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MASTER THESIS

Investigation of the occurrence, removal and transformation of emerging contaminants in Drinking Water Treatment Plants

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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

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ABSTRACT

Water is inseparably connected with the development and preservation of life on Earth, and it is of priceless value for livings organisms. The available resources of this vital molecule are nowadays threatened by the numerous and variable chemicals that are used in modern societies. Although many of these chemicals are detected in different environmental compartments, they remain unregulated and are characterized as emerging contaminants. Despite the fact that efforts regarding the protection of water bodies have resulted in the publication of Directives in the European Union, the environmental problem still raises scientific community's concern. Once released in the environment, emerging contaminants may end up in surface and ground water used for drinking water production. The processes applied at Drinking Water Treatment Plants for water purification are designed to remove conventional contaminants, and chlorination is widely used for disinfection purposes. Consequently, emerging contaminants occurring in raw water may be detected in drinking water after treatment or even transformed into unknown products as a result of chlorination. In addition, through chlorination, a wide variety of disinfection by-products can be produced from substances occurring naturally in drinking water. With the scope to investigate the occurrence and assess the removal and transformation of emerging contaminants in Drinking Water Treatment Plants, raw water samples were collected at the entrance of Aspropyrgos Drinking Water Treatment Plant. Samples were analyzed by RPLC-ESI-QToF-MS both in positive and negative ionization mode after the application of a generic sample preparation protocol based on solid phase extraction. Validation of the applied method was performed using a representative group of all compounds included in the database. Direct injection analysis was also tested to evaluate the obtained limits of detection for a representative number of compounds. Samples were screened for more than 2,400 multi-class compounds including their metabolites and transformation products.

SUBJECT AREA: Environmental Analytical Chemistry

KEYWORDS: emerging contaminants, drinking water, chlorination, wide-scope target screening, high resolution mass spectrometry

ΠΕΡΙΛΗΨΗ

Το νερό είναι άρρηκτα συνδεδεμένο με την ανάπτυξη και την διατήρηση της ζωής στην Γη και είναι ανεκτίμητης αξίας για τους ζωντανούς οργανισμούς. Τα διαθέσιμα αποθέματα αυτού του ζωτικού μορίου απειλούνται στις μέρες μας από τα πολυάριθμα και ποικίλα χημικά που χρησιμοποιούνται στις σύγχρονες κοινωνίες. Παρόλο που πολλά από αυτά τα χημικά ανιχνεύονται σε διαφορετικά περιβαλλοντικά διαμερίσματα, παραμένουν μη νομοθετημένα και χαρακτηρίζονται ως αναδυόμενοι ρύποι. Παρά το γεγονός ότι οι προσπάθειες για την προστασία των υδάτινων σωμάτων έχουν οδηγήσει στην έκδοση Οδηγιών στην Ευρωπαϊκή Ένωση, το περιβαλλοντικό πρόβλημα ακόμα εγείρει το ενδιαφέρον της επιστημονικής κοινότητας. Αφού απελευθερωθούν στο περιβάλλον, οι αναδυόμενοι ρύποι μπορεί να καταλήξουν σε επιφανειακά και υπόγεια ύδατα που χρησιμοποιούνται για την παραγωγή πόσιμου νερού. Οι διαδικασίες που εφαρμόζονται στις Μονάδες Επεξεργασίας Πόσιμου Νερού για τον καθαρισμό του νερού είναι σχεδιασμένες για την απομάκρυνση των συμβατικών ρύπων και η χλωρίωση χρησιμοποιείται ευρέως με σκοπό την απολύμανση. Συνεπώς, αναδυόμενοι ρύποι που εντοπίζονται στο ανεπεξέργαστο νερό μπορεί να ανιχνεύονται και στο επεξεργασμένο πόσιμο νερό ή ακόμα και να μετασχηματίζονται σε άγνωστα προϊόντα ως αποτέλεσμα της χλωρίωσης. Επιπλέον, μέσω της χλωρίωσης, μια μεγάλη ποικιλία παραπροϊόντων από την απολύμανση μπορεί να παραχθεί από συστατικά που βρίσκονται φυσικά στο νερό. Με σκοπό την διερεύνηση της παρουσίας και την αξιολόγηση της απομάκρυνσης και του μετασχηματισμού αναδυόμενων ρύπων στις Μονάδες Επεξεργασίας Πόσιμου Νερού, συλλέχθηκαν δείγματα ανεπεξέργαστου νερού στην είσοδο της Μονάδας Επεξεργασίας Πόσιμου Νερού του Ασπροπύργου. Τα δείγματα αναλύθηκαν με RPLC-ESI-QToF-MS τόσο σε θετικό όσο και σε αρνητικό ιοντισμό μετά από την εφαρμογή ενός γενικού πρωτοκόλλου προκατεργασίας δειγμάτων που βασίζονταν στην εκχύλιση στερεάς φάσης. Προηγήθηκε επικύρωση της εφαρμοζόμενης μεθόδου με την χρήση μιας αντιπροσωπευτικής ομάδας αναλυτών όλης της βάσης δεδομένων. Επίσης, δοκιμάστηκε η ανάλυση μέσω απευθείας ένεσης για την αξιολόγηση των ορίων ανίχνευσης για έναν αντιπροσωπευτικό αριθμό αναλυτών. Τα δείγματα σαρώθηκαν για περισσότερες από 2,400 ενώσεις διαφόρων κατηγοριών συμπεριλαμβανομένων μεταβολιτών τους και προϊόντων μετασχηματισμού.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Περιβαλλοντική Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: αναδυόμενοι ρύποι, πόσιμο νερό, χλωρίωση, ευρείας εφαρμογής στοχευμένη σάρωση, υψηλής διακριτικής ικανότητας φασματομετρία μαζών

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PREFACE

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THEORETICAL PART

CHAPTER 1
INTRODUCTION

1.1 Emerging contaminants

The growth of the global population in combination with the technological development have made the production and use of chemicals essential aspects of today's global economy and modern living [1]. Therefore, 204 million compounds are included in the Chemical Abstract Database (CAS) while the inventory of the European Chemicals Agency (ECHA, 2019) contains more than 100,000 chemicals [1]–[3]. The wide variety of these compounds reflects the diversity of the products that are used nowadays, and their discharge in the environment has raised the concern of scientists [1], [4]. Only a few of these substances have been characterized as Priority pollutants (PPs) and are monitored routinely [1]. Consequently, during the last decades, the focus of environmental research has shifted from PPs and persistent organic pollutants (POPs) to emerging contaminants (ECs) [1], [5].

The terms ECs or emerging pollutants (EPs) (also named as chemicals of emerging concern, micropollutants or organic micropollutants) refer to substances and to their metabolites that are not covered by the current legislation regarding water quality (surface, groundwater or drinking water (DW)). However, some of them are detected in aquatic ecosystems at very low concentrations and may pose risks both to environmental ecosystems and to human safety and health. In contrast to PPs, ECs have not been excessively studied yet, since they are not included in routine environmental monitoring programs. Therefore, they may be candidates for future legislation depending on the data derived from studies concerning their occurrence in environmental compartments and their ecotoxicity [5], [6].

ECs originate both from human activities and natural procedures, and they include diverse groups of compounds like pharmaceuticals and personal

care products (PPCPs), drugs of abuse (DoAs) and their metabolites, steroids and hormones, endocrine disrupting compounds (EDCs), plant protection products (PPPs), surfactants, poly- and perfluoroalkyl substances (PFAS), phosphoric ester flame retardants, industrial additives and agents, siloxanes, artificial sweeteners and gasoline additives [5]. ECs are primarily released in the environment through urban wastewater discharges since the conventional processes applied at Wastewater Treatment Plants (WWTPs) do not or only partially remove them [1], [6]. They also end up in the aquatic environment through agricultural activities (*Figure 1*). Once disposed, ECs may undergo transformation due to biotic and abiotic factors [5]. They can also spread further in the water circle and even end up in DW if they reach the resources used for its production, since the procedures applied at Drinking Water Treatment Plants (DWTPs) are not designed to secure their removal. It is crucial, then, to investigate the occurrence of ECs in water bodies (WB) used for DW production and in DW itself, since the latter can be a substantial route of human exposure to ECs [6], [7]. According to NORMAN (Network of reference laboratories, research centers and related organizations for monitoring of emerging environmental substances), more than 700 ECs including their metabolites and transformation products (TPs) have been detected until today in Europe's aqueous systems [1], [8].

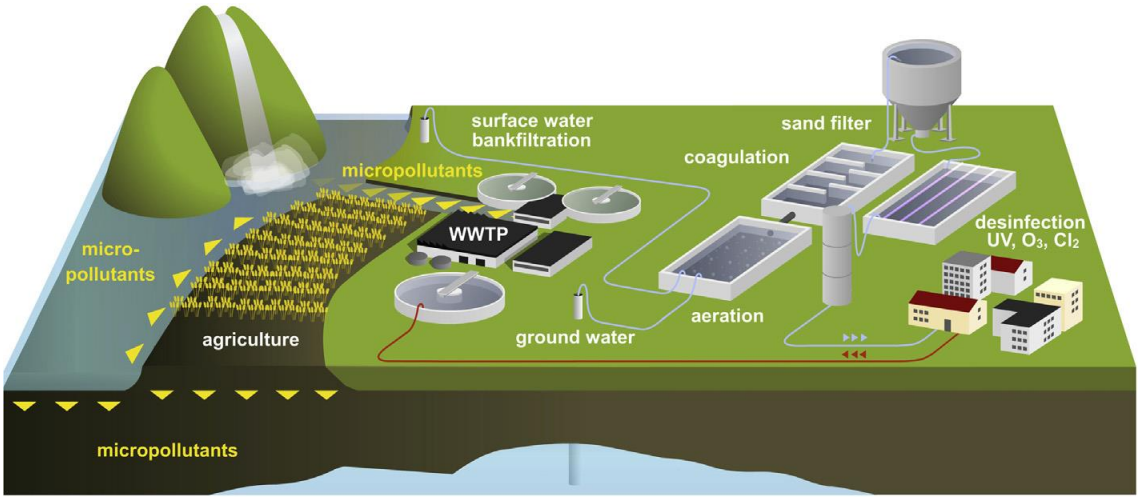


Figure 1 The main pathways through which ECs end up in WB and DW [9]**.**

The determination of ECs in environmental samples is an analytical challenge due to the numerous categories of compounds with different physicochemical characteristics, to their low concentrations and to the complexity of the environmental matrices [5], [10]. Analytical methods and techniques that can analyze simultaneously numerous and multi-class ECs are needed. The advances in analytical chemistry and especially in high resolving power mass analyzers have contributed to the development of wide-scope multi-residue screening methods and to the feasible detection of ECs in numerous complex environmental sample matrices [5].

