working and needed a replacement. The presence of the analyses was confirmed using the m/z ratio, shown in Figure 41. The m/z of all the compounds was observed to be stable during the year and in all the cases the Δppm is below 10, which reduces significantly the possibility of false positive detections.

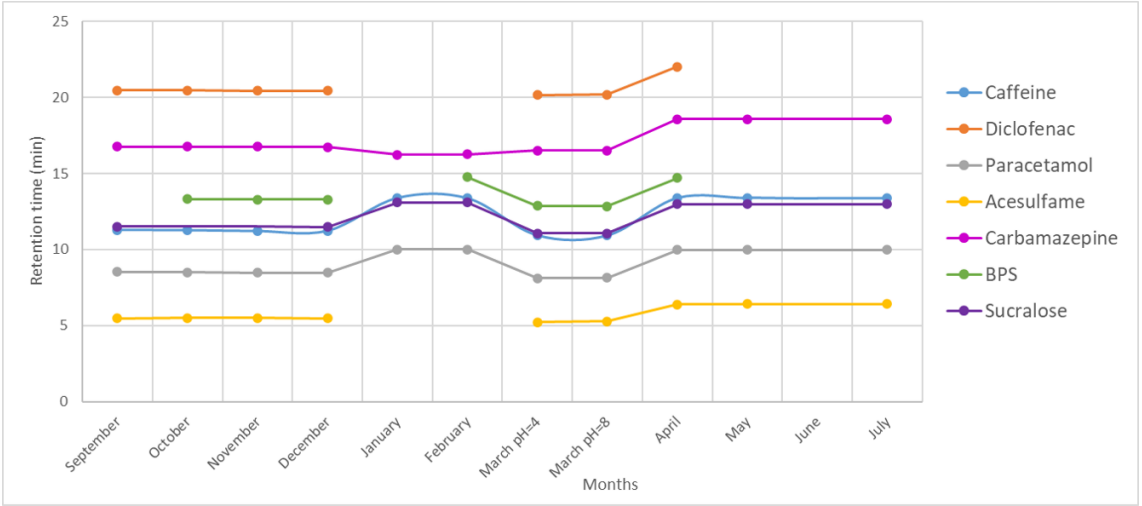


Figure 40: Representation of the retention time of the markers in the influent wastewater sample.

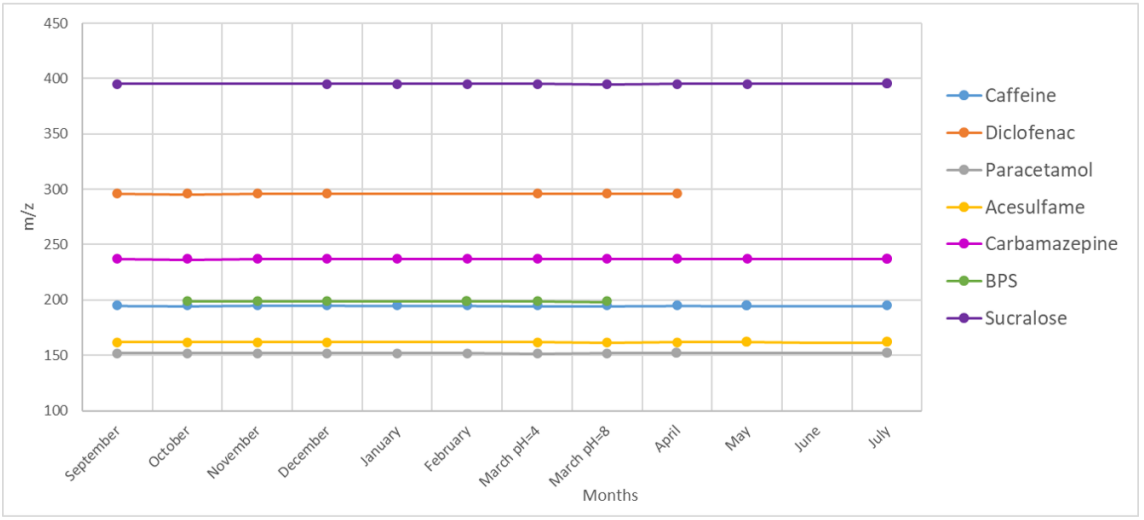


Figure 41: Representation of the m/z ratio of the markers in the influent wastewater sample.

Table 12: Table with the lipophilic character of the markers present in the influent wastewater.

Analyte	Acesulfame	Sucralose	Caffeine	Paracetamol	BPS	Carbamazepine	Diclofenac
log P [6]	-1.33	-1.00	-0.07	0.46	1.65	2.45	4.51
order log P	1	2	3	4	5	6	7
normalised t_R shift	0.203	0.168	0.204	0.207	0.141	0.136	0.090
order t_R	3	4	2	1	5	6	7

As can be observed in Figure 40, the shift is not equal for all the compounds. It was hypothesised that the analytes with less lipophilic character would be more affected, as they are less retained in the column. To test this hypothesis, Table 12 presents the normalised shift of t_R and the log P of each marker. The normalised shift of each sample was computed by dividing the size of its shift by its mean retention time. The log P value indicates the partition coefficient water/octanol, which shows the lipophilic and hydrophilic character of the compound. The ranking in terms of t_R and log P are also shown in Table 12. The two rankings are more or less equal, thus supporting the hypothesis. However, the order is not perfect in the less lipophilic compounds. This could be related to the pH of the mobile phase, which was not evaluated but can have a significant effect on the ionization of each compound.

8.4 Influence of the pH of the sample on the detection

The goal of this section is to study whether the detection of the compound is affected by the pH of the sample. The reason of considering two different pHs is that studies in the literature found that some compounds are detected better in acidic mediums [71, 119, 168]. More acidic pH slows down degradation of the compounds caused by microbiological activities, as lower pH destroys bacteria. Besides an acidic compound with a pKa$\sim$4 is neutralised at low pH and ionised at high pH environments. Thus using SPE all ionised compounds may not be pre-concentrated. In Figure 42, the areas at a different pH for each sample

[6] Values extracted from PubChem:https://pubchem.ncbi.nlm.nih.gov/

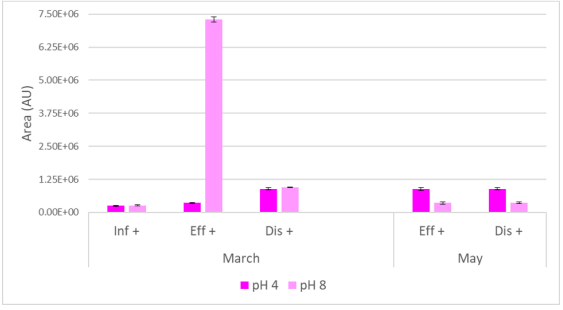
are displayed. This study was conducted using only two months, March and May. The error bars are the standard deviation of the two replicates performed.

The results for carbamazepine are shown in Figure 42a. In general pH=4 seemed to work better for this marker, as the areas were larger. This is particularly so for the samples taken in May. In the effluent sample of March, however, the marker was better detected at pH=8. The error bars are below 10% for all the cases. Probably, the reason behind these differences on detection is the effect the matrix has, because it was the only parameter that changed for the different samples.

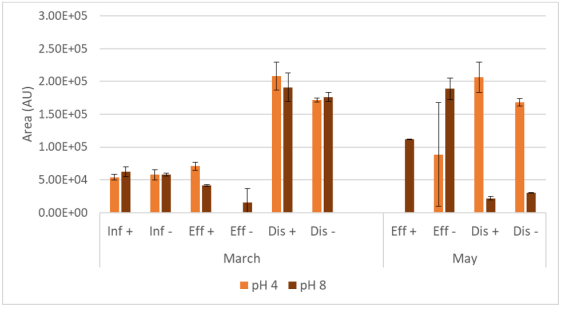
Diclofenac is presented in Figure 42b. From the samples taken in March, differences were only observed in the effluent sample in the negative ionisation mode, where pH=8 worked better, the analyte was not detected using the positive ionisation mode. Regarding the samples taken in May, diclofenac was detected better in pH=8 for the effluent samples and pH=4 for the disposal samples. The error bars are below 10%, except for the effluent sample in May using the negative ionisation mode. In general, no difference can be observed between the two pH, it depends on the sample and probably, again, the matrix has a big effect.

The results concerning sucralose are shown in Figure 42c. In the effluent sample of March, the pH=8 seems to worked better, but it has the higher deviation between the two replicates. For the other samples,the areas presented little difference when changing pH.

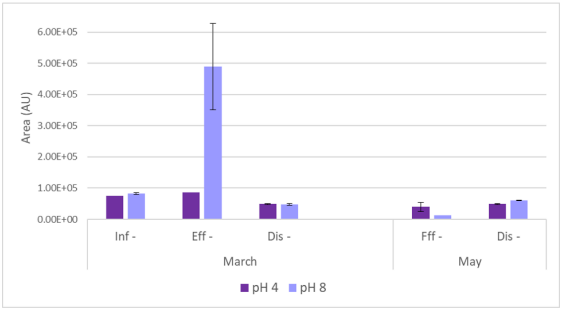
In Figure 42d, the results for acesulfame, BPS, caffeine and paracetamol are presented. In these four cases, there was only one sample available, which makes the results not representatives. More samples would be needed to improve this section of the study. There is not much difference in the detection of acesulfame and paracetamol for both pHs. For caffeine acidic pHs help improve the detection. For BPS, however, pH=8 was observed to work significantly better.



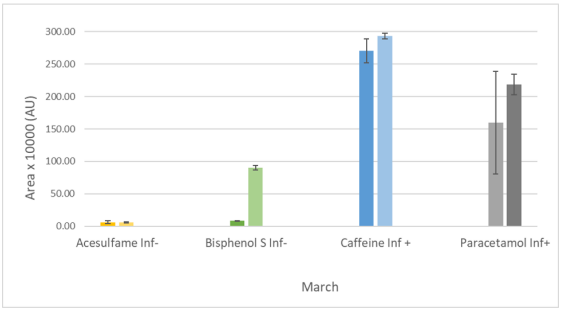
(a) Carbamazepine



(b) Diclofenac



(c) Sucralose



(d) pH=4 to the left and pH=8 to the right.

Figure 42: Comparison of sample's pH for the different markers.

Observing that the results between the two pHs are pretty similar and depend on the sample, a decision table was built, shown in Table 13. For each sample a + is assigned to the pH for which the area is larger.

Table 13: Decision table for the comparison of the two pHs. Each '+' indicate that the detection with the corresponding pH was better or equal than with the other

	pH=4	pH=8
Carbamazepine-March	++	+++
Carbamazepine-May	++	
Diclofenac-March	+++++	+++++
Diclofenac-May	++	++
Sucralose-March	++	+++
Sucralose-May	++	++
Acesulfame	+	+
Bisphenol S		+
Caffeine	+	
Paracetamol	+	+
	18	18

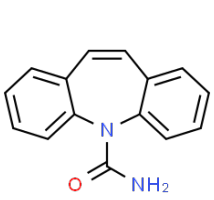
If the detection area is similar, both pHs are given the +. The different number of + for each marker reflect the fact that more samples are available for those markers. In total, 19 samples were studied. Of these, the detection with pH=4 was clearly better for 5 samples; likewise the detection was better in 5 samples with pH=8. In the rest, the detection was similar.

The effect that the pH can have on the marker also depends on their chemical structure and pK_a. Figure 43 shows the chemical structure of all the markers and Table 14 shows their pk_a and if they are ionised in the different pHs. Compounds that are able to ionise can have significant changes in retention time when the pH is modified. With reverse phased columns, the ions are less retained than the neutral compounds. In our case, most of the markers do not change to their ionised form between pH 4 and 8, which solve the problem of false positive detection for a peak with less retention time. Only diclofenac and BPS present differences, due that one of the pH is similar to their pK_a, causing the compound to be half in their acid form.

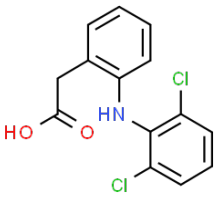
Table 14: Table with the pK_a of the markers.

Marker	CBZ	DCF	SUC	ACE	BPS	CAF	PARAC
pK_a[7]	-3.8 & 15.96	4.2	$\sim$15	2.0	8.2	14.0	9.4
pH=4	Not ionised	Half ionised	Not ionised	Ionised	Not ionised	Not ionised	Not ionised
pH=8	Not ionised	Ionised	Not ionised	Ionised	Half ionised	Not ionised	Not ionised

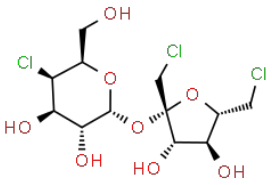
[7] Values extracted from PubChem:https://pubchem.ncbi.nlm.nih.gov/



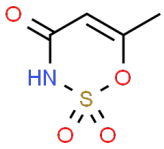
(a) Carbamazepine



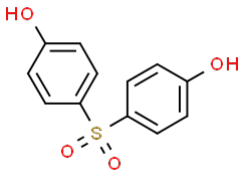
(b) Diclofenac



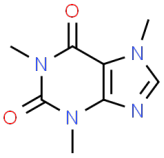
(c) Sucralose.