1.2 Drinking water

1.2.1 General

Water (H_2O) is an essential molecule for regulating crucial equilibrations in Nature and hence, is fundamental for maintaining life on Earth. It is estimated that Earth has 1.4 x 10^{18} m^3 of water of which more than 97% is in oceans. Approximately 35 x 10^{15} m^3 of Earth's water is fresh water of which 0.3% is held in lakes, rivers and reservoirs. The remainder of the fresh water is stored in glaciers, permanent snow and groundwater aquifers. Earth's atmosphere also contains approximately 13 × 10^{12} m^3 of water. Living organisms have a water content that ranges between 60% and 95%. Human body is composed of about 60% of water depending on the age, and in order to sustain health people should consume 1.5 - 2.5 L of water per person daily. The vital role of DW makes it one of the most precious resources on Earth [11].

According to the European Union (EU) Directive 2020/2184, 'water intended for human consumption' means:

> (a) all water, either in its original state or after treatment, intended for drinking, cooking, food preparation or other domestic purposes in both public and private premises, regardless of its origin and whether it is supplied from a distribution network, supplied from a tanker or put into bottles or containers, including spring waters;

(b) all water used in any food business for the manufacture, processing, preservation or marketing of products or substances intended for human consumption [12].

In order to evaluate potential risks to human health, DW should be monitored for its physical, biological and chemical constituents. Hence, the major aspects of DW quality are chemical, microbiological and radiological and those affecting water acceptability in appearance, taste and odor. According to the World Health Organization (WHO), each year, 3.4 million people, mostly children, die from water related diseases, whereas it is estimated that improving water quality can reduce the global disease burden by approximately 4%. Additionally, access to DW is not guaranteed for every individual neither in the EU nor around the world. Interestingly, it is estimated that 65 million EU citizens (approx. 8% of EU population), are served by relatively small water suppliers, considered to implement inferior water quality control compared to that of large water suppliers, whereas two million are without water service. Therefore, safeguarding the quality of DW and assuring access to it are fundamental aspects of public health's protection [13].

1.2.2 Production of drinking water

At conventional DWTPs, physicochemical processes are applied to convert raw water to DW (*Figure 2*). Their major aims are to eliminate pathogens, reduce turbidity and improve both the taste and the odor of finished DW [9].

Prior to the treatment steps applied in DWTPs, bank filtration is used as an effective pretreatment step which purifies raw water before entering the DWTP.

Typically, the following processes are applied for DW production:

- Aeration
- Coagulation, flocculation and sedimentation
- Rapid and slow sand filtration
- Advanced oxidation and disinfection

- Adsorption onto activated carbon

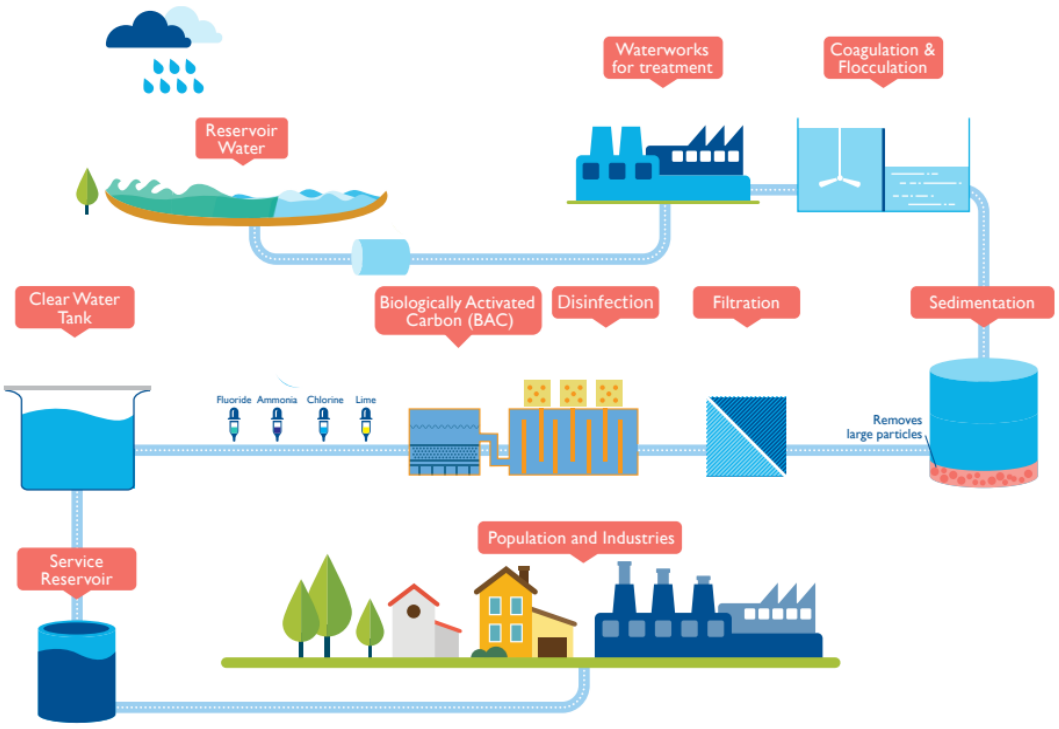


Figure 2 Treatment process at a conventional DWTP [14]**.**

- Aeration aims to increase the dissolved oxygen in the water and to physically strip gases such as methane, hydrogen sulfide and excess carbon dioxide. Oxygen participates as an electron acceptor in chemical and biological redox reactions. Therefore, it can contribute to the removal of reduced iron and manganese species along with the growth of heterotrophic bacteria, particularly during the subsequent filtration processes. [9].

- Coagulation, flocculation and sedimentation are designed to precipitate metal hydroxides and reduce turbidity caused by suspended organic and inorganic particles. Organic compounds associated with the surfaces of suspended particles and flocs can also be removed during sedimentation, which is the principal mechanism for organic pollutants' removal during this process. In general, hydrophobic chemicals with relatively low water solubility and relatively high $\log K_{ow}$ (>6) can be

removed in this step, while the majority of polar and semi-polar compounds remain partitioned in the aqueous phase [9].

- Sand filtration (rapid and slow) is performed to remove or transform inorganic species and residual particles remaining after sedimentation. Sand filters are routinely colonized by native microbial populations that attach to sand grain surfaces and grow on steady fluxes of nutrients in raw water. Despite the fact that both in-laboratory and full-scale experiments have shown the potential of sand filters to remove organic pollutants, several research topics concerning their optimal operational strategy taking into account both parent compounds and their transformation products and the mechanisms of action of microbial communities remain unaddressed [9].
- Advanced oxidation and disinfection Disinfection has been used since early 1900s in DW and wastewater treatment [15]. Both disinfection and advanced oxidation are applied in order to remove color and odor from finished DW and to inactivate pathogens so that it will be biologically stable during storage, distribution and consumption. These processes rely either on the addition of a chemical oxidant like hydrogen peroxide, free chlorine, chloramines, or ozone or on the application of Ultra violet (UV) radiation. Chlorination as a disinfection procedure is described in detail in *Paragraph 1.2.2.1*.

Instead of chlorination, ozonation or other advanced oxidation techniques (e.g. combining ozonation with hydrogen peroxide and/or UV radiation to produce a high yield of reactive hydroxyl radicals) are also used in DWTPs. As a selective oxidant, ozone favors electron rich organic functional groups and has a strong affinity to pollutants containing phenol, aniline or deprotonated-amine substructures. On the contrary, hydroxyl radicals as non-selective oxidants display diffusion-limited reactivity with nearly all organic molecules and can non-selectively oxidize a wide range of natural and anthropogenic organic compounds regardless of substructure.

Although disinfection and advanced oxidation can contribute to the removal of ECs, their high operational and maintenance costs along with the reported formation of persistent and potentially toxic disinfection/oxidation byproducts make the evaluation of cost versus benefit difficult [9].

- Adsorption onto activated carbon In some DWTPs, activated carbon filters are employed as a tertiary treatment step to eliminate remaining growth substrates and thereby minimize bacterial regrowth in the water distribution system. In particular, granular activated carbon (GAC) filters can additionally remove non-polar and semi-polar to polar ECs from water. Residual disinfection/oxidation products formed either biologically in sand filters or chemically during disinfection/oxidation can be also removed by activated carbon filters. However, compounds that are retained in the filters may pose a risk as water is exposed to them, unless a desorption process remobilizes the compound in the effluent. Operation and maintenance of activated carbon filters can be cost-prohibited in some locations and consequently, their use is not considered as a sustainable technology [9].

1.2.2.1 Chlorination

Chlorine-based reagents are widely used in DW production since they can be efficient against different pathogenic bacteria and conditional pathogens [15]. Oxidative chlorine species can be selective or non-selective [9]. Chlorine as gaseous chlorine or hyperchlorite are frequently used in DWTPs. Chlorine hydrolyzes in water and forms hypochlorous acid according to the reaction (*eq. 1*):

$$Cl_2 + H_2O \rightarrow HClO + Cl^- + H^+ \text{ (eq. 1)}$$

Hypochlorous acid is a weak acid that dissociates in aqueous solutions as following (*eq. 2*):

$$HClO \leftrightarrow ClO^- + H^+ \text{ (eq. 2)}$$

with a dissociation constant K_{HOCl} = 2.9 • 10^{-8} (pKa = 7.54 at 25°C). At the typical pH range for DW treatment conditions (6-9), hypochlorous acid and hypochlorite are the main chlorine species present.