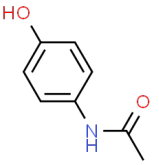
(d) Acesulfame



(e) BPS



(f) Caffeine



(g) Paracetamol

Figure 43: Chemical structure of the markers detected.

Summarising the results, we cannot conclude on which pH works better. The differences observed between the same analyte in different months might be caused by the matrix effect, the matrices are probably different depending on the month. More samples need to be analysed to obtain a proper conclusion.

8.5 Semi-targeted and targeted analysis comparison

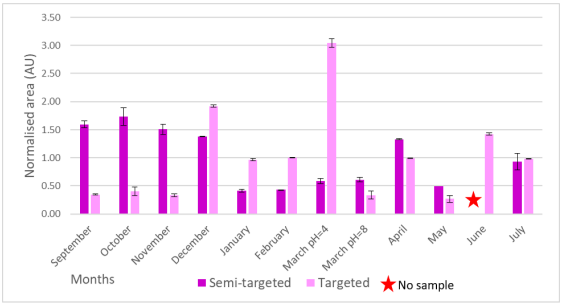
Even though, the techniques and concentration levels detected are different, it is interesting to compare the same method and samples with two different instruments.

Semi-targeted analysis is a qualitative analysis looking at ppm and ppb level. Targeted analysis is a quantitative analysis looking mostly at ppt levels. This means that compounds detected at ppt levels will not appear in the semi-targeted analysis.

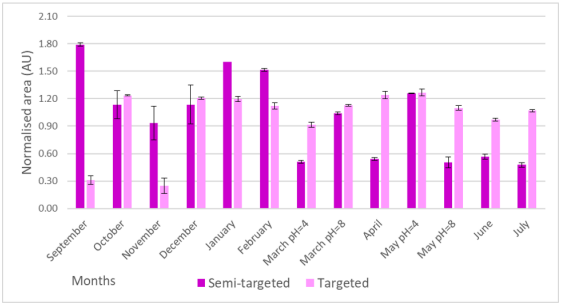
The graphics of this section, Figures 44, 45, 46 and 47, show the normalised area of the compound found for each sample using, semi-targeted analysis (left) and targeted (right). For semi-targeted analysis, there was no sample in June, due to the break down of the instrument. In combining both techniques, four scenarios could be observed: (1) if the analyte is detected by both instruments, then the presence of the marker in the sample is confirmed; (2) if it is only detected in the targeted analysis, then it can be inferred that the marker is present at a concentration below the limit of detection of the semi-targeted analysis; (3) if it is only detected using the semi-targeted analysis, then it is probably a false positive detection; and (4) if it is not detected by neither technique, then it is probably not present at significant concentrations.

The results of the comparison for carbamazepine are presented in Figure 44. The analyte was detected with both semi-targeted and targeted analysis for the influent (Figure 44a), effluent (Figure 44b) and disposal (Figure 44c) samples. For the April disposal wastewater sample, the marker was not detected using semi-targeted analysis, probably due to low concentration of the compound in the sample. This confirms the presence of carbamazepine in all the wastewater samples. The error bars are below 10% for all the samples.

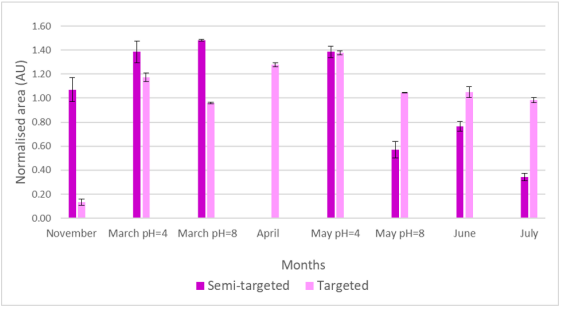
The comparison for diclofenac is presented in Figure 45. For the influent sample, shown in Figure 45a, the marker was only detected using semi-targeted analysis in 7/11 samples. In the effluent and disposal



(a) Influent.



(b) Effluent.



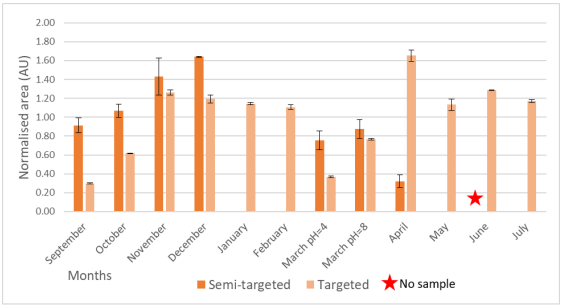
(c) Disposal.

Figure 44: Comparison of the semi-targeted and targeted analysis for Carbamazepine.

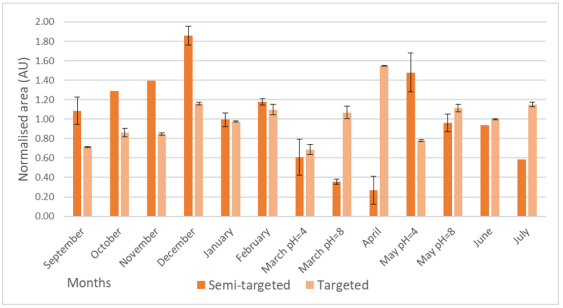
samples, shown in Figure 45b and Figure 45c, the compound was detected using both strategies. The error bars are smaller than 10% in all the samples. The presence of the marker was confirmed by the detection using the semi-targeted and targeted analysis. In the samples where the analyte was only detected by targeted analysis, probably indicates a low concentration of the compound in the wastewater.

The comparison for sucralose is presented in Figure 46. The situation is very similar to the previous two markers, diclofenac and carbamazepine. The error bars are below 10%. For the three samples, the presence of the sucralose is confirmed by the detection using both strategies.

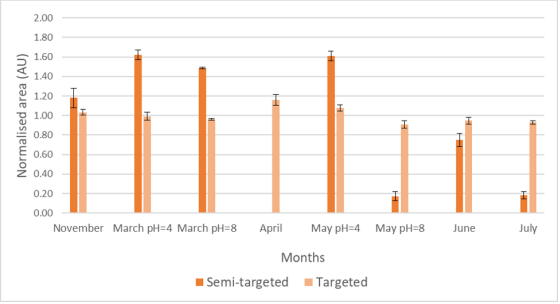
Figure 47 shows the results from acesulfame (Figure 47a), caffeine (Figure 47b) and paracetamol (Figure 47c). They were all detected only in the influent sample. The presence of the analytes was confirmed in all the samples by the both strategies. For paracetamol, shown in Figure 47c, the presence of the marker was confirmed by the detection with the two strategies, except in May, where the analyte was not detected in non-targeted analysis. In the effluent and disposal samples, the markers were not detected by either technique. The error bars are below 10% in all the cases except October and December for acesulfame and paracetamol.



(a) Influent.

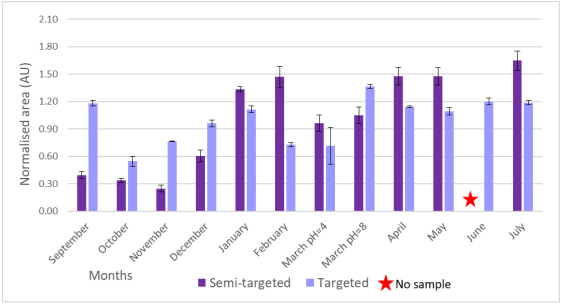


(b) Effluent.

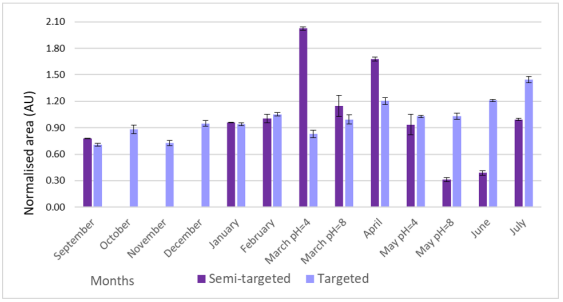


(c) Disposal.

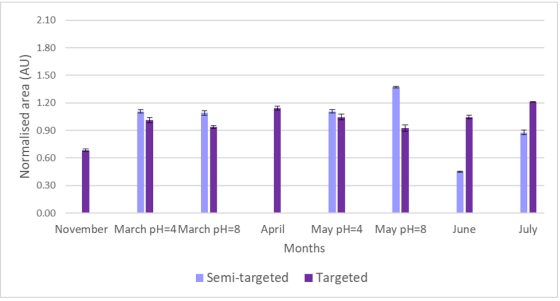
Figure 45: Comparison of the semi-targeted and targeted analysis for diclofenac.



(a) Influent.

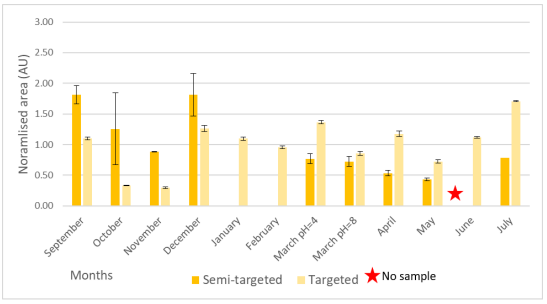


(b) Effluent.

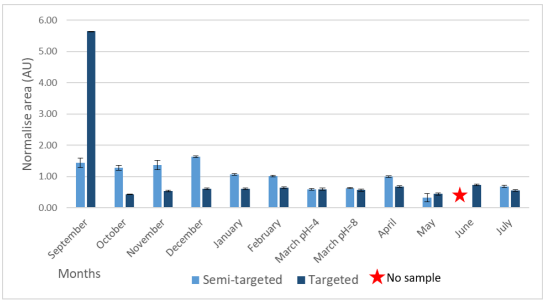


(c) Disposal.

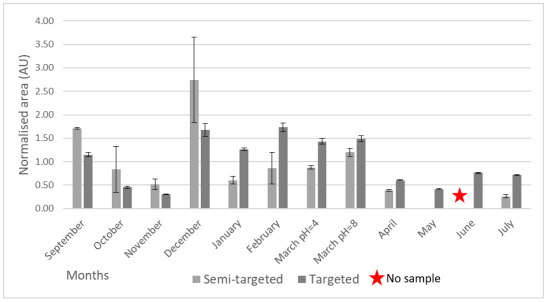
Figure 46: Comparison of the semi-targeted and targeted analysis for sucralose.



(a) Acesulfame.



(b) Caffeine.



(c) Paracetamol.

Figure 47: Comparison of the non-targeted and targeted analysis for the influent samples of the indicated marker.

In general, the presence of the markers is confirmed by the detection using both of the strategies.

8.6 Principal Component Analysis

In this section, the samples will be observed and discussed looking at their similarities between the different months and types of samples, influents and effluents.

First, a dendrogram is shown on Figure 48. A dendrogram is a classification system which uses Euclidean distances to represent similarities between objects. The more similar two objects are, the closer they are linked at the bottom of the dendrogram.

As it is observed in Figure 48, the first separation of the dendrogram (left branch) splits the effluents of September, January and February from the rest of the samples, and shows that January and February present more similarities between each other. The second separation (right branch) splits the rest of the months in two big groups. In general, it can be observed that most influents and effluents are separated by at least one branch, which highlights the differences among both types of samples. A closer inspection revealed that September, January and February present the most differences between their influent and effluent samples, being far away from each other. In contrast, November and July are the closer together between influent and effluent, showing that they are similar between both types of samples.

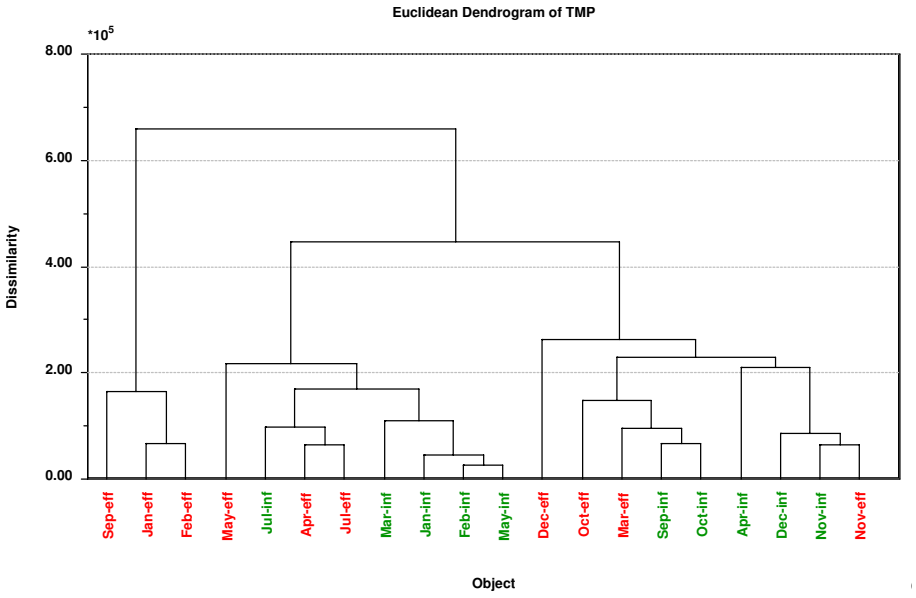


Figure 48: Dendrogram of the objects.

Another typical chemometric method used to observe similarities between elements is called principal component analysis (PCA). PCA is an exploratory method that aims to obtain a description of the data using new uncorrelated variables, called principal components (PC). PC are linear combinations of all the original variables, they are orthogonal between each other and they are in the direction explaining most of the data variation.

The PCs are given as vectors of loadings or scores. The loading vectors represent a basis for the variable space, while the score vectors represent a basis for the object space. Plotting the objects on the loading vectors provides a picture of the relationships between objects, while plotting the variables on the score vectors shows the relationships between variables. The reproduced variable space and object space can be combined into one plot, called biplot.

The similarities between objects or variables are observed by their proximity (the closer together, the more similar) and the angle (if the angle between the origin and the objects/variables are the same). Also, ninety degrees angle between the loadings describes complementary information.

Before doing the PCA analysis, it is interesting to look at the sum plot of the data. It describes the sum of all the variables for each object. In our case, it shows the addition of the four markers for each month and type of sample, influent or effluent. The greater the peak, the bigger the area of the chromatographic peaks. This sum of the variables, together with variations between the variables, will be used to create a model. The matrix used to create the model will be 5 variables (one per each marker and the sum) x 20 samples (10 influent and 10 effluent).

In Figure 49, it can be observed the sum plot, which shows that the samples with larger marker signal are observed for September effluent, December effluent, January effluent and February effluent. The sum is calculated by adding the areas of the four analytes. The months with lower areas are January influent, February influent, March influent and May influent. Having the area of the peaks bigger in the effluent, means that the area of the markers is higher in the effluent sample, which does not make sense chemically because the WWTP is supposed to remove part of the compounds. The increase in area is not caused only by one of the markers, it is a general tendency for few of them in some of the samples.

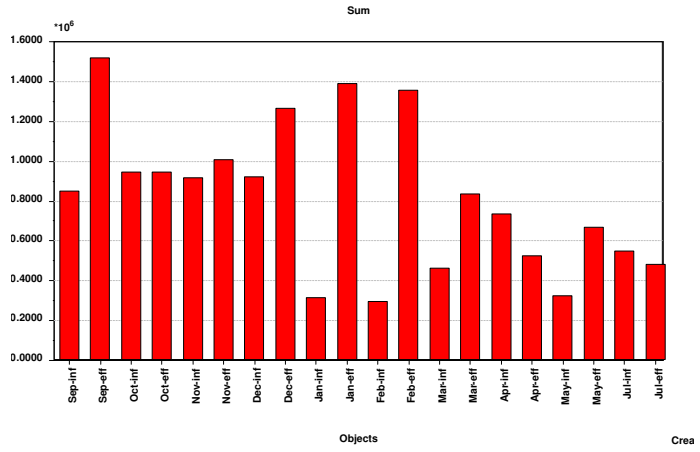


Figure 49: Sum plot of the variables.

In this case, a PCA with 2 PC was modelled. The first PC represented 93.00% of the variance, the second PC a 5.96% and the 1.31% are residuals.

The score plot, presented in Figure 50, shows the position of the objects on the two principal components. It can be observed that the first component represents most of the data and that it separates most of the influent and effluent samples. On the right side of the graph (positive PC1 values), effluents of January, February and September are clearly separated. On the left side (negative PC1 values), there are the influent samples of January, February, March, May and July. The second component separates May, November and December effluent samples, as well as, November and December influent samples. The contribution of each object in the two main principal components can be observed on Figure 51. It shows that the first PC, coloured in red, represents most of the data, which is in agreement with the previous discussion. The first PC describes the large variation of the effluent samples of September, January and February. The second PC, coloured in blue, describes mostly November and December samples (both influent and effluent), and the effluent sample of May. The residuals, coloured in pink, are the information from the variation that the two PCs do not represent. In this case, part of the information from the effluent sample of October is not represented. Summarising, this score plot shows the similarities between some of the months from the same type of sample, most of the effluents are represented mostly on the right side of the first PC and half of the influent samples are grouped in the left side of the first PC.

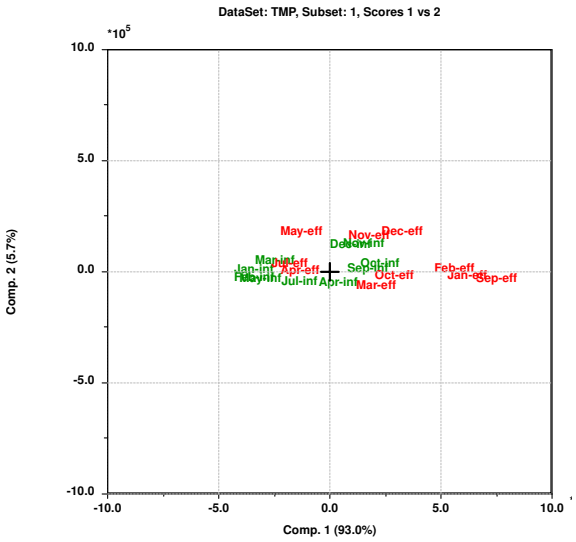


Figure 50: Score plot of the two principal components.

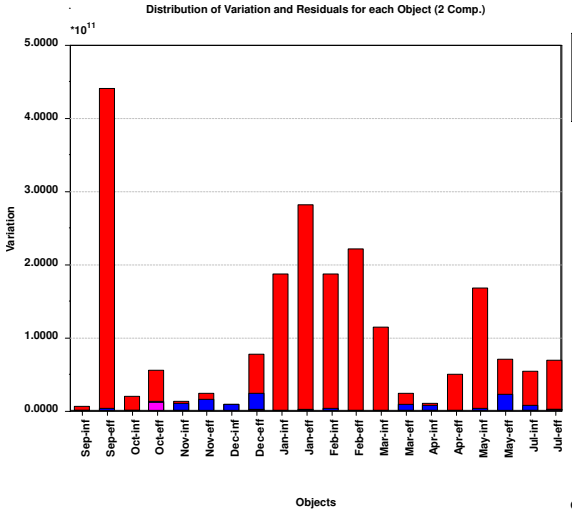


Figure 51: Contribution of the objects on the principal components.

The loading plot, presented in Figure 52, shows the position of the four variables in the two main PC. Carbamazepine (CBZ) is represented by the first PC, observed in the right of the graph. Looking at the graphical representation of the variables' contribution, shown in Figure 53, it can be confirmed that all the variance carbamazepine is represented by the first PC. Diclofenac + and Diclofenac - are similar to each other, being close together and having a similar angle from the origin, meaning that they provide similar information. Their variability is represented by part of the first PC and part of the second PC, as shown in Figure 6. Sucralose presents small variation and it is represented by both PCs. Generally, it can be observed that the four markers are separated, demonstrating their differences between each other, with the exception of both diclofenacs, which are grouped together. These suggest that carbamazepine, sucralose and diclofenac provide complementary information whereas diclofenac+ and diclofenac- show a similar trend

in all samples, which could make sense because they are the same molecule but with a different ionisation state.

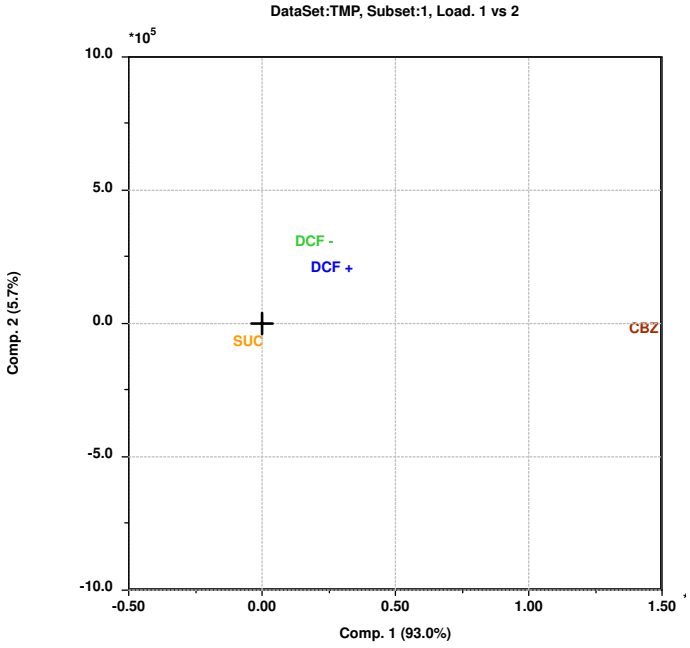


Figure 52: Loading plot of the two principal components.

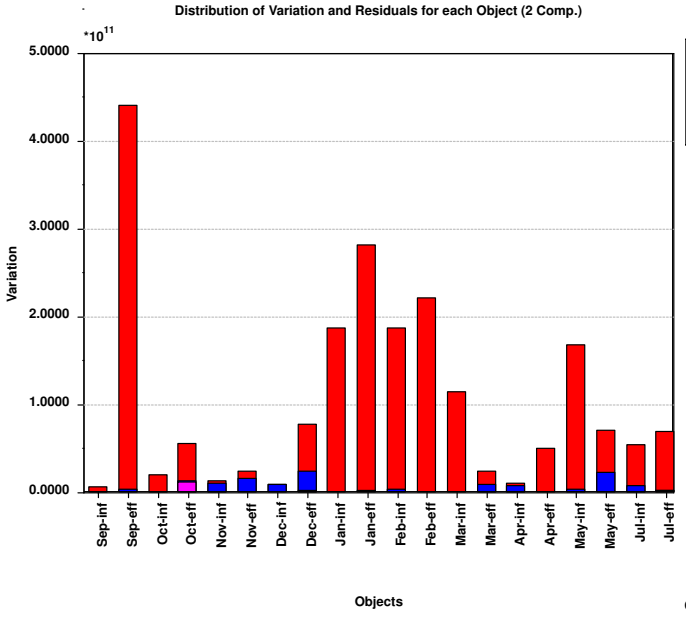


Figure 53: Contribution of the objects on the principal components

Finally, the biplot, which combines the projection of variable and object space onto two PCs in the same plot, is displayed in Figure 54. The biplot is used to observe which objects present similar variability

to the different variables. In this case, it can be observed that none of the objects contain only carbamazepine, which is isolated on the right. The effluents of January, February and September present more carbamazepine than the others, being closer to the marker. It can be observed that the effluent samples unexpectedly contain more carbamazepine than their respective influent samples. Diclofenac and the samples from November and December, both influent and effluent, are similar to each other. The influent sample from April and the marker sucralose are alike, having the same angle from the origin of coordinates. Summarising, samples displayed in the right contain more carbamazepine and less sucralose than the ones on the left. Besides that, samples presented higher on the graph contain more diclofenac. In general, it can be observed that most of the months are not only represented by one of the markers and that their variance is a mixture.

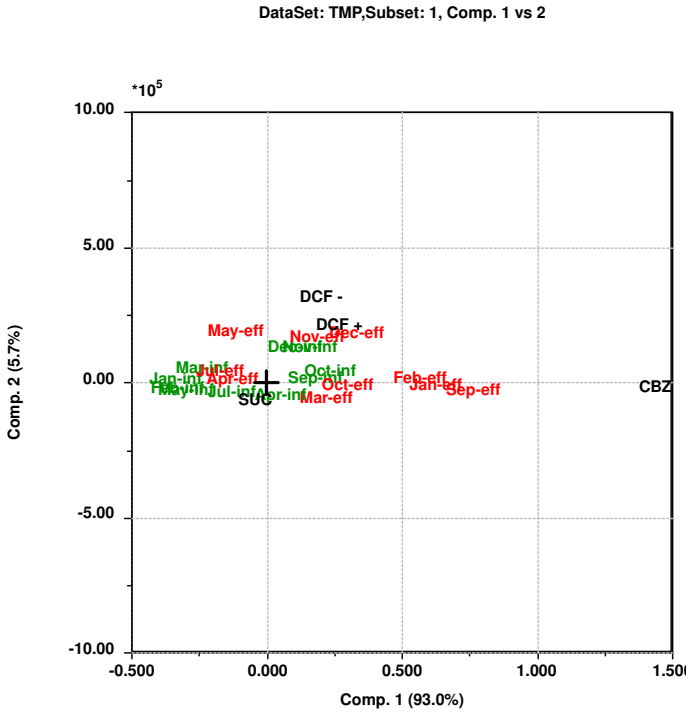


Figure 54: Loading plot of the two principal components.