In waters with high bromine content bromination reactions also take place. In particular, chlorine oxidizes bromide (*eq. 3*) and produces aqueous bromine (HOBr + OBr^-):

$$HClO + Br^- \rightarrow HOBr + Cl^-,\ k=1.55 \bullet 10^3\ M^{-1}s^{-1} \quad \text{(eq. 3)}$$

Aqueous bromine dissociates in water as following (*eq. 4*):

$$HOBr \leftrightarrow OBr^- + H^+ \quad \text{(eq. 4)}$$

and presents a pKa value of 8.9 (25°C), which indicates that HOBr is a weaker acid than HOCl [16].

Chlorine is the most widely used disinfectant around the world due to its advantages [15]. In particular, it is widely available, inexpensive and reacts with various inorganic and organic micropollutants present in raw water [15], [16]. However, the main disadvantage of chlorine-based disinfection reagents derives from the fact that they are responsible for the formation of various DBPs and TPs [17], [18]. The formation of these products is discussed in the following paragraph (*Paragraph 1.2.2.2*).

1.2.2.2 Emerging contaminants' TPs and Disinfection by-products

Once released in the environment, ECs are subject to various transformation processes due to biotic and abiotic factors resulting in the formation of TPs and/or their elimination depending on their persistence and mobility in the different environmental compartments. Biotransformation products are metabolites of microbial, animal and human origin. The abiotic factors include both reactions that take place in the natural environment (hydrolysis, photolytic and photocatalytic degradation) and during water treatment processes [19].

In particular, during chlorination, the most widely applied disinfection procedure for DW production worldwide, it is known that chlorine-based disinfectants can react with ECs present in water. Hypochlorous acid (HClO)

which is mainly responsible for pathogens deactivation both as HClO and ClO^- can react with organic compounds through addition, substitution and oxidation reactions generating the respective TPs. Additionally, chlorine radicals may react with ECs present in aqueous matrices producing chlorinated organic compounds which can be harmful, biologically active and persistent [19],[18].

Apart from ECs that can be present in source water (surface water, groundwater) used for DW production, natural organic matter (NOM), bromide and iodide are also present. Disinfectants can also react with these constituents and produce a variety of disinfection by-products (DBPs) [17], [18]. According to their structure, DBPs are generally divided into categories such as trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs), halonitromethanes (HNMs), halogenated acetamides (HAMs) [18]. Unlike ECs which can be detected at ng L^{-1} levels in source water and usually are not detected in finished DW, DBPs are always present in finished disinfected DW usually at μg L^{-1} levels [17]. Additionally, they have been associated with several adverse health effects from long-term exposure like cancer, miscarriage and birth defects. Although more than 700 DBPs have been reported in literature, they represent less than the 50% of total organic halogen produced by water chlorination and yet the rest is unidentified [17], [18].

Since the majority of DBPs produced during DW production are still undetermined and considering the fact that disinfectants' reactions can produce genotoxic TPs with even greater toxicity than the parent compound, the effect of chlorination on DW quality needs to be excessively investigated [17]–[19].

1.2.3 Regulations

The necessity to safeguard Earth's water resources and to protect public health should be priorities for both individuals and countries. To achieve the abovementioned goals, the EU has published various Directives throughout the years regarding water resources. In particular, the Directive 2000/60/EC

of 23 October 2000 establishes a framework for Community action in the field of water policy. According to it, Member States may establish safeguard zones for water bodies used for DW production and shall ensure their protection. The major aims of these actions are to avoid the deterioration in their quality in order to reduce the level of purification treatment required in the production of DW. A list of priority substances in the field of water policy consisting of 45 multi-class substances that should be monitored routinely is defined [20]. The Regulation (EU) 2020/741 of 25 May 2020 determines the minimum requirements for water reuse and it entered in force in 26th June 2023. It sets the minimum requirements regarding the quality of treated urban wastewater that will be used in agricultural irrigation [21]. Since agriculture activities are one of the main pathways of water resources' pollution, they can affect the quality of DW. At the same time, the need to ensure that the quality of water intended for human consumption fulfills certain requirements led to the publication of the Directive 2020/2184 of 16 December 2020 which repealed the Directive 98/83/EC. The objectives of this Directive are to protect human health from the adverse effects of any contamination of water intended for human consumption by ensuring that it is wholesome and clean and to improve access to water intended for human consumption. To accomplish that scope, it establishes a set of microbiological and chemical parametric values and their acceptable limits in DW. These parametric values and their limits are presented in **Table 11** and **Table 12** in *Annex I* [12].

The Directive 2020/2184 also includes the 5 currently regulated DBPs in DW in EU (total THMs, HAAs, bromate, chlorite, chlorate with maximum contaminant levels 100, 60, 10, 250, 250 μg L^{-1} correspondingly) [12], [22]. Worldwide, while more than 700 DBPs have been reported in DW, less than 20 are currently regulated although it is reported that many of the unregulated DBPs are apparently more toxic than the regulated ones [22]. Despite the fact that there is legislation regulating chemicals, there is little mention about their TPs since there is a gap of knowledge about their occurrence and toxicity [19]. Further investigation regarding unregulated

chemicals, leads to publication of Environmental quality standards (EQSs). Although the compliance with EQS is not mandatory, along with PPs list, form the basis for further research, for reviewing the DW standards and for future legislation [13].

Apart from EU Directives and EQS, since the protection of DW and WB is an issue of global concern, WHO has also published guidelines that set recommended values regarding the concentrations of selected compounds in water matrices. Generally, the limits established in the Directive 2020/2184 are in accordance with the recommended values of WHO guidelines [13].

CHAPTER 2
ANALYTICAL TECNIQUES AND LITERATURE REVIEW

2.1 Analytical techniques

In the following paragraphs, the most commonly applied sample preparation methods and analytical techniques for the determination of ECs and their TPs in aqueous matrices are described.

2.1.1 Sample preparation

- Solid phase extraction

Solid phase extraction (SPE) is the most frequently used sample preparation method for clean-up, pre-concentration and extraction of compounds of interest from aqueous matrices. Typically, it is performed using disposable cartridges or disks. It is used as an alternative to liquid-liquid extraction (LLE) since it overcomes many of its drawbacks. The advantages of SPE are related to low solvent consumption and low cost. In addition, it offers good pre-concentration factors enabling the detection of analytes at trace levels in the samples. SPE can be performed in offline or online mode (automated process) [23], [24].

In general, the SPE sample preparation procedure consists of five steps (*Figure 3*):

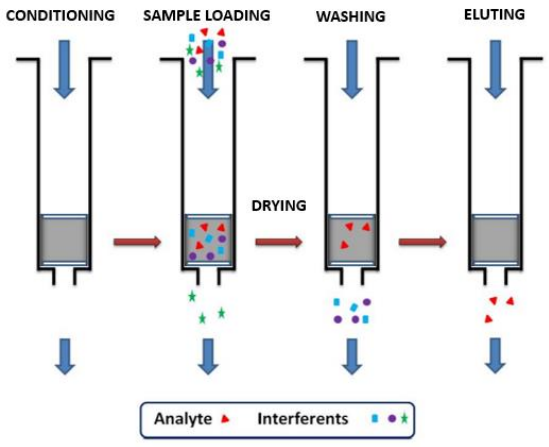


Figure 3 SPE procedure [24]**.**

1. Conditioning The sorbent material is wetted and its functional groups are activated by passing through adequate volume of solvents. Any impurities contained in the sorbent material or the packaging are removed and the void volume is filled with solvent. Conditioning is performed at a low flow rate and the sorbent material should not dry between the conditioning and the following steps because the analytes will not be efficiently retained and thus, poor recoveries will be obtained [23].

2. Loading Sample loading can be performed by gravity, pumping, aspirated by vacuum or by an automated system. The sample flow rate should maintain a balance between low (to permit the efficient retention of analytes) and high (to avoid excessive duration). Target compounds are retained onto the sorbent material by various mechanisms depending on their physicochemical properties. Hence, the selection of the sorbent material is the most crucial step of the procedure. Some of the most common SPE sorbent materials are octadecyl-bonded silica (C_{18}-silica), Hydrophilic Lipophilic Balanced (HLB), weak anion/cation exchange (WAX, WCX) and mixed-mode anion/cation exchange (MAX, MCX) (*Figure 4*) [23], [24].

3. Drying Cartridges are left to dry under vacuum in order to remove any traces of water from the sorbent material [23].

4. Washing (optional) The removal of interferences is accomplished by passing through the cartridge a low elution strength solvent. In this way, matrix components that may have been retained in the sorbent material are eliminated without displacing the compounds of interest [23].

5. Eluting Compounds of interest are eluted from the sorbent material with known volume of solvent(s) or elution solution(s) and the eluents are collected in test tubes. The flow rate should be appropriately adjusted to ensure efficient elution [23].

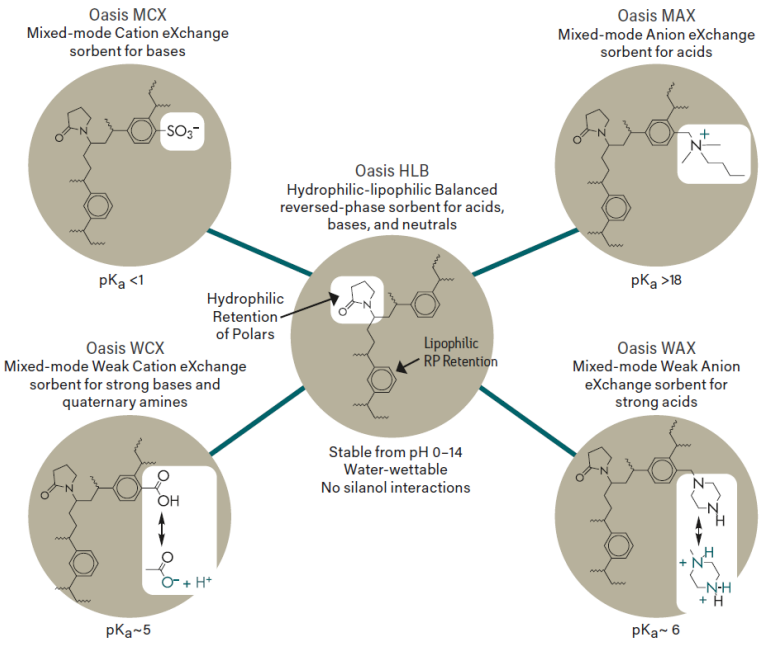


Figure 4 Examples of common sorbent materials in SPE [25]**.**

- <u>Vacuum-assisted evaporative concentration (VEC)</u>

Vacuum-assisted evaporative concentration (VEC) is a simple method used for sample concentration. Briefly, the evaporation of the aqueous sample is performed using an appropriate apparatus with which temperature, pressure and orbital movement of the sample are controlled. Sample evaporation can be done either to dryness or to a determined final volume. The advantages of VEC are its simplicity and minimum supervision combined with the fact that it requires no sample pre-treatment [26], [27].