If there was a seasonal effect, we would expect the colder months group together, and the same with the warmer months. However, such pattern cannot be clearly seen. While January and February are grouped together for the effluent sample; for the influent, they are grouped with warmer months instead of not with November and December, which have a group with the two types of samples. In this statistical analysis, it can be observed that usually the effluent samples contain more of the selected chemicals than the influent, which is completely unexpected because the WWTP is designed to partially remove some of them. Nevertheless, a larger amount of samples would be needed to confirm this fact.

8.7 Is the WWTP eliminating the compounds?

In this section, the efficiency of the WWTP will be evaluated. To do it, a paired t-test was performed, comparing the influent and effluent samples for all the markers.

Table 15: Results of the paired t-test comparing the influent and effluent samples.

Analyte	Diclofenac +	Diclofenac -	Carbamazepine	Sucralose
t	-2.60	-1.31	-2.48	4.12
t crit	2.57	2.57	2.26	2.36

In the case of the markers that were detected only on the influent sample, the WWTP was able to decrease the concentration enough so it could not be detected using our method. These markers were acesulfame, caffeine, paracetamol and BPS.

For the other four markers, diclofenac in both ionisation modes, carbamazepine and sucralose, the paired t-test was performed. This statistical procedure was used to determine whether the mean difference between the influent and effluent samples was zero or significantly different than 0. It cannot be assumed that the area will be smaller after the WWTP, because, as said before, it was observed in the literature that lipophilic compounds tend to be higher in the effluent [107].

The results of this test are presented in Table 15. For diclofenac, it can be observed that the ionisation mode affected the detection. With negative ionisation mode, the paired t-test shows that the areas obtained in the influent and the effluent are significantly different, as indicated by the t value obtained, which is larger than the critical value in absolute value. However, for the positive ionisation mode, it shows that the areas are not significantly different, which implies that the compound was not efficiently removed from the WWTP. More samples would be needed to study the possible effect of the charge of the ions. Carbamazepine present the same behaviour as diclofenac +, having the areas significantly different between the influent and the effluent samples. For sucralose, the t-test also shows significant differences.

Having a negative t value implies that the areas for the effluent samples had a higher value than for the influent samples. It can be observed with diclofenac and carbamazepine. As previously said, it can be caused by the higher lipophilicity of the compounds, which increases the difficulty of removal.

WWTP were built to decrease the concentration of the contaminants not to eliminate them completely. It should be important to always try to increase the efficiency of these systems to discharge water which is less contaminated as possible. In this case, the WWTP does not seem to do a good job decreasing the concentration of some of the compounds detected in this project. More research should be performed on the removal of anthropogenic chemical compounds to improve the quality of water released into the natural environment.

8.8 Discussion of sampling

In the results, a large variability has been observed and that is probably masking some of the effects studied in this work. The source of this variability is unknown. More samples would be needed to analyse it in depth.

One of the causes of this variation could be inappropriate sampling: a more robust sampling protocol is needed to have a greater understanding of the distribution of the markers. To obtain more representative samples, composite sampling could be an option. In our case, temporal variations are difficult to asses without composite samples, due to the differences in the wastewater that arrives at the treatment plant depending on the time of the day. In the morning, the wastewater will contain more drugs because of the typical accumulation of them during the night in the human body. Consequently, during the middle of the day and evening, the concentration of products in the water will decrease. By averaging over the

day, composite sampling would also enhance the correlation between influent and effluent samples. As discussed in Section8.1, one of the issues encountered was that the influent and effluent waters collected were different, due to the processing time in the WWTP. It must be considered, however, that the chemical stability of the analytes could be affected, adding uncertainty to the analysis [9]. An alternative would be to ensure that samples are taken always at the same times and compensate for the daily influx variations, which would likely reduce the variability of the analytes.

Also, although seasonality has not been observed, the large variability present could be masking it. In order to assess it better, more samples would be needed, which could include both taking samples several days a month and extending the study period for longer than a year. This would ensure that the selected markers do not present seasonality.

9 Conclusions

The main goal of this project was to find a combination of markers that can be used worldwide to study anthropogenic wastewater contamination. The possible markers were selected because of their large usage by humans internationally. Seven of them were pharmaceuticals (carbamazepine, gabapentin, diclofenac, metformin, methylphenidate, metoprolol and paracetamol), two stimulants (amphetamine and nicotine) and the human metabolite of nicotine (continine), three compounds contained in various food products (acesulfame, caffeine and sucralose), one from personal care products (methyl-paraben), one herbicide (atrazine) and five bisphenols used in different plastic products (BPA, BPAF, BPE, BPF and BPS). The monitoring of all these compounds would help to analyse the habits of the population and improve the efficiency of removal of them in the WWTP.

Three sampling points were analysed, using HPLC-MS, before the WWTP (influent), just after (effluent) and the discharging point to the sea (disposal). Seven of the selected compounds (carbamazepine, diclofenac, paracetamol, acesulfame, caffeine, sucralose and BPS) were detected in the influent, and 3 (carbamazepine, diclofenac and sucralose) in the effluent and disposal.

A good marker should be present in different types of samples without presenting any seasonal effect. Hence, different parameters were studied to analyse the possibility of a seasonal effect of the detection of the compounds. For that, the difference in areas was statistically analysed and a principal component analysis study was performed. The results showed that there was not a seasonal effect for any marker. However, more samples are needed to properly confirm this theory.

Two different pHs of the sample were also studied, to see if more acidic pH slow down degradation of the containing molecules. The study was performed only for two months, which limited the representativeness. For this reason, no conclusions could be extracted from this section. Future studies could address this issue with one year of sampling to help extracting a conclusion.

Markers can be a really important tool to help detecting water pollution caused by wastewater. The combination suggested in this project could provide not only with information about the contamination of fresh water, but also about the correct functioning of the WWTP.

10 Literature

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11 Annexes

11.1 Annex 1- Examples on how to do semi-targeted analysis

Examples

1- July. Influent1_pos1. Caffeine

TIC:

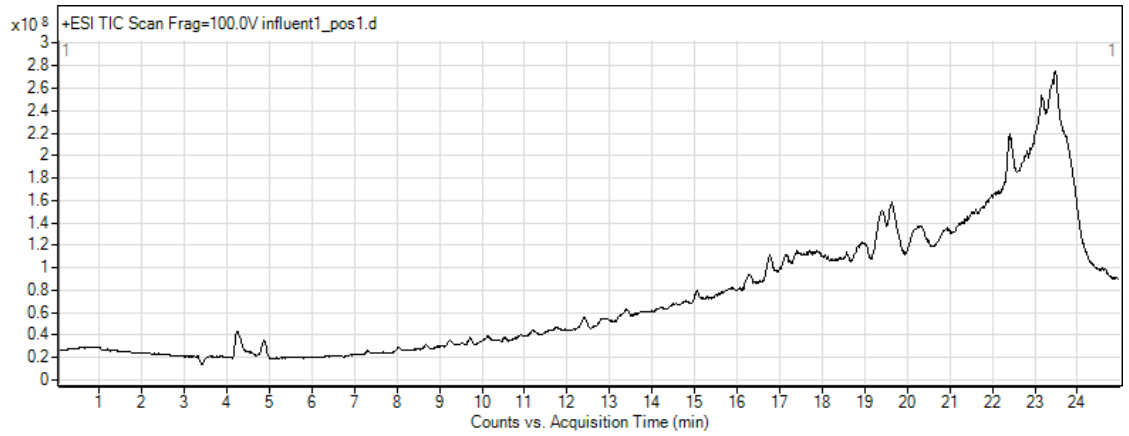

Figure 55: Total ion chromatogram of the sample called "July, Influent1_1_pos1" in the positive ionisation mode.

Extract 195.0882, which is the monoisotopic mass of Caffeine in the positive ionisation mode, $[M+H]^+$.

EIC:

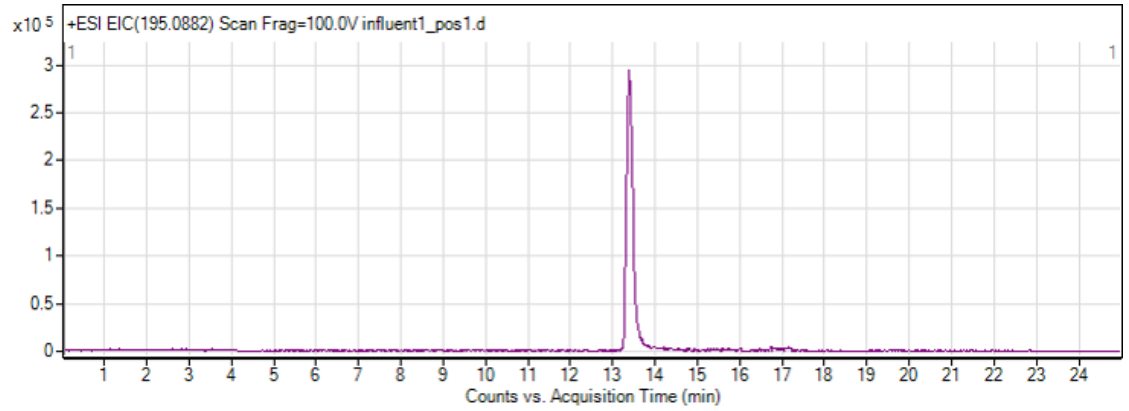

Figure 56: Extracted ion chromatogram with m/z value of 195.0882.

The peak matches with the retention time obtained from the standard of caffeine. To check if the signal is given by caffeine, we need to check the MS spectrum.

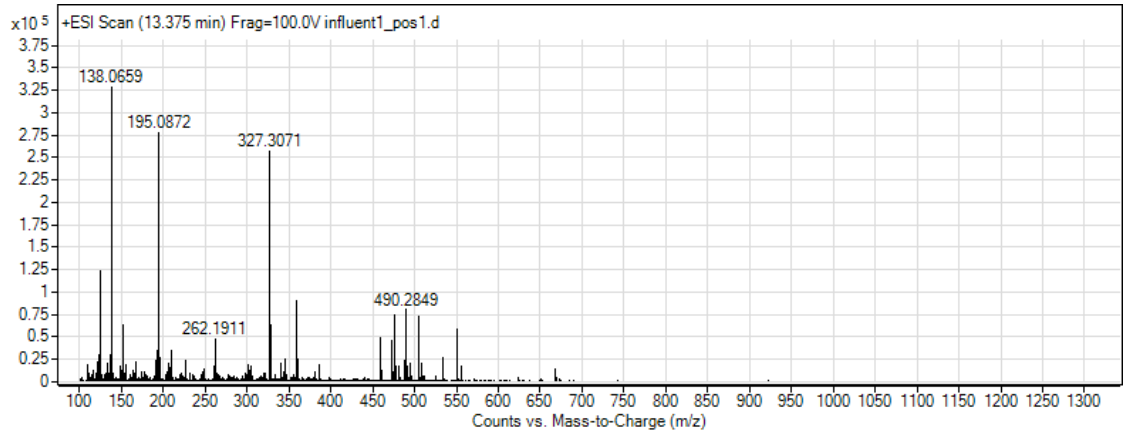

Figure 57: Mass spectrometry spectrum from the peak at 13.375 min of retention time.

There are three main peaks. It is needed to check the signal given by them to see if they have the same retention time, extracting the EIC. Select a peak, right click, and extract EIC at maxima ranges. For 138, the peak matches with the peak obtained in the first extracted chromatogram. They have the same retention time.

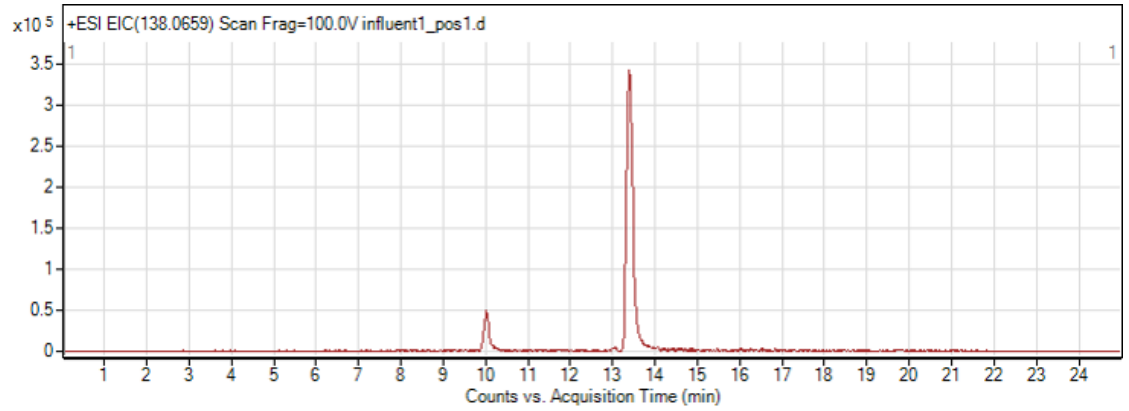

Figure 58: Chromatogram extracted by m/z 138.0659.