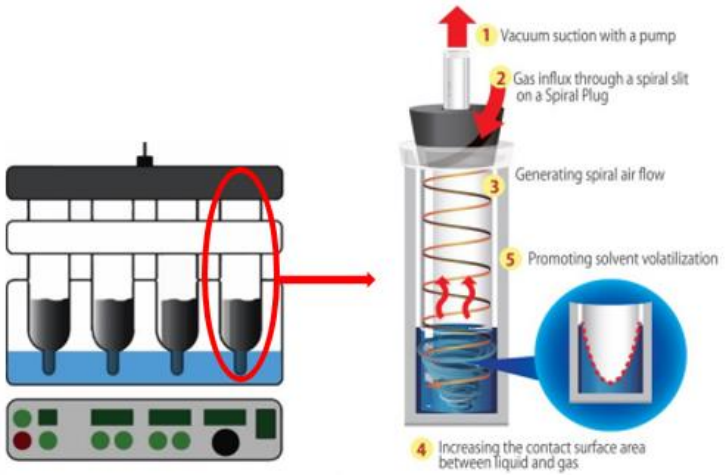


Figure 5 Schematic setup of a typical VEC system [26]**,** [27]**.**

- Direct injection

In direct injection analysis, the sample is immediately injected into the chromatographic system. Only the addition of internal standards is required and no sample clean-up or preconcentration is performed. Hence, it can be applied in clean matrices since strong matrix effect can lead to signal enhancement or suppression [28].

2.1.2 Identification and quantification

During the last decades, high resolution mass spectrometry (HRMS) has become a valuable tool in identifying ECs and their TPs in aqueous matrices. While Gas chromatography (GC)-HRMS is not yet as a popular choice as Liquid chromatography (LC)-HRMS, in recent years its complementary use is increasingly proposed [19], [22]. In the following paragraphs the main principles of liquid chromatography LC, GC and mass spectrometry (MS) are described.

2.1.2.1 Ultra High Performance Liquid chromatography

In Ultra High Performance Liquid chromatography (UHPLC), separation of analytes is achieved due to the different partitioning that compounds present between a stationary and a mobile phase. A typical LC instrument like the one demonstrated in *Figure 6* consists of reservoirs for the solvents, a pump, a sample injection valve, an analytical column and a detector. The main difference with HPLC is the use of short columns with particles of small diameter and, hence, the different pressures achieved [29].

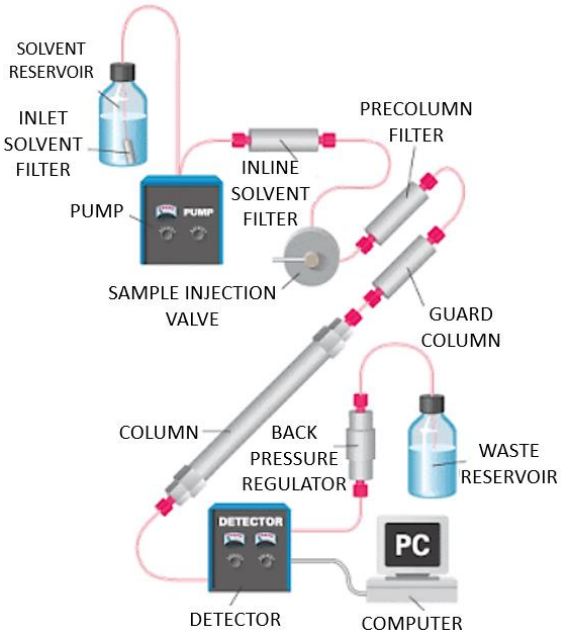


Figure 6 The main components of a typical LC system [30]**.**

The selection of the stationary phase determines the mechanism of interaction with the compounds [29]. For some applications, different stationary phases (typically reversed phase and ion exchange) are combined in one column, overcoming the limitations that each chromatographic method presents towards the retention of analytes. That type of chromatography is known as mixed-mode liquid chromatography (MMLC) [31]. The most common columns in UHPLC are C_{18} and C_8 and hence, UHPLC is mainly performed in reverse phase (RP) mode. The structure of the sorbent material of a C_{18} column is depicted in *Figure 7*. In particular, the use of a non-polar column combined with a mobile phase of aqueous and organic solvents permits the separation. Methanol and acetonitrile are commonly used solvents and typically formic or acetic acid are added. Gradient elution programs are applied for more efficient and faster separation [29].

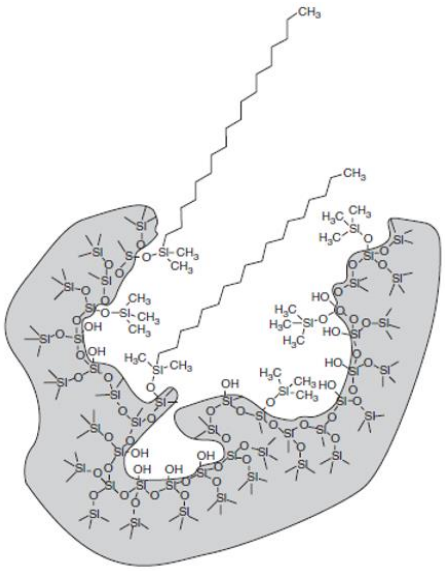


Figure 7 Sorbent material of a C_{18} column [32]**.**

Moreover, Hydrophilic Interaction Liquid Chromatography (HILIC) is frequently used in environmental analysis. It is applied for the separation of polar compounds as an alternative to normal phase liquid chromatography (NPLC). The mobile phase consists of a high percentage of organic solvent (typically acetonitrile) while containing a minimum percentage of water for the hydration of the stationary phase. The stationary phase is hydrophilic and there are several options available depending on their capacity to promote different interactions. The most frequently used columns in environmental analysis are bare silica, zwitterionic, amide and diol. Although the mechanism of HILIC in not yet fully understood, it is characterized as mixed-mode since it involves many types of interactions like partitioning, hydrogen and dipole-dipole forces and electrostatic interactions [33].

Less frequent is the use of Supercritical fluid chromatography (SFC). The mobile phase of SFC is typically comprised of compressed carbon dioxide (CO_2). Liquid solvents such as methanol are commonly used to optimize SFC separation and to moderate the elution of compounds from the stationary phase. SFC is considered as a green technology because of its relatively low consumption of organic solvents. SFC enables the separation of chiral and achiral molecules and of small molecules from complex mixtures [34].

2.1.2.2 Gas chromatography

In GC the separation of a mixture in its components is based on their different boiling points. During a GC separation, the sample is vaporized and injected right into the head of a chromatographic column. Elution is achieved due to the flow of an inert gaseous mobile phase, which does not interact with analyte's molecules; its only function is to transport the analyte through the column. Column temperature is an important parameter that must be controlled, during a GC separation. Thus, the column is housed in a thermostated oven. The optimum column temperature depends on the boiling point of the target compounds and the degree of separation required. Roughly, a temperature equal to or slightly above the average boiling point of a sample results in a reasonable elution time. For samples with a broad boiling range of their analytes, it is often desirable to use a temperature program, whereby the column temperature is increased either continuously or gradually as the separation proceeds [29].

There are two types of GC depending on the stationary phase: Gas-Solid Chromatography (GSC) and Gas-Liquid Chromatography (GLC). In GSC, the stationary phase is solid and retention of analytes takes place as a result of their adsorption/desorption from the solid phase. In GLC, the stationary phase is liquid and holds on a finely divided inert solid support. The retention of analytes is based on solute partitioning between mobile (gas) and liquid phase. GLC is more commonly used in sciences and simply is mentioned as GC [29].

A GC instrument (*Figure 8*) consists of simple components. Usually helium, hydrogen or nitrogen gas compressed in cylinders are used as the carrier gas. A GC column is attached to the injection port and samples are introduced into the carrier gas stream at a temperature sufficient to ensure vaporization of all analytes. Typically, the sample is introduced with a microliter syringe which is forced through a rubber septum at the injection port. An attached detector right after the column exit monitors each individual compound that elutes from the column. The detector must be insensitive to

the carrier gas, while detecting the eluted analytes. A recording of its response to the analytes with time forms a chromatogram [29].

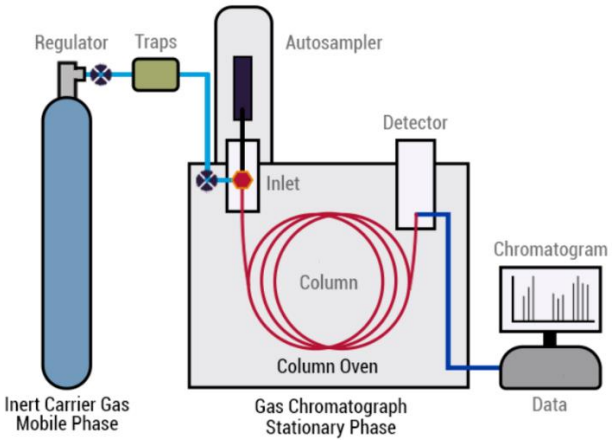


Figure 8 A typical GC system [35]**.**

2.1.2.3 Mass spectrometry

Mass spectrometry's fundamental principle is the separation of ions in gaseous phase according to their mass to charge ratio (m/z). A mass spectrometer consists of the following parts: sample inlet, ionization source (gas-phase or desorption source), mass analyzer (low or high resolution mass analyzer), ions detector, vacuum pump [29]. In the following paragraphs the principal of operation of the most commonly used ionization sources and mass analyzers are briefly presented.

2.1.2.3.1 Ionization sources

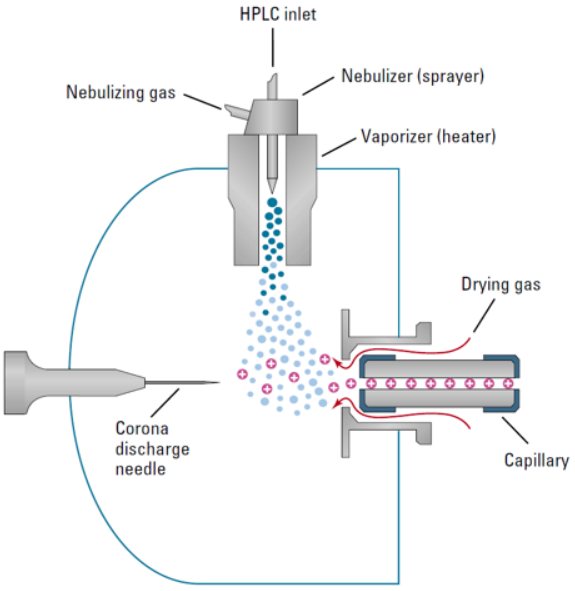
Electron Impact ionization (EI)

In EI source, molecules are bombarded with a high-energy pack of electrons. This produces positive ions, negative ions and neutral species. The positive ions are directed towards the analyzer by electrostatic repulsion. The high energy of the electron pack produces many fragments. These fragments are very useful in identifying the molecular species entering the mass spectrometer. Mass spectra for many libraries of MS data have been collected using EI sources since it is the most common ionization source in mass spectrometers [29].

Atmospheric Pressure Chemical Ionization (APCI)

In APCI (*Figure 9*), ionization involves three primary steps: i) conversion of the analyte to a gas-phase species (nebulization and desolvation), ii) ionization of the analyte through gas-phase ion–molecule reactions at atmospheric pressure and iii) extraction of the ions into the mass spectrometer with simultaneous exclusion of most of the non-ionized species [32], [36].

It can occur in both positive and negative mode. Ionization in positive-ion mode occurs by reaction of the analyte with protonated solvent molecules, generally giving rise to an abundant protonated analyte molecule $[M+H]^+$ or adduct ions like $[M+NH_4]^+$ if ammonium salts are added to the solvent. In the negative-ion mode, ions are generated by proton abstraction by oxygen ions or by the formation of adducts with anions such as acetate or chloride present in the sample or solvent [36].



$N_2 + e^- \rightarrow N_2^{+\cdot} + 2e$ (eq. 5)

$N_2^{+\cdot} + 2N_2 \rightarrow N_4^{+\cdot} + N_2$ (eq. 6)

$N_4^{+\cdot} + M \rightarrow M^{+\cdot} + 2N_2$ (eq. 7)

$N_4^{+\cdot} + H_2O \rightarrow H_2O^{+\cdot} + 2N_2$ (eq. 8)

$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + OH^{\cdot}$ (eq. 9)

$H_3O^+ + nH_2O \rightarrow H_3O^+(H_2O)_n$ (eq. 10)

$H_3O^+(H_2O)_n + M \rightarrow [M+H]^+ + nH_2O$ (eq. 11)

Figure 9 Schematic illustration of APCI source [36]**,** [37]**.**

When the reagent gas is nitrogen, electrons produced from the corona discharge needle could ionize nitrogen plasma, creating N_2^{*+} and N_4^{*+} (*eq. 5* and *eq.6*). These primary ions collide with the vaporized solvent molecules to form secondary reactant gas ions (e.g. H_3O^+ and $(H_2O)nH^+$ (*eq. 7*)). These

reactant gas ions then undergo repeated collisions with the analyte resulting in the formation of analyte ions. With the presence of water, H_2O^{*+} is formed by the charge transfer, which then it reacts with another molecule of water to produce H_3O^+ (*eq. 8* and *eq. 9*). The formed H_3O^{*+} would further react with more water molecules to give water clusters $H_3O^+(H_2O)n$, which ionizes the analyte molecule by the proton transfer. The high frequency of collisions results in a high ionization efficiency and thermalization of the analyte ions. This results in spectral data typically rich in predominantly molecular species and adduct ions with very little fragmentation, categorizing the APCI in low energy (soft) ionization sources [36].

Electro Spray Ionization (ESI)

In ESI, a solution is sprayed into a high electric field at atmospheric pressure. Charged droplets result from the nebulization of the solution in an electric field, with both solvent and analyte ions being detected. An illustration of the ionization process is presented in *Figure 10*. ESI is a liquid-phase ionization technique, which does not require the evaporation of a neutral analyte, but rather the formation of preformed ions in solution. Therefore, ESI is the method of choice for the ionization of analytes that would easily thermally decompose. In order to achieve analyte ions in solution, the composition of the sample solution (or LC mobile phase) has to be adjusted to convert a neutral analyte into an ion in solution. Basic analytes, for example, are ionized by the addition of an acid to the solution. The electrospray process in greatly limited in terms of flow rate that can be nebulized. The use of a nebulizing gas allows higher flow rates to be used. However, higher flow rates also require the use of heat, for example, by application of a concurrent, countercurrent or cross flow of hot gas, for the desolvation of charged droplets in order to promote the release of analyte ions into the gas phase. The high efficiency of the technique and its compatibility with LC and other liquid separation methods gave rise to much interest in the pharmaceutical applications of the technique. In addition to increasing sensitivity, ESI effectively extends the mass range of analytes amenable to MS by more than an order of magnitude to beyond 150 kDa [29], [32].

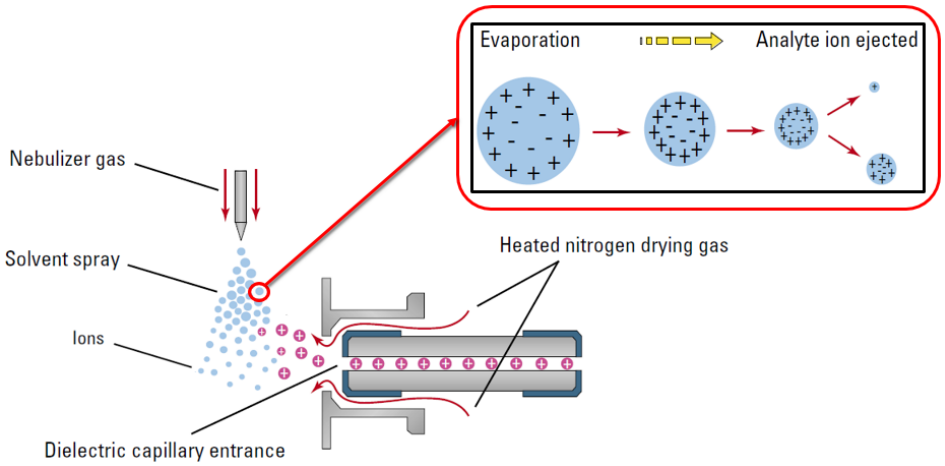


Figure 10 ESI source [37]**.**

2.1.2.3.2 Mass analyzers

Depending on the accuracy of the mass measurement, MS can be divided into two categories: low resolution MS (LRMS) and high resolution MS (HRMS). The most common LRMS instruments are Quadrupole, triple Quadrupole and Ion Trap, whereas the most common HRMS instruments are Time of Flight and Orbitrap.

- Quadrupole (Q)

A Q analyzer (*Figure 11*) consist of four parallel rods and uses a combination of radio frequency (RF) alternating current (AC) and direct current (DC) voltages as a mass filter, for separating ions. The positive DC voltage is applied on two opposite rods, and the same value of the negative DC voltage is applied on the remaining two rods. The AC is connected to all four rods. Combined DC and RF potentials on the quadrupole rods can be set to pass only a selected m/z ratio. All other ions do not have a stable trajectory through the quadrupole mass analyzer and will collide with the quadrupole rods, never reaching the detector. The single quadrupole is certainly the simplest, cheapest, most robust and ubiquitous mass analyzer in research and development laboratories, but it suffers from a limited sensitivity, resolving power and mass accuracy [29], [32].

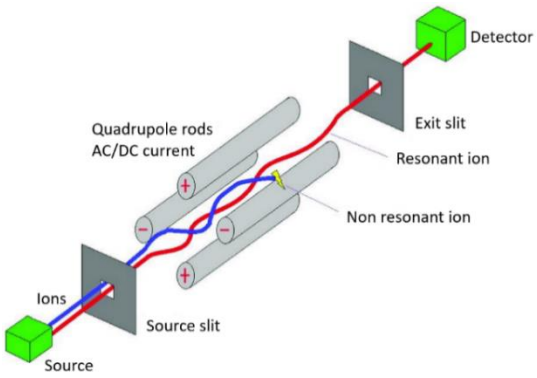


Figure 11 Quadrupole [29]**.**

- Triple Quadrupole (QqQ)

A QqQ mass spectrometer (*Figure 12*) offers MS/MS in which the first and third Qs act as mass filters, while the second Q is used for fragmentation of the precursor ion through interaction with a collision gas (usually nitrogen or argon). The main MS/MS scan modes are product ion, precursor ion, neutral loss, single reaction monitoring (SRM), multiple reaction monitoring (MRM), and MS^n scans. The main benefits of analysis in MS/MS mode are minimum matrix component interferences, and at the same time, thanks to the possibility of selecting suitable precursor and product ions, possible identification and quantification of the abovementioned contaminants even at (ultra)trace concentrations [32].