The peak at 195, also matches with the previous ones, Figure 56 and 58.

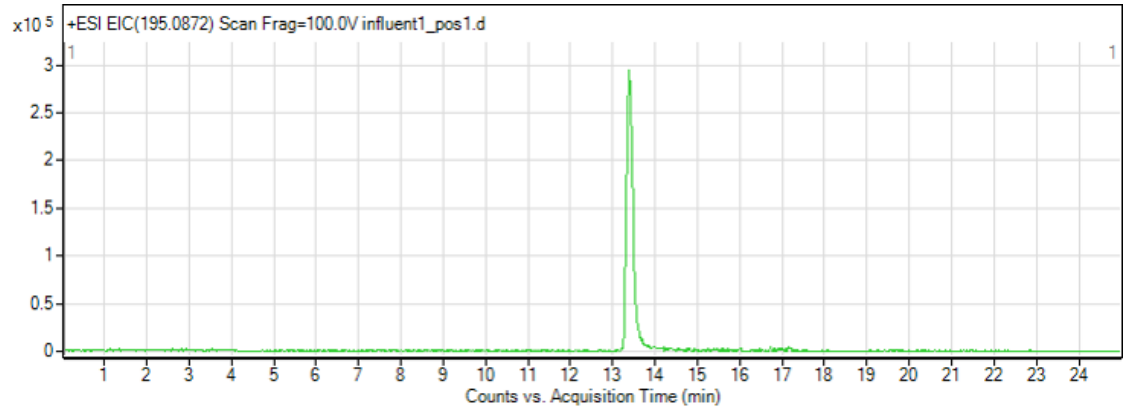

Figure 59: Chromatogram extracted by m/z 195.0875.

The peak at 327 does not match.

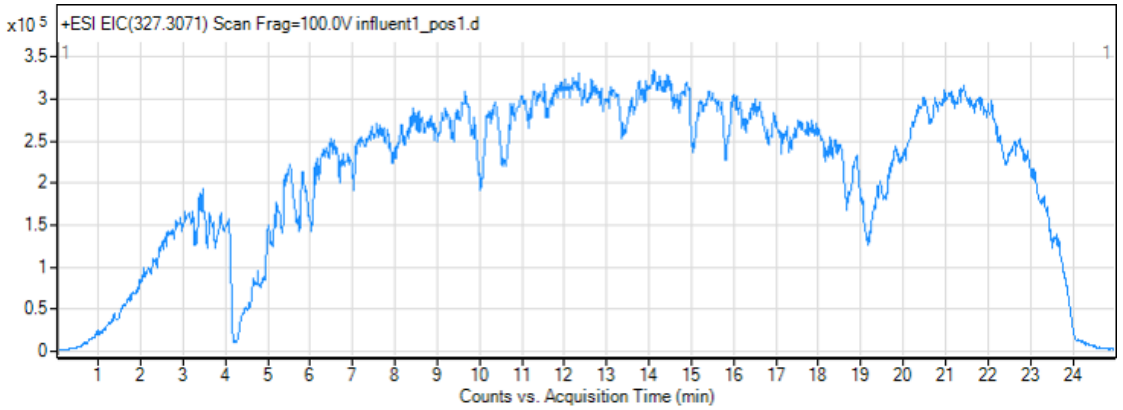

Figure 60: Chromatogram extracted by m/z 327.3071.

Now, we know that the peak obtained in the first EIC, Fig 56 is given by two m/z, 138 and 195. The peak 138 could be a fragment of the caffeine molecule and this reason could explain why they both have the same retention time. In the literature (cite), we can find that the molecule usually breaks giving a fragment of 138. This means that the peak found at 13 min is from caffeine.

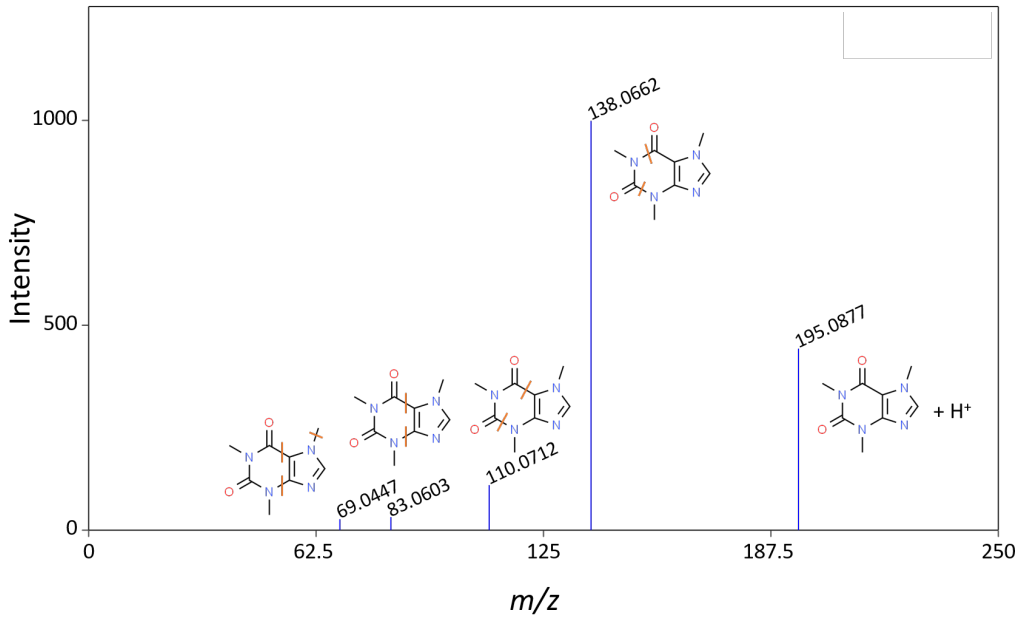

Figure 61: Mass spectrometry spectrum of caffeine with the peaks labelled with the molecule fragmented.

2- November. influent1_Neg1. Paracetamol.

TIC:

Figure 62: Total ion chromatogram of the sample called "November, Influent1_neg1" in the negative ionisation mode.

Extract 150.0555, which is the monoisotopic mass for paracetamol in negative ionisation mode, $[M-H]^-$.

EIC:

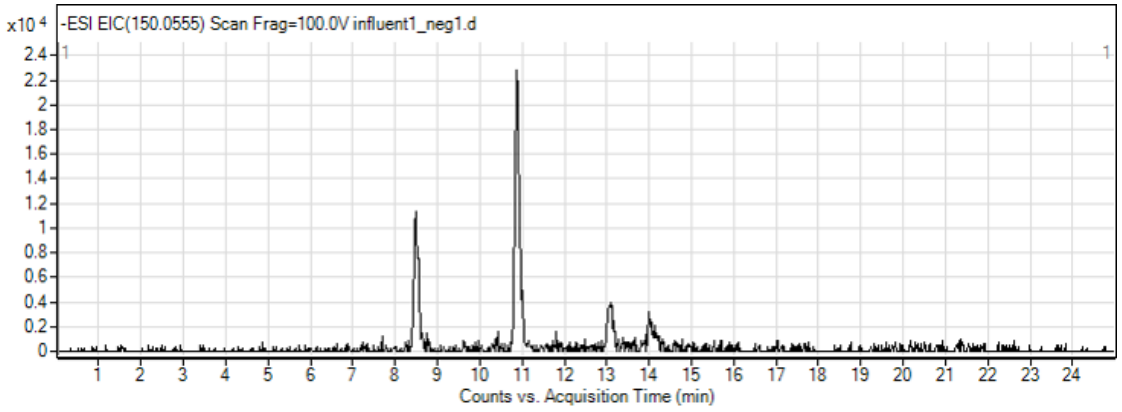

Figure 63: Extracted ion chromatogram with m/z value of 150.0555.

There are two big peaks that give signal with this m/z. From standards, we know that the retention time for paracetamol is approximately 8.1 min. To check if the peak at 8.5 min is paracetamol, it is needed to check the MS spectrum.

Select at the chromatogram the maximum of the peak and check the MS given.

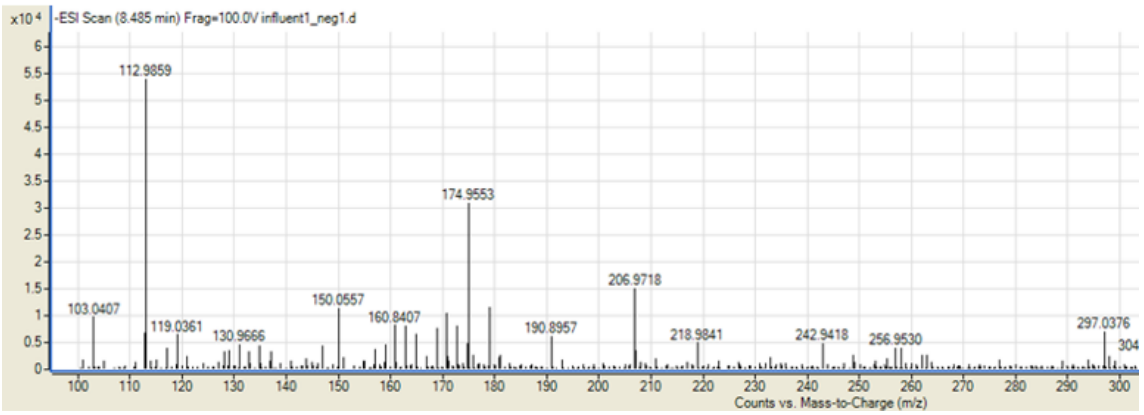

Figure 64: Mass spectrometry spectrum from the peak at 8.485 min of retention time zoomed between 100 and 300 mass vs charge.

At first sight, a peak at 150 cannot be seen, we need to zoom in that section. To zoom, right mouse click and drag. Select a peak, right click, and extract EIC at maxima ranges. We need to check it the chromatogram given has de same retention time as the peak at the first EIC.

The 112.9859 peak gives a chromatogram which does not match with the peak at 8.5 min. So, we can say that the signal in the chromatogram is not from a molecule with m/z =112.

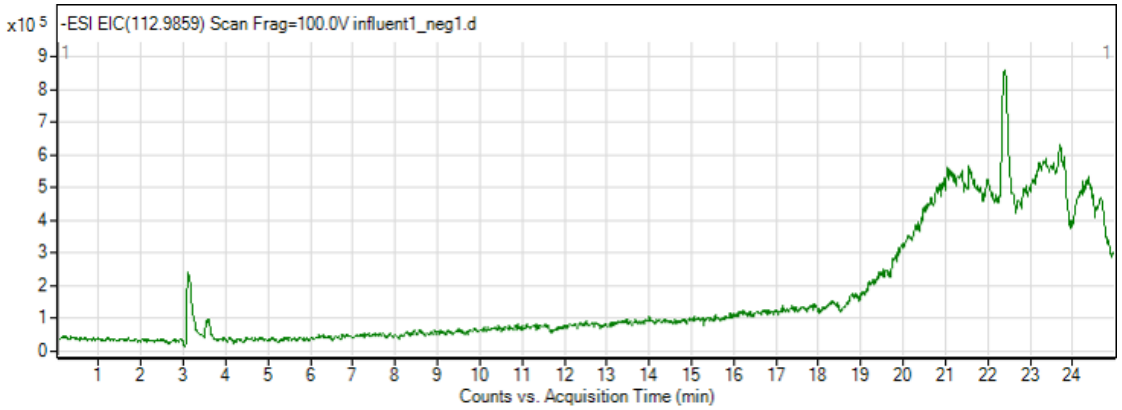

Figure 65: Chromatogram extracted by m/z 112.9859.

The second big peak is at 150. And it matches perfectly with the first chromatogram extracted, Figure 63.

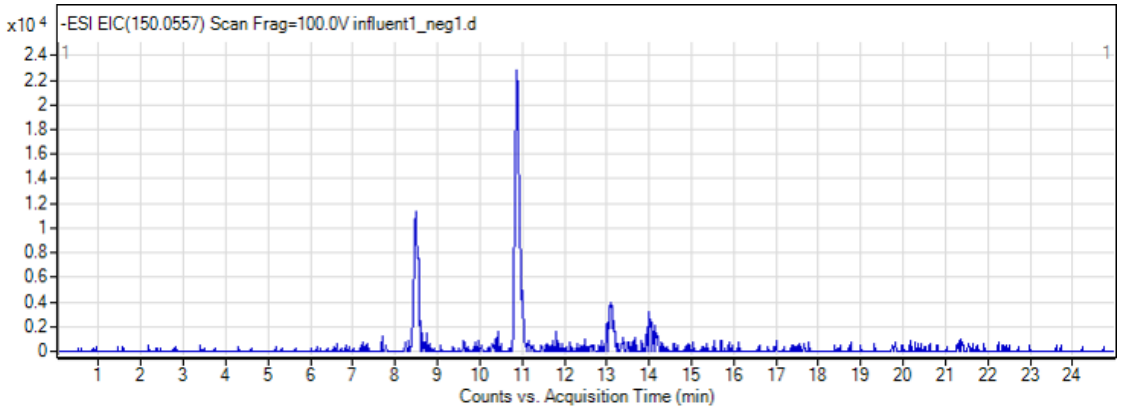

Figure 66: Chromatogram extracted by m/z 150.0557.