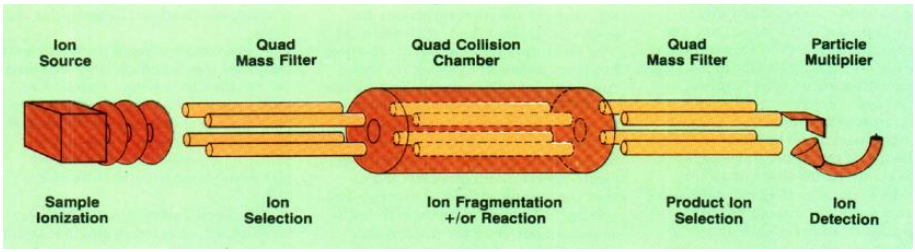


Figure 12 Conceptual diagram of the QqQ mass spectrometer [38]**.**

- Ion Trap (IT)

An IT comprises two endcap electrodes and a ring electrode, all of hyperbolic or hemispherical cross-section. The end-cap electrodes contain small-diameter holes for allowing ions to enter and leave the trap. Ions are confined inside the trap by a radio frequency field of constant frequency but variable

power. The ions may be detected, according to their m/z ratio by applying voltages sufficient to eject them from the trapping field [32].

- Time of Flight (TOF)

The operation of a TOF mass analyzer (*Figure 13*) is based on the fact that ions with the same energy but different masses travel with different velocities. Basically, ions formed by a short ionization event are accelerated by an electrostatic field to a common energy and travel over a drift path to the detector. The lighter ones arrive before the heavier ones, and a mass spectrum is recorded. Measuring the flight time for each ion allows the determination of its mass. This cycle is repeated with a repetition rate that depends on the flight time of the highest mass to be recorded. The enhancement in the mass resolution is obtained by using a reflectron, which is a series of ring electrodes with increasing voltage that creates retarding fields. The higher-energy ions reaching the reflectron area penetrate more deeply inside, and this results in extension of the time until they are reflected. Due to this phenomenon, the ions of the same m/z value with different initial energies hit the detector at almost the same time. The flight times of the ions separated in a field-free region are proportional to the square root of the respective m/z value [29], [32].

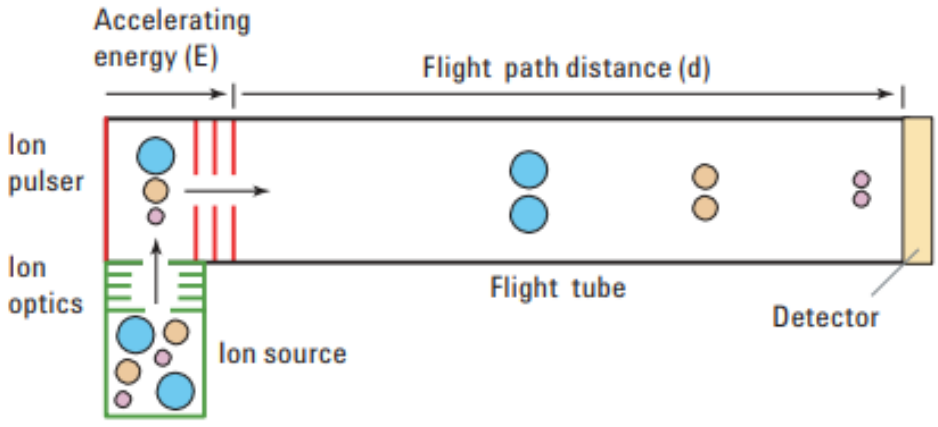


Figure 13 TOF mass analyzer [39]**.**

- Orbitrap

The Orbitrap mass analyzer (*Figure 14*) essentially consists of three electrodes. Outer electrodes have the shape of cups facing each other and electrically isolated by a hair-thin gap secured by a central ring made of a dielectric. A spindle-like central electrode holds the trap together and aligns it via dielectric end-spacers. When voltage is applied between the outer and the central electrodes, the resulting electric field is strictly linear along the axis and thus oscillations along this direction will be purely harmonic. At the same time, the radial component of the field strongly attracts ions to the central electrode [40].

Ions are injected into the volume between the central and outer electrodes essentially along a tangent through a specially machined slot with a compensation electrode (a “deflector”) in one of the outer electrodes. With voltage applied between the central and outer electrodes, a radial electric field bends the ion trajectory toward the central electrode while tangential velocity creates an opposing centrifugal force. With a correct choice of parameters, the ions remain on a nearly circular spiral inside the trap, much like a planet in the solar system. At the same time, the axial electric field caused by the special conical shape of electrodes pushes ions towards the widest part of the trap initiating harmonic axial oscillations. Outer electrodes are then used as receiver plates for image current detection of these axial oscillations. The digitized image current in the time domain is Fourier-transformed (FT) into the frequency domain in the same way as in Fourier-Transform Ion Cyclotron Resonance (FTICR) and then converted into a mass spectrum [40].

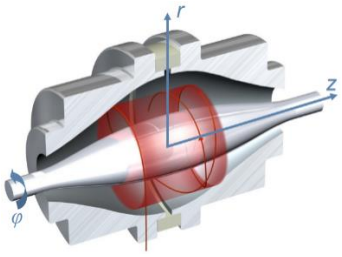


Figure 14 Orbitrap mass analyzer [40]**.**

2.1.2.4 Hybrid instruments: QToF

The coupling of two different analyzers is known as a hybrid instrument. An example of a widely used hybrid instrument is QqTOF (*Figure 15*) where the first quadrupole is used for initial mass selection and focusing, the second serves as a collision cell where fragments are produced which will be subsequently separated by the third mass analyzer, TOF. Interfering peaks from ions having the same nominal mass can be resolved due to the high resolving power of QqTOF, (typical range 20,000-40,000), thus improving the signal to noise ratio [29].

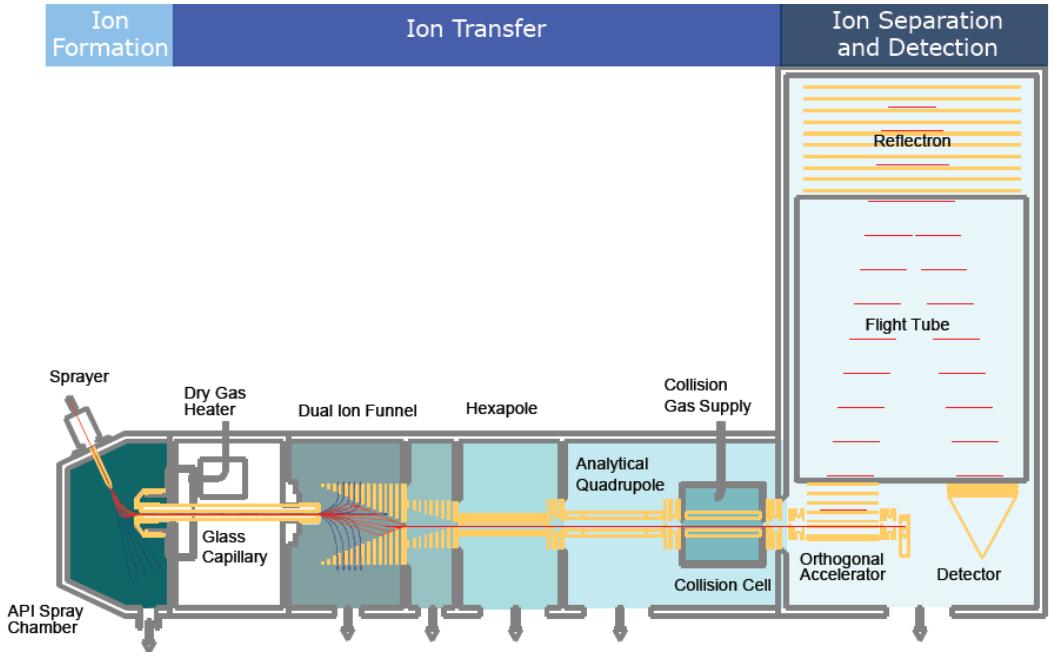


Figure 15 Scheme of QqTOF instrument (Maxis Impact, Bruker) [41]**.**

2.1.2.5 Data acquisition modes

Two data acquisition modes are available in HRMS: Data Dependent Acquisition mode (DDA) and Data Independent Acquisition mode (DIA).

In DDA mode, there is firstly a full scan which is defined as the survey scan and data are processed “on-the-fly” to determine the candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. If the selection criteria are met, MS/MS analysis is then triggered and MS/MS scans (data-dependent) are performed. With this acquisition, ‘clean’ spectra structural information is obtained in one injection.

However, if the number of candidates of interest is big, the number of scans is decreased, so there are less data points that affect the detectability of the chromatographic peak [42].

With DIA mode, there is no need to pre-select the precursor ion. Full-scan spectra at different collision energies are obtained in one injection. This acquisition provides simultaneously accurate mass data of parent compounds and fragment ions in a single run using two scans, one at low and one at high collision energy. By applying low energy in the collision cell, no fragmentation is performed. A full-scan spectrum is obtained that provides information for the parent ion (the (de)-protonated molecule) and, in some cases, the adduct ions and the in-source fragments. By applying high energy in the collision cell, fragmentation is performed and a spectrum similar to MS/MS experiments is obtained. This approach is called all-ions MS/MS or bbCID (broadband collision-induced dissociation), according to the QToF manufacturer [42].

2.2 Identification approaches

Three different screening post-acquisition approaches can be used for data processing derived from LC-HRMS or GC-HRMS analysis: target, suspect, and non-target screening. These three approaches should not be considered as different but as complementary to each other in order to identify ECs and their TPs in DW [43].

- **Target screening** is the easiest and most straightforward approach comparing to the others. It refers to the screening of a predetermined number of analytes for which reference standards are available. It often requires little to no prioritization and it can be performed in an automated way for many compounds using a database [43].
- **Suspect screening** requires prior knowledge for the compounds of interest since it is based on the comparison of the ionized masses in the sample with the masses of compounds suspected to be present in the sample. Suspect compounds for which both name and structure are known are gathered in a list. Suspect lists can be created from literature

search and by using software able to predict suspect compounds. It does not require a reference standard from the beginning and has great potential of retrospective screening [43].

- Unlike target and suspect screening, which require previous knowledge about the expected chemicals, **non-target screening** is based on component prioritization and does not consider a (tentative) structure from the beginning. The masses of interest are firstly prioritized following various approaches and using sophisticated tools. The prioritized masses undergo the very time-consuming process of structure elucidation and, afterwards, further evaluation is done following the same workflow and tools like in suspect screening [43]. Advances in the available software are required to overcome the challenges that non-target screening is facing regarding data treatment [44].

2.3 Levels of confidence

2.3.1 Confidence in target screening

The confirmation of positive findings in target screening can be performed by attributing identification points (IPs). According to the 2002/657/EC guideline, 4 IPs are required for unequivocal confirmation, and for HRMS instruments with resolution higher than 10,000, the precursor ion earns 2 IPs and the product ions earn 2.5 IPs. This means that one single HRMS/MS transition can confirm the detection of a substance, which is risky when there are several co-eluting isomers. Another fact is that resolving power may largely vary between HRMS instruments, which makes the definition of general criteria difficult. More precise criteria for the use of mass accuracy and mass resolution have to be implemented to define clearly the requirements for a reliable confirmation in LC-HRMS [5], [42].

2.3.2 Confidence in suspect and non-target screening

The wide use of HRMS not only in the environmental chemistry but also in other research fields resulted in the occurrence of various levels of confidence regarding the obtained results among different substances and

studies. Consequently, the identification of small molecules such as ECs and their TPs via suspect and non-target HRMS, requires a common system to communicate concisely and accurately the achieved level of confidence. In order to ease the communication of confidence, *Schymanski et al*. proposed a five-level system (*Figure 16*) [45].

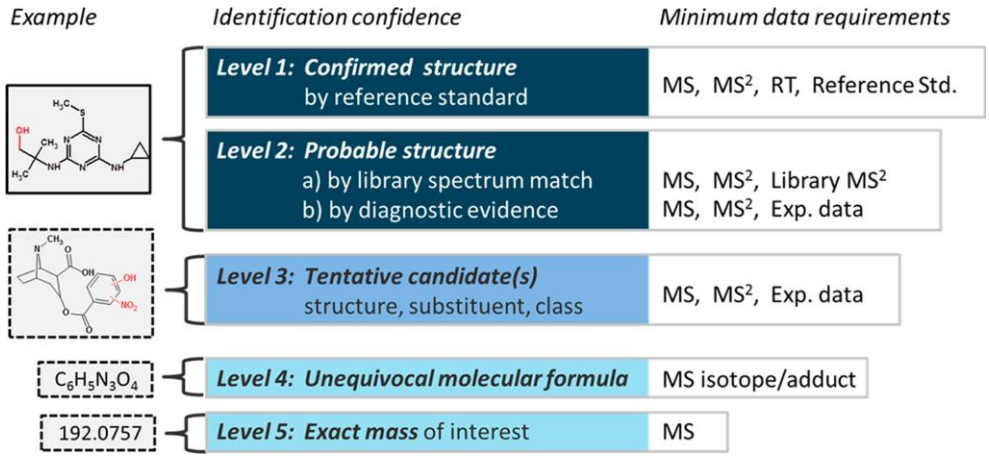


Figure 16 Proposed identification confidence levels in HRMS analysis [45]**.**

Level 5 Exact mass (m/z): refers to an m/z that can be measured in a sample but there is a lack of information even for the assignment of a formula. This level should be applied only to limited masses of particular interest.

Level 4 Unequivocal molecular formula indicates that the assignment of a formula based on spectral information is possible but insufficient evidence exists to propose possible structures.

Level 3 Tentative candidate(s) This level indicates a “grey zone” where evidence exists for possible structure(s) but there is insufficient information for exact structural proposal.

Level 2 Probable structure describes the case where the proposal of an exact structure was possible due to the available evidence.

2a Library At this level it is possible to match library or spectrum data with the structure. The validity of the match depends strongly on the acquisition parameters of the different spectra and the decision criteria must be clearly presented.

2b Diagnostic. The experimental evidence is in accordance with only one structure but no standard or literature information is available for confirmation. Evidence include diagnostic MS/MS fragments, ionization behavior, parent compound information and experimental context.

Level 1 Confirmed structure refers to the ideal situation where a reference standard of the substance of interest is available. The assignment is performed by measuring the reference standard using MS, MS/MS, retention time matching and an orthogonal method if possible [45].

CHAPTER 3
LITERATURE REVIEW: DETERMINATION OF ECs AND THEIR TPs IN DWTPs

So far in literature many studies concerning the occurrence and transformation of ECs in various aqueous matrices (surface water, groundwater, wastewater, DW, tap water) have been reported [19]. Considering the scope of this research, our literature review focused mainly on source (surface water, DWTP input) and DW samples (DWTP output, tap water) analyzed by HRMS techniques. The most representative and relevant research studies are presented in **Table 1**.

As previously described in *Paragraph 1.1*, the determination of ECs in environmental samples constitutes a great challenge for analytical chemistry. The application of a generic sample preparation protocol followed by a highly selective and sensitive analytical technique is required [1]. In general, the analytical methods developed for this scope aim at analyzing a limited number (<100) of multi-class compounds ([6], [10], [46]–[51]) or a greater number (from 100 to 500) of target compounds typically belonging to fewer classes (up to two different classes) [52], [53]. Few methodologies have been applied for the determination of more than 500 compounds belonging to either two or various classes ([26], [54], [55]) while there are some methods designed for 100 to 300 multi-class compounds [4], [56]. At the same time, very few wide-scope target screening HRMS methodologies aiming at analyzing more than 1,000 multi-class compounds in surface and DW samples have been reported [57], [58].

Tröger et al. and *Padhye et al.* conducted the most relevant studies to our research scope [4], [46]. In particular, *Tröger et al.* developed a multi-residue method for the determination of 134 multi-class ECs in water samples. The method was applied to samples collected at different stages of DW production including surface water, water before and after the different treatment steps applied at a conventional DWTP, DW after its production

and tap water. The aim of this research work was to investigate the occurrence of ECs and their fate during the hydrological pathway from source to tap water and to explore the efficiency of the treatment processes applied at the DWTP regarding their removal [4]. Similarly, *Padhye et al.* examined the seasonal variation of the occurrence and removal of PPCPs and EDCs in water samples. Target analytes were chosen to be representative of a wide range of physicochemical properties and samples were collected at the entrance and at the exit of a DWTP and at sampling points between the different treatment steps [46].

Considering the investigation of the quality of DW, *Machado et al.* [6] and *Rodil et al.* [7] conducted research that provided an overall assessment of DW quality in a country and in an area respectively. In more detail, *Machado et al.* conducted the first nationwide study in Brazil regarding the presence of ECs in source and DW [6] while *Rodil et al.* investigated the occurrence of ECs in sewage, surface and DW in A Coruña, Galicia, Spain, during one-year period. More than 50 ECs were investigated in the water circle of the area providing a comprehensive snapshot of A Coruña [7]. Correspondingly, *Diamanti et al*., *Ruff at al.* and *Hollender et al.* investigated the occurrence of ECs in river water used for DW production in order to assess the chemical pollution status of the Dniester River ([58]), evaluate the temporal and spatial variation of ECs in the Rhine River ([56]) and monitor numerous ECs in three rivers in Switzerland [54].

In the following paragraphs, current trends regarding the sample treatment and the analytical techniques used for water analysis are discussed.

Table 1 Determination of ECs and their TPs in aqueous matrices through target analysis.

No	Matrix	Target compounds	Sample treatment	Technique	Results	Reference
1	**Drinking water** (DWTP input & output) Wastewater (WWTP influent & effluent) Tap water	**134** multi-class ECs	**SPE** (Oasis HLB & Bond-Elut ENV)	**UHPLC-QToF-MS** (positive: Acquity UPLC HSS T3-C_{18} negative: Acquity UPLC BEH-C_{18})	**41** ECs (drug-related compounds [3], food additives [2], pesticides [2], PPCPs [23], PFAS [11]) were detected in at least one sample (input & output of the DWTP & tap water). Concentrations from sub ng L^{-1} levels to almost 80 ng L^{-1}.	[4]
2	**Drinking water** (DWTP input & output & samples after each treatment step) Surface water	**30** multi-class ECs	**SPE** (HLB)	**LC-ESI-Qtrap-MS/MS** (positive & negative: Ascentis RP-Amide)	**21** ECs (pharmaceuticals [15], PCPs [5], herbicide [1]) were detected in at least one sample (input & output of the DWTP). Average concentrations ranging from 0.1 to 140 ng L^{-1}.	[46]
3	**Drinking water** (tap water) Surface water	**16** multi-class ECs	**SPE** (Oasis HLB)	**LC-ESI-QqQ-MS/MS** (Zorbax SB-C_{18})	**5** ECs (Caffeine, atrazine, triclosan, bisphenol-A, phenolphthalein) were detected in at least one sample. Average concentrations ranging from < 3.0 ng L^{-1} to 3540 ng L^{-1}.	[6]
4	**Drinking water** (DWTP input & output, tap water) Surface water Wastewater (WWTP influent & effluent)	**53** multi-class ECs	**SPE** (Oasis HLB)	**LC-ESI-QqQ-MS/MS** (positive: Symmetry Shield RP18 negative: Luna Phenyl-Hexyl)	**24** ECs (pharmaceuticals [6], OPs [8], herbicides [4], bactericide [1], insect repellents [2], UV filters [3]) were detected in at least one sample (DW & surface water). Concentrations ranging from <10 ng L^{-1} to 150 ng L^{-1}.	[7], [47]