The next big peak is at 174.9553. The chromatogram extracted does not match with the first one, Fig. 63.

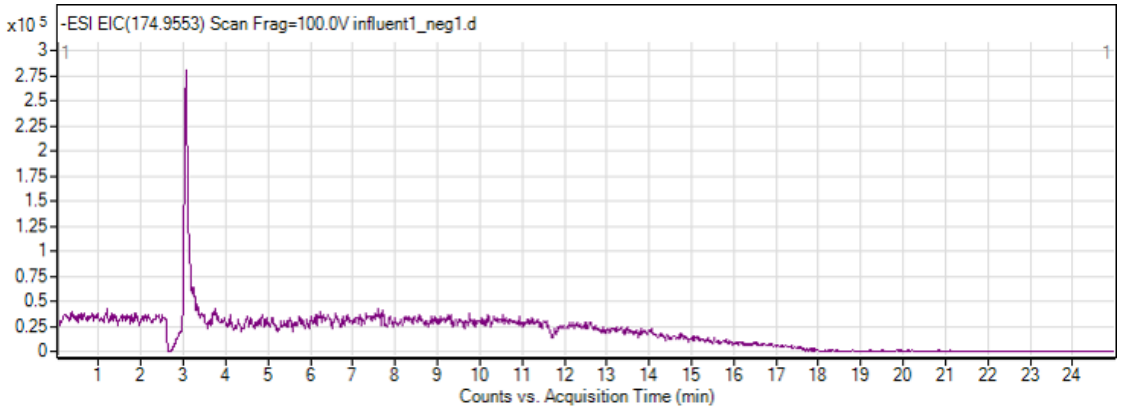

Figure 67: Chromatogram extracted by m/z 174.9553.

The last big peak is at 206.9718. The chromatogram does not match.

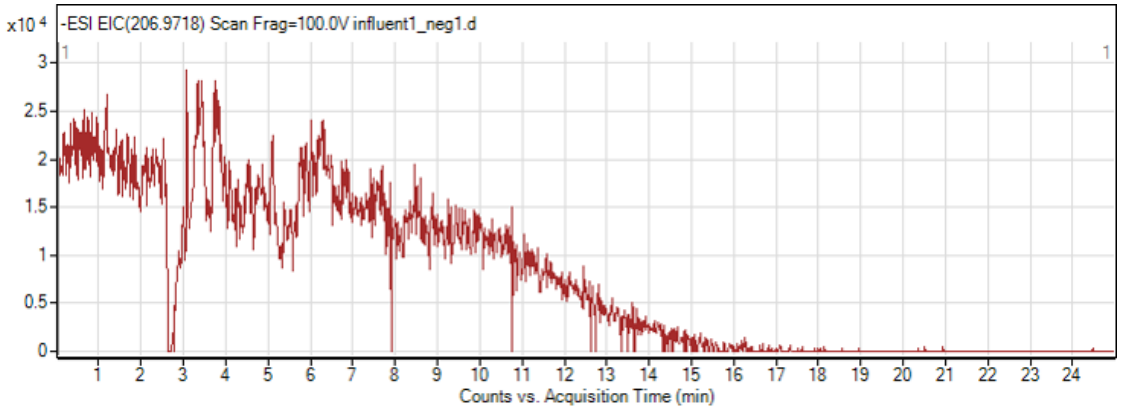

Figure 68: Chromatogram extracted by m/z 206.9718.

With all of this information, we can say that the peak at 8.5 and 11 min is given by a m/z of approximately 150. Now, it should be checked what is present in the peak at 11 minutes. Click at the maximum intensity and in the MS it can be observed a peak at 151.0393. This peak does not belong to Paracetamol because the Δppm is too big.

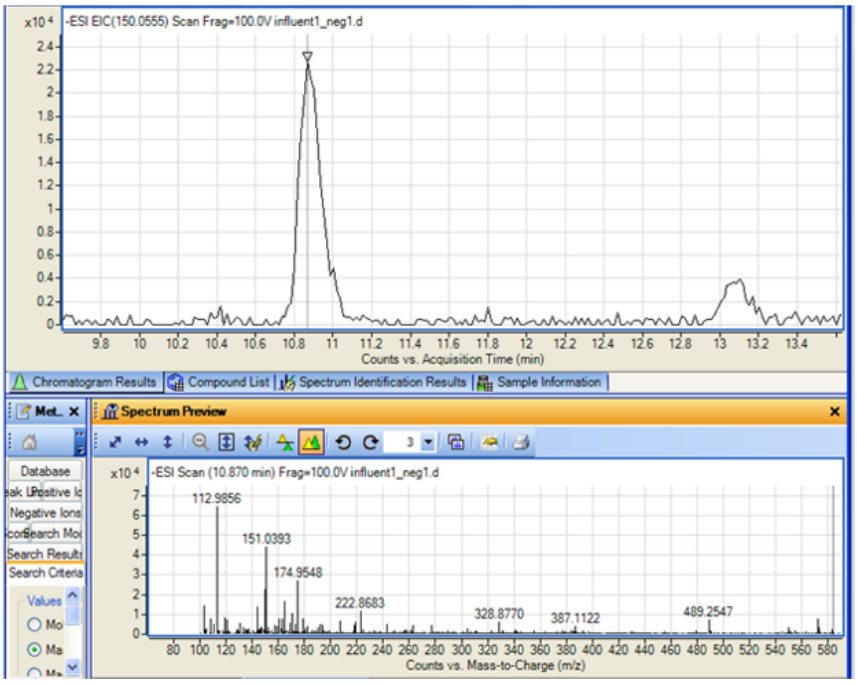

Figure 69: Chromatogram at retention time of 11 minutes and mass spectrometry spectrum of this peak.

$$\Delta ppm = \frac{m/z_{\text{theoretical}} - m/z_{\text{experimental}}}{m/z_{\text{theoretical}}} * 10^6 = \\ = \frac{150.0555 - 151.0393}{150.0555} * 10^6 = -6556.24 \quad (2)$$

This means that the peak at 8.5 is Paracetamol.

3- March. Influent1_ph_4_neg1. Acesulfame.

TIC:

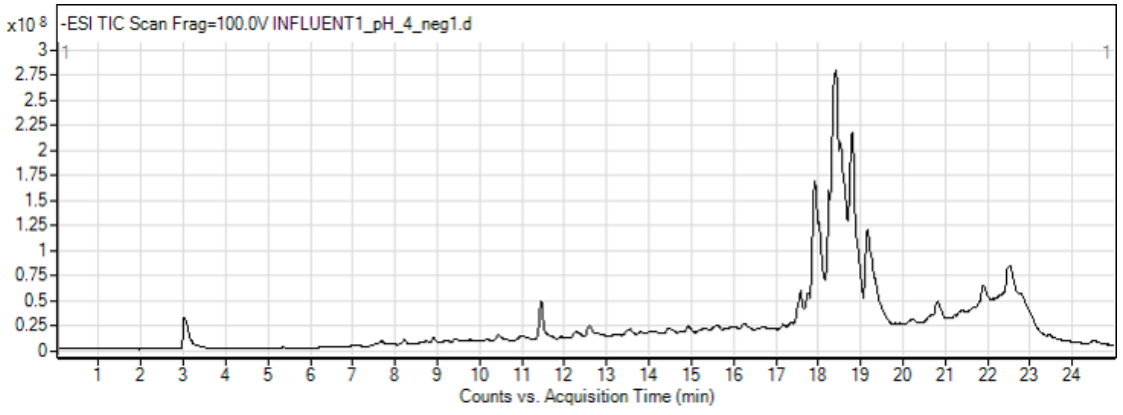

Figure 70: Total ion chromatogram of the sample called "March, Influent1_ph_4_neg1" in the negative ionisation mode.

Extract 161.9861, which is the monoisotopic mass for acesulfame in negative ionisation mode, $[M\text{-}H]^-$.

EIC:

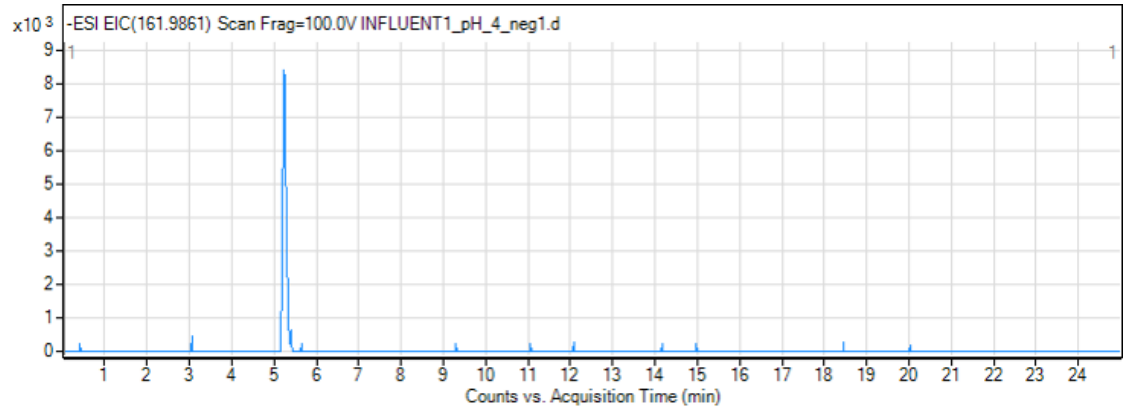

Figure 71: Extracted ion chromatogram with m/z value of 161.9861.

A peak at 5.5 min matches with the retention time obtained using standards. To check if the peak at 5.5 min is acesulfame, it is needed to check the MS spectrum. Select at the chromatogram the maximum of the peak and check the MS given.

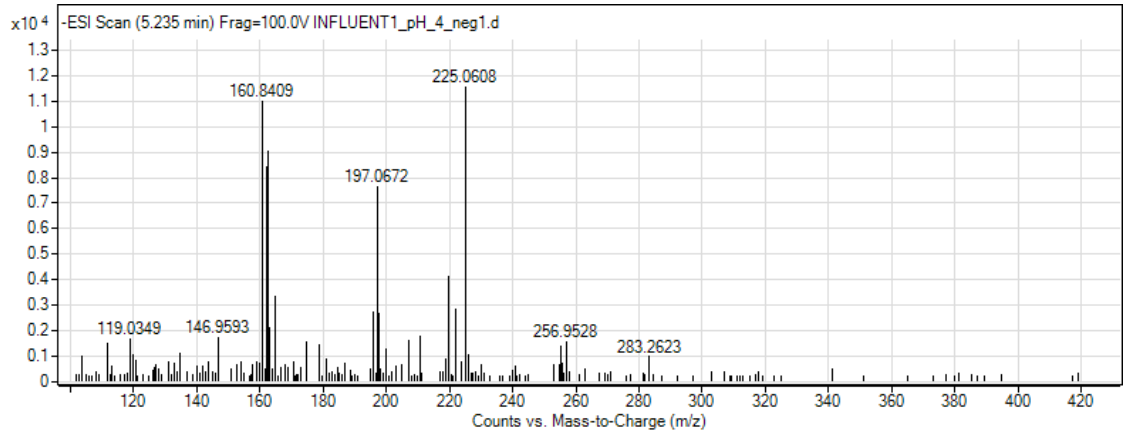

Figure 72: Mass spectrometry spectrum from the peak at 5.235 min of retention time.

There are a lot of big peaks and they are not close to m/z 161.9861. To make the spectrum clearer, the peaks coming from the background should be subtracted. To do it, click on the chromatogram just before the peak starts. In the Spectrum preview, right click and move to background spectra. Then, click at the maximum intensity in the chromatogram, right click on the spectrum preview and copy to user's spectra. In "Data navigator", right click on the scan under the user spectra and subtract the background spectrum and a MS spectrum obtained is more clear.

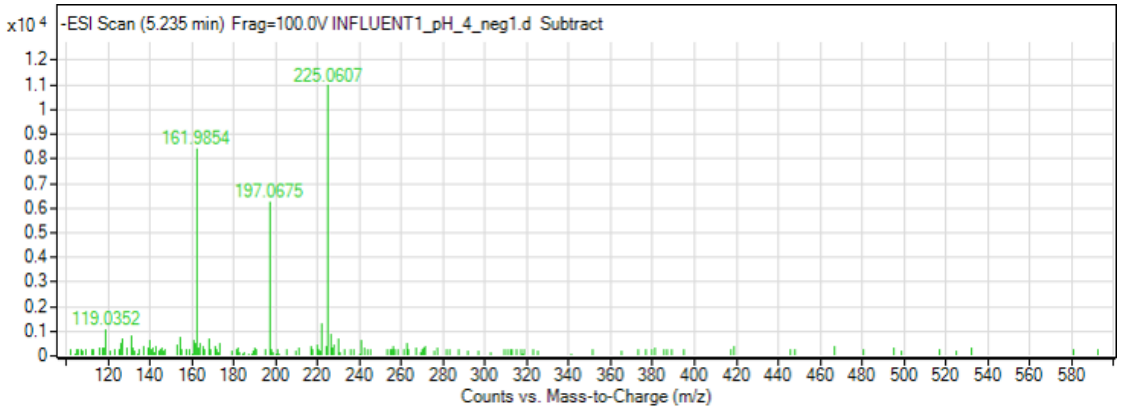

Figure 73: Mass spectrometry spectrum from the peak at 5.235 min of retention time without the background peaks.

There are 3 main peaks. The first one, 161, matches with the first one obtained at the first extraction, Figure 71.

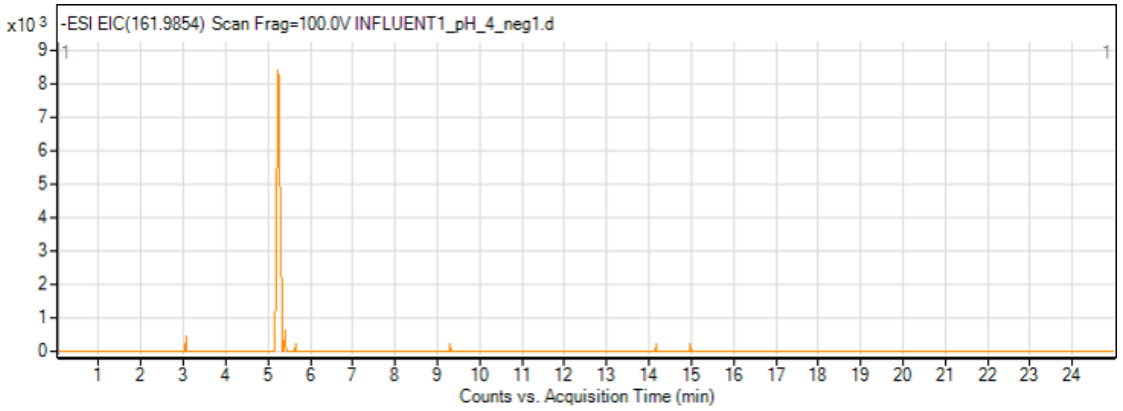

Figure 74: Chromatogram extracted by the m/z 161.9854.

The second one, 197, does not match properly.

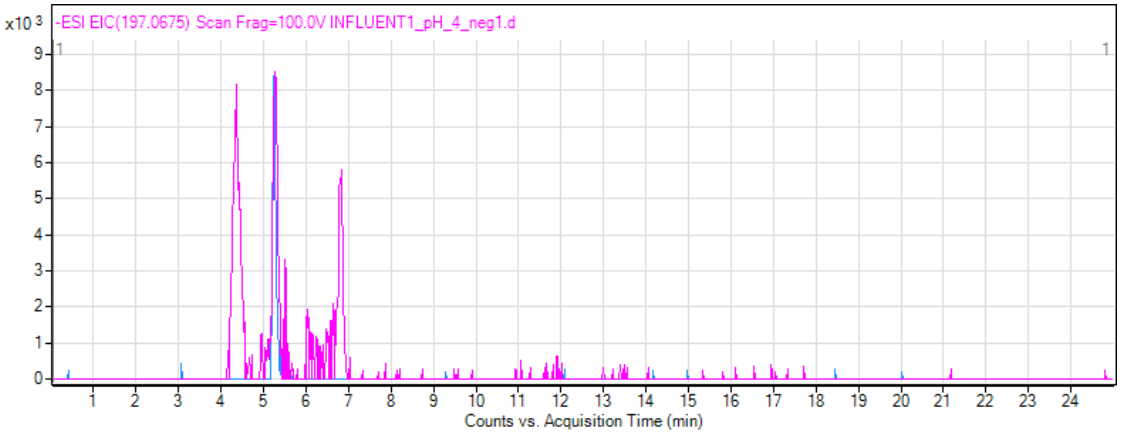

Figure 75: Chromatogram extracted by the m/z 197.0675 in pink and chromatogram extracted by the m/z 161.9861 in blue.

The third one, at the first glance, they match. But if you zoom in, you can see that they have slightly different retention time, hence, they do not come from the same molecule.

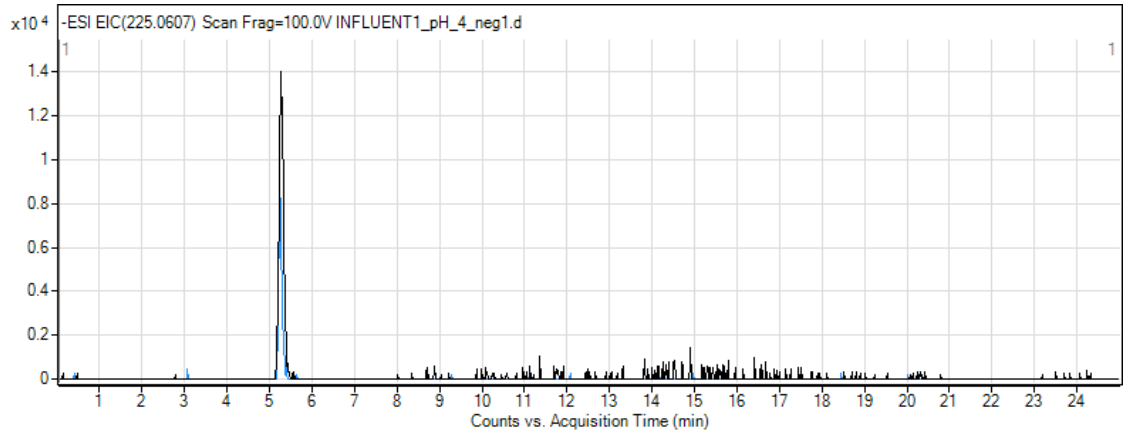

Figure 76: Chromatogram extracted by the m/z 225.0607 in black and chromatogram extracted by the m/z 161.9861 in blue.

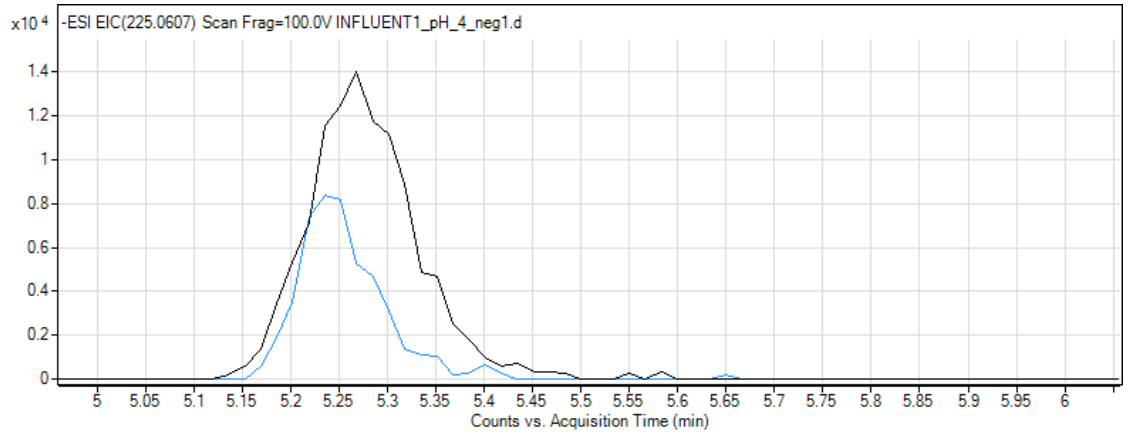

Figure 77: Chromatogram extracted by the m/z 225.0607 in black and chromatogram extracted by the m/z 161.9861 in blue, zoomed between 5 and 6 min.

In conclusion, the peak found at the first EIC belongs to acesulfame.

11.2 Annex 2- Tables with the raw results

In the following the raw results for each marker are summarised in Tables 16 and 17. In these, x indicate that the analyte was not detected, pos indicates that it was detected using the positive ionisation mode, neg indicates that it was detected using the negative ionisation mode and (1) indicates that it was only detected in one of the replicates.

Table 16: Table with the analytes detected (1)

Analytes			Acesulfame	Amphetamine	Atrazine	BPA	BPAF	BPE	BPF	BPS	Caffeine	Carbamazepine
September		Inf	neg	x	x	x	x	x	x	x	pos	pos
		Eff	neg	x	x	x	x	x	x	x	x	pos
October		Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	x	x	x	x	x	x	x	x	x	pos
November		Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	neg	x	x	x	x	x	x	neg	x	pos
		Dis	x	x	x	x	x	x	x	x	x	pos
December		Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	x	x	x	x	x	x	x	x	x	pos
January		Inf	neg (1)	x	x	x	x	x	x	x	pos	pos
		Eff	x	x	x	x	x	x	x	x	x	pos
February		Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	x	x	x	x	x	x	x	x	x	pos
March	pH=4	Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	x	x	x	x	x	x	x	x	x	pos
		Dis	x	x	x	x	x	x	x	x	x	pos
	pH=8	Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	x	x	x	x	x	x	x	neg	pos	pos
		Dis	x	x	x	x	x	x	x	x	x	pos
April		Inf	neg	x	x	x	x	x	x	neg	pos	pos
		Eff	x	x	x	x	x	x	x	neg	x	pos
		Dis	x	x	x	x	x	x	x	x	x	x
May		Inf	neg	x	x	x	x	x	x	x	pos	pos (1)
	pH=4	Eff	x	x	x	x	x	x	x	x	x	pos
		Dis	x	x	x	x	x	x	x	x	x	pos
	pH=8	Eff	x	x	x	x	x	x	x	neg	x	pos
		Dis	x	x	x	x	x	x	x	neg	x	pos
June		Eff	x	x	x	x	x	x	x	neg	x	pos
		Dis	x	x	x	x	x	x	x	neg	x	pos
July		Inf	neg (1)	x	x	x	x	x	x	x	pos	pos
		Eff	x	x	x	x	x	x	x	neg	x	pos
		Dis	x	x	x	x	x	x	x	neg	x	pos

Table 17: Table with the analytes detected (2)

Analytes			Diclofenac	Gabapentin	Metformin	Methyl paraben	Methylphenidate	Metoprolol	Nicotine	Cotinine	Paracetamol	Sucralose
September		Inf	both	x	x	x	x	x	x	x	both	neg
		Eff	both	pos	x	x	x	x	x	x	x	neg
October		Inf	both	x	x	x	x	x	x	x	both	neg
		Eff	both	x	x	x	x	x	x	x	x	x
November		Inf	both	pos	x	neg	x	x	x	x	both	neg
		Eff	both	x	x	x	x	x	x	x	x	x
		Dis	pos	x	x	x	x	x	x	x	x	x
December		Inf	both	x	x	x	x	x	x	x	both	neg
		Eff	both	x	x	x	x	x	x	x	x	neg (1)
January		Inf	neg	x	x	neg	x	x	x	x	pos	neg
		Eff	both	x	x	x	x	x	x	x	x	neg
February		Inf	both	x	x	neg	x	x	x	x	both	neg
		Eff	both	x	x	x	x	x	x	x	x	neg
March	pH=4	Inf	both	x	x	neg	x	x	x	x	pos	neg
		Eff	both	x	x	x	x	x	x	x	x	neg
		Dis	both	x	x	x	x	x	x	x	x	neg
	pH=8	Inf	both	x	x	neg	x	x	x	x	both	neg (1)
		Eff	both	x	x	x	x	x	x	x	pos	neg
		Dis	both	x	x	x	x	x	x	x	x	neg
April		Inf	both	x	x	x	x	x	x	x	pos	neg
		Eff	both	x	x	x	x	x	x	x	x	neg
		Dis	neg	x	x	x	x	x	x	x	x	x
May		Inf	x	x	x	x	x	x	x	x	pos	neg
	pH=4	Eff	both	x	x	x	x	x	x	x	x	neg
		Dis	both	x	x	x	x	x	x	x	x	neg
	pH=8	Eff	both	x	x	neg	x	x	x	x	x	neg (1)
		Dis	both	x	x	x	x	x	x	x	x	neg
June		Eff	both	pos	x	x	x	x	x	x	x	neg
		Dis	both	x	x	x	x	x	x	x	x	neg
July		Inf	neg(1)	x	x	neg	x	x	x	x	pos	neg
		Eff	both	x	x	x	x	x	x	x	x	neg
		Dis	both	x	x	x	x	x	x	x	x	neg

Table 18: Raw results for acesulfame.