No	Matrix	Target compounds	Sample treatment	Technique	Results	Reference
5	Surface water (river water)	**302** multi-class ECs and their TPs	**SPE** (Oasis HLB, Strata X-AW, Strata X-CW, Isolute ENV$^+$)	**LC-ESI-LTQ-Orbitrap-MS** (positive & negative: Xbridge RP C_{18})	**132** ECs (pharmaceuticals and their TPs [59], pesticides and their TPs [44], biocides and their TPs [7], artificial sweeteners [4], illicit drugs and their TPs [5], industrial chemicals [3], corrosion inhibitors and others [10]) were detected at least in one sample. Concentrations ranging from 1 ng L^{-1} to 3044 ng L^{-1}.	[56]
6	Surface water (river water) Groundwater Wastewater (WWTP effluent)	**88** polar multi-class ECs and their TPs	online-**SPE** (Oasis HLB, Strata X-AW, Strata X-CW, Isolute ENV$^+$)	**LC–ESI-QqQ-MS/MS** (positive & negative: Atlantis T3)	**36** ECs (pesticides, biocides, pharmaceuticals, corrosion inhibitors, artificial sweeteners and their TPs) were detected at least in one sample (groundwater). Concentrations ranging from 0.1 ng L^{-1} to 600 ng L^{-1}.	[48]
7	**Drinking water** Surface water (river water) Wastewater (WWTP effluent)	**72** multi-class ECs	online-**SPE** (C_{18} Hypersil Gold)	**UHPLC–HESI-Q-Orbitrap-MS** (positive & negative: C_{18} core–shell Accucore RP-MS)	Not applied in real samples. Analysis time: 0.2 min per analyte Sample volume: 1mL LODs: down to 0.01 µg L^{-1}	[49]
8	**Drinking water** (tap, fountain, well water)	**21** multi-class ECs and their TPs	**SPE** (Oasis HLB)	**UHPLC-ESI-QqQ-MS/MS** (positive & negative: Kinetex XB-C_{18})	**13** ECs (pharmaceuticals and their TPs [8], pesticides [4], industrial chemicals [1]) were detected in tap water samples. Concentrations up to 7.87 ng L^{-1}.	[10]

No	Matrix	Target compounds	Sample treatment	Technique	Results	Reference
9	Surface water Groundwater	**1500** multi-class ECs (LC:1597 compounds, GC: 525 compounds)	**SPE** (Oasis HLB)	**LC-ESI-QToF-MS** **(**Acquity UPLC HSS T3 , Acquity UPLC BEH C_{18}) **LC-ESI-QqQ-MS** (Acquity UPLC BEH C_{18}) **GC-APCI-QToF-MS** (fused silica DB-5MS column) **GC-EI-ToF-MS**	**53** ECs (pesticides [34], antioxidants [2], cosmetics [2], drugs of abuse [2], insect repellent [1], musk [1], PAHs [2], pharmaceuticals [1], plasticizer [1], preservatives [4], UV filters [3]) were detected through the screening procedure (LC & GC). **33** ECs (pesticides and their TPs [24], pharmaceuticals [7], drugs of abuse and their metabolites [2]) were quantified.	[57]
10	Surface water (river water)	**2273** multi-class ECs	**SPE** (Oasis HLB disks)	**LC-ESI-QToF-MS** (positive & negative: Acclaim RSLC C_{18})	**106** ECs (pesticides and their TPs [44], pharmaceuticals and their TPs [40], drugs of abuse [4], stimulants and their TPs [4], industrial chemicals [11], sweeteners [3]) were detected at least in one river water sample. Concentrations ranging from 1.64 ng L^{-1} to 7708 ng L^{-1}.	[58]
11	Surface water (river water)	**526** multi-class ECs	**SPE** (Oasis HLB, Strata X-AW, Strata X-CW, Isolute ENV^{+}, ENVI carb)	**LVI-LC-ESI-Q-Orbitrap-MS** (positive & negative: (XBridge C_{18})	**123** ECs were detected and quantified at least in one sample. Median concentrations of all quantified ECs were 9.0, 6.9, 5.6 ng L^{-1} in the three sampling locations.	[54]

No	Matrix	Target compounds	Sample treatment	Technique	Results	Reference
12	Surface water Wastewater (WWTP influent & effluent)	**590** multi-class ECs	**VEC** **SPE** (Oasis HLB, Strata X-AW, Strata X-CW, Isolute ENV^{+}, ENVI carb)	**LVI-LC-ESI-Q-Orbitrap-MS** (positive & negative: Atlantis T3)	SPE and VEC performed equally well (≥ 70% recovery) for a vast number of analytes (n = 327). **121** ECs were quantified in surface water samples. Concentrations down to sub ng L^{-1} levels.	[26]
13	Surface water Groundwater Wastewater (WWTP influent & effluent)	**150** multi-class ECs	**SPE** (C_{18})	**GC-QToF-MS** (fused silica HP- 5MS capillary column)	**24** ECs were detected at least in one surface & groundwater sample. Qualitative screening method.	[59]
14	**Tap water**	**539** compounds (pesticides and drug residues)	online-**SPE** (Oasis HLB)	**UHPLC-HESI-LTQ-FT-Orbitrap-MS** (HSS T3 column)	**34** compounds (pesticides and their TPs [30], drugs [4]) were detected in tap water samples. Concentrations ranging from 0.5 ng L^{-1} to 10 ng L^{-1}.	[55]
15	**Drinking water** Surface water Groundwater Wastewater	**100** pharmaceuticals including some of their degradation products	**SPE** (Oasis HLB)	**LC-ESI-QToF-MS** (positive & negative: ZORBAX Eclipse XDB-C_8)	**36** pharmaceuticals were most frequently detected in surface water samples. Average concentrations ranging from 10 ng L^{-1} to 455 ng L^{-1}.	[50]
16	**Tap water** Surface water (river water)	**36** EDCs (estrogens, progestogens, phenols, and their metabolites)	**SPE** (Oasis HLB)	**UHPLC-QToF-MS** (positive & negative: Acquity UPLC HSS T3)	**3** compounds (estriol, estrone, bisphenol-A) were detected at least in one sample. Concentrations ranging from 1.0 ng L^{-1} to 690 ng L^{-1}.	[51]

No	Matrix	Target compounds	Sample treatment	Technique	Results	Reference
17	Surface water (river water) Wastewater (WWTP effluent)	**198** pharmaceuticals and pests in surface water **170** pharmaceuticals and pests in wastewater	**SPE** (Strata X)	**LC-ESI-QToF-MS** (positive & negative: Acquity UPLC BEH Phenyl)	**18** ECs were detected and semi-quantified at least in one surface water sample. Concentrations ranging from > 0.01 µg L^{-1} to > 1.0 µg L^{-1}. Qualitatively validated method.	[53]

The sample preparation protocols designed for water samples' analysis usually include a sample filtration step to remove any particulate matter from samples [48], [49], [26]. In addition, pH adjustment (acidification) can be required to charge appropriately the target compounds or for sample preservation [7], [10], [47], [55]. Especially for DW analysis, either for target analysis or for kinetic studies, residual Cl can be quenched [16], [60]. Despite the fact that various quenching agents (sodium thiosulfate, sodium sulfite, ammonium chloride, ascorbic acid) have been reported to be used, careful selection of the reagent is needed since their addition can affect the compounds of interest [10], [60].

Regarding sample treatment, either online or offline SPE is applied for the extraction of target compounds [2], [4], [6], [10], [16], [25]–[28], [30], [32], [17]–[24]. While the high degree of automation of online SPE reduces significantly the total analysis time, it also reduces flexibility compared to offline SPE [48], [43]. The physicochemical properties of the target analytes determine the selection of the sorbent material. While the use of specific SPE sorbents like Strata-X and C_{18} aim at a limited number of compounds with similar properties [53], [59], HLB is mostly used in multi-residue methods due to its ability to retain a wide range of compounds [43]. In order to achieve even broader chemical enrichment, *Ruff et al.* and *Huntscha at al.* combined four sorbent materials (Oasis HLB, Strata X-AW, Strata X-CW, Isolute ENV+) at specific quantities in one in-house made cartridge [48], [56]. Mixed-bed multilayered cartridges are a popular alternative to HLB cartridges in wide-scope target screening since they achieve the extraction of neutral, anionic and cationic ECs of a broad range by exploiting the different retention mechanisms of each sorbent [43], [48], [56]. These cartridges have been also successfully used by *Gago-Ferrero et al.* in wide-scope target analysis of more than 2,000 ECs in wastewater samples [5]. Moreover, *Hollender et al.* and *Mechelke et al.* added a fifth sorbent material (ENVI carb) in the abovementioned cartridges in order to achieve the efficient enrichment of non-polar to very polar compounds [26], [54]. For the extraction of a large sample volume (2 L), SPE HLB disks have been used in an automated extraction system [58]. In addition, *Mechelke et al.* evaluated the performance of VEC as an alternative sample treatment method to the SPE

using the three layered mixed-bed cartridges consisting of five different sorbent materials. VEC performed equally well with SPE (≥ 70% recovery) for a great number of compounds (327 compounds). Since VEC requires significantly less sample volume and minimum volume of organic solvents combined with its lower cost and limited need for supervision, it is proposed as an environmentally friendlier and efficient alternative sample preparation method to SPE [26], [43]. Moreover, *Albergamo et al.* were the first research group that applied and published the combination of direct injection with LC-HRMS. Their aim was to determine 33 polar micropollutants in surface water used for DW production and in groundwater by combining direct injection analysis with RPLC-(+/-)ESI-QToF-MS using a core-shell biphenyl column. The method was successfully validated and efficient for the detection of micropollutants at environmentally relevant concentrations (limits of detection ranging from 9 to 93 ng/L). The application of the developed and validated method revealed the occurrence of ECs in surface and groundwater samples ranging from 28 to 1000 ng/L [28].

LC coupled with HRMS is the main technique of choice for wide-scope target screening in water samples [19], [43]. LC separation is mostly achieved under RPLC conditions, using mainly C_{18} analytical columns and a gradient program of mobile phases [43]. Since highly polar compounds are not well retained in RPLC conditions and, hence, not well separated, *Hollender et al.* and *Mechelke et al.* proposed the use of a polar C_{18} analytical column in large volume injection (LVI) RPLC [26], [54], [43]. ESI and heated ESI (HESI) are mainly used as ionization sources operating both in positive and negative modes