Acesulfame		t_R	Area	m/z_{theo}	m/z_{exp}	Δppm
Influent						
September		5.48	148,164	161,9861	161.9873	-7.10
October		5.50	102,576	161,9861	161.9848	8.03
November		5.51	71,839	161,9861	161.9861	0.00
December		5.48	148,230	161,9861	161.9863	-0.93
January		Not detected				
February		Not detected				
March	pH=4	5.23	62,669	161,9861	161.9858	2.16
	pH=8	5.27	59,079	161,9861	161.9845	9.26
April		6.34	46,355	161,9861	161.9847	8.64
May		6.42	35,414	161,9861	161.9850	6.49
July		6.41	63,981	161,9861	161.9845	9.57
Mean		4.70	67,119	161,9861	161.9866	3.37
Effluent						
September		5.45	7,389	161,9861	161.9874	-7.72
Other months		Not detected				

Table 19: Raw results of bisphenol S.

Bisphenol S		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent						
October		13.33	63,191	199.0759	199.0765	-3.22
November		13.30	107,054	199.0759	199.0761	-1.01
December		13.28	84,480	199.0759	199.0763	-2.21
February		14.77	103,601	199.0759	199.0764	-2.41
March	pH=4	12.86	83,046	199.0759	199.0754	2.61
	pH=8	12.87	924,961	199.0759	199.0747	6.02
Other monts		Not detected				
Mean		13.40	227,722	199.0759	199.0759	-0.03
Effluent						
November		13.28	15,783	199.0759	199.0747	6.23
March	pH=8	12.67	404,991	199.0759	199.0759	0.20
April		12.66	341,023	199.0759	199.0777	-9.24
May	pH=8	12.98	96,443	199.0759	199.0754	2.41
June		13.25	60,922	199.0759	199.0761	-1.00
July		12.87	18,513	199.0759	199.0763	-2.01
Other months		Not detected				
Mean		12.95	133,953	199.0759	199.0760	-0.43
Disposal						
May	pH=8	12.66	377,196	199.0759	199.0748	5.42
June		13.50	84,673	199.0759	199.0758	0.40
July		12.90	22,137	199.0759	199.0759	0.00
Other months		Not detected				
Mean		13.02	69,144	199.0759	199.0757	0.97

Table 20: Raw results of caffeine.

Caffeine		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent						
September		11.28	6,659,784	195.0882	195.0875	3.84
October		11.27	5,927,378	195.0882	195.0871	5.64
November		11.21	6,355,021	195.0882	195.0877	2.56
December		11.23	7,576,922	195.0882	195.0878	2.05
January		13.41	4,927,894	195.0882	195.0875	3.34
February		13.38	4,685,868	195.0882	195.0874	3.85
March	pH=4	10.93	2,701,857	195.0882	195.0869	6.41
	pH=8	10.92	2,930,500	195.0882	195.0871	5.39
April		13.40	4,609,275	195.0882	195.0874	3.85
May		13.39	1,519,247	195.0882	195.0874	4.11
July		13.39	3,167,423	195.0882	195.0874	4.36
Mean		12.17	4,641,926	195.0882	195.0874	4.12
Effluent						
May	pH=8	11.20	703.469	195.0882	195.0882	0.00
Other months		Not detected				

Table 21: Raw results of carbamazepine

Carbamazepine		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent						
September		16.76	675,949	237.1028	237.1017	4.43
October		16.76	732,246	237.1028	237.1017	4.85
November		16.76	638,015	237.1028	237.1023	1.90
December		16.75	583,905	237.1028	237.1025	1.06
January		16.25	174,842	237.1028	237.1030	-0.84
February		16.2585	181,893	237.1028	237.1023	2.11
March	pH=4	16.53	247,667	237.1028	237.1032	-1.48
	pH=8	16.52	258,511	237.1028	237.1020	3.38
April		18.57	563,705	237.1028	237.1022	2.32
May		18.56	207,148	237.1028	237.1036	-3.37
July		18.57	395,664	237.1028	237.1024	1.69
Mean		17.12	423,595	237.1028	237.1025	1.46
Effluent						
September		16.74	1,257,148	237.1028	237.1019	3.59
October		16.75	796,659	237.1028	237.1024	1.69
November		16.76	657,720	237.1028	237.1027	0.42
December		16.74	797,766	237.1028	237.1030	-0.84
January		16.52	1,125,173	237.1028	237.1020	3.37
February		16.53	1,061,684	237.1028	237.1031	-1.27
March	pH=4	16.52	359,143	237.1028	237.1014	5.91
	pH=8	16.42	729,354	237.1028	237.1038	-4.22
April		16.43	380,221	237.1028	237.1017	4.43
May	pH=4	Not detected				
	pH=8	16.60	354,328	237.1028	237.1019	3.80
June		16.94	398,457	237.1028	237.1019	4.01
July		16.55	335,018	237.1028	237.1028	0.21
Mean		16.63	687,722	237.1028	237.1024	1.76
Disposal						
November		16.75	689,916	237.1028	237.1022	2.32
March	pH=4	16.69	892,496	237.1028	237.1017	4.85
	pH=8	16.71	955,004	237.1028	237.1013	6.33
April		Not detected				
May	pH=4	16.69	892,496	237.1028	237.1017	4.85
	pH=8	16.42	367,678	237.1028	237.1020	3.38
June		17.19	492,863	237.1028	237.1019	3.80
July		16.55	221,139	237.1028	237.1026	0.85
Mean		16.71	644,513	237.1028	237.1019	3.77

Table 22: Raw results of diclofenac using ESI +.

Diclofenac, ESI +		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent						
September		20.45	65,076	296.0245	296.0236	2.88
October		20.45	75,995	296.0245	296.0231	4.73
November		20.44	101,906	296.0245	296.0243	0.68
December		20.43	116,926	296.0245	296.0237	2.70
March	pH=4	20.16	53,825	296.0245	296.0248	-1.01
	pH=8	20.18	62,354	296.0245	296.0234	3.88
April		22.01	22,976	296.0245	296.0233	3.89
Other months		Not detected				
Mean		20.59	45,369	296.0245	296.0240	1.61
Effluent						
September		20.39	126,799	296.0245	296.0232	4.39
October		20.45	150,792	296.0245	296.0244	0.34
November		20.45	163,079	296.0245	296.0238	2.37
December		20.46	216,941	296.0245	296.0247	-0.51
January		20.16	116,208	296.0245	296.0237	2.70
February		20.17	131,036	296.0245	296.0257	-4.05
March	pH=4	20.17	70,891	296.0245	296.0230	5.24
	pH=8	19.97	41,678	296.0245	296.0246	-0.34
April		19.97	31,295	296.0245	296.0239	2.03
May	pH=4	Not detected				
	pH=8	20.26	112,122	296.0245	296.0239	2.20
June		20.54	109,675	296.0245	296.0236	3.04
July		20.20	68,523	296.0245	296.0250	-1.86
Mean		20.26	111,523	296.0245	296.0241	1.30
Disposal						
November		20.46	151,612	296.0245	296.0239	2.20
March	pH=4	20.31	207,978	296.0245	296.0230	5.07
	pH=8	20.34	191,077	296.0245	296.0288	5.74
April		Not detected				
May	pH=4	20.36	206,430	296.0245	296.0230	5.07
	pH=8	19.96	21,982	296.0245	296.0244	0.34
June		20.81	96,209	296.0245	296.0233	4.05
July		20.20	23,668	296.0245	296.0249	-1.52
Mean		20.35	128,422	296.0245	296.0236	2.99

Table 23: Raw results of diclofenac using ESI-.

Diclofenac, ESI -		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent						
September		20.45	75,577	294.0089	294.0107	-6.29
October		20.45	109,631	294.0089	294.0078	3.91
November		20.43	175,983	294.0089	294.0081	2.89
December		20.45	171,241	294.0089	294.0083	2.04
January		22.09	37,873	294.0089	294.0095	-2.04
February		Not detected				
March	pH=4	20.18	57,809	294.0089	294.0078	3.91
	pH=8	20.18	57,834	294.0089	294.0063	9.00
April		22.01	31,558	294.0089	294.0093	-1.36
May		Not detected				
July		20.92	26,165	294.0089	294.0091	-0.68
Mean		20.92	67,606	294.0089	294.0085	1.26
Effluent						
September		20.41	99,349	294.0089	294.0087	0.68
October		Not detected				
November		20.47	188,043	294.0089	294.0060	9.86
December		20.44	218,535	294.0089	294.0065	8.16
January		20.18	105,390	294.0089	294.0079	3.57
February		20.20	119,409	294.0089	294.0087	0.68
March	pH=4	Not detected				
	pH=8	19.99	15,151	294.0089	294.0109	-6.80
April		19.97	41,830	294.0089	294.0114	-8.50
May	pH=4	20.23	88,677	294.0089	294.0082	2.38
	pH=8	20.30	188,740	294.0089	294.0078	3.74
June		20.57	163,288	294.0089	294.0088	0.34
July		20.20	38,071	294.0089	294.0074	5.07
Mean		20.27	115,135	294.0089	294.0084	1.74
Disposal						
November		Not detected				
March	pH=4	20.35	171,677	294.0089	294.0091	-0.85
	pH=8	20.36	176,446	294.0089	294.0086	1.02
April		20.21	94,748	294.0089	294.0070	6.46
May	pH=4	20.35	168,579	294.0089	294.0095	-2.21
	pH=8	19.98	30,430	294.0089	294.0068	7.14
June		20.81	141,101	294.0089	294.0095	-2.04
July		20.20	18,206	294.0089	294.0089	0.17
Mean		20.32	114,454	294.0089	294.0084	1.38

Table 24: Raw data of gabapentin.

Gabapentin	t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent					
November	12.71	514,408	172.1338	172.1328	5.81
Other months	Not detected				
Effluent					
September	12.72	273,224	172.1338	172.1326	6.97
June	15,49	117,636	172.1338	172.1328	5.52
Other months	Not detected				

Table 25: Raw results of paracetamol.

Paracetamol		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent ESI +						
September		8.55	3,115,712	152.0712	152,0703	5.59
October		8.52	1,529,669	152.0712	152,0701	7.23
November		8.49	942,418	152.0712	152,0701	7.23
December		8.49	4,997,657	152.0712	152,0705	4.93
January		10.02	1,100,714	152.0712	152,0704	5.26
February		10.01	1,565,714	152.0712	152,0704	5.26
March	pH=4	8.13	1,597,366	152.0712	152,0699	8.22
	pH=8	8.14	2,181,170	152.0712	152,0702	6.25
April		9.98	698,492	152.0712	152,0703	5.59
May		Not detected				
July		9.98	478,340	152.0712	152,0701	6.91
Mean		9.03	1,820,725	152.0712	152,0703	6.25
Infuent ESI -						
September		8.54	310,945	152.0712	152,0724	-8.00
October		8.53	177,976	152.0712	152,0702	6.67
November		8.49	98,555	152.0712	152,0706	4.00
December		8.49	680,245	152.0712	152,0716	-2.34
March	pH=8	8.13	332,856	152.0712	152,0701	7.33
Other months		Not detected				

Table 26: Raw results of sucralose.

Sucralose		t_R	Area	m/z $_{theo}$	m/z $_{exp}$	Δppm
Influent						
September		11.51	31,170	395.0067	395.0086	-4.94
October		11.51	26,473	395.0067	395.0067	0.00
November		Not detected				
December		11.48	47,367	395.0067	395.0058	2.41
January		13.09	104,611	395.0067	395.0069	-0.51
February		13.09	115,127	395.0067	395.0068	-0.13
March	pH=4	11.06	75,420	395.0067	395.0054	3.17
	pH=8	11.08	84,595	395.0067	395.0039	7.09
April		12.98	115,866	395.0067	395.0073	-1.40
May		12.98	115,866	395.0067	395.0073	-1.40
July		12.98	129,152	395.0067	395.0070	-0.63
Mean		12.18	84,565	395.0067	395.0066	0.37
Effluent						
September		11.46	33,276	395.0067	395.0084	-4.43
October		Not detected				
November		Not detected				
December		11.47	33,120	395.0067	395.0041	6.58
January		11.38	40,945	395.0067	395.0050	4.43
February		11.39	42,842	395.0067	395.0064	0.76
March	pH=4	11.06	76,162	395.0067	395.0028	9.87
	pH=8	10.83	48,912	395.0067	395.0095	-7.22
April		10.83	71,439	395.0067	395.0070	-0.64
May	pH=4	11.12	39,926	395.0067	395.0066	0.13
	pH=8	11.29	13,241	395.0067	395.0076	-2.18
June		11.67	18,572	395.0067	395.0050	4.30
July		11.09	42,402	395.0067	395.0066	0.26
Mean		11.24	38,403	395.0067	395.0063	0.91
Disposal						
November		Not detected				
March	pH=4	11.37	49,027	395.0067	395.0053	3.42
	pH=8	11.40	48,225	395.0067	395.0065	0.63
April		Not detected				
May	pH=4	11.37	49,027	395.0067	395.0050	4.43
	pH=8	10.82	60,706	395.0067	395.0046	5.19
June		11.73	19,934	395.0067	395.0053	3.55
July		11.09	38,766	395.0067	395.0057	2.54
Mean		11.30	37,955	395.0067	395.0056	3.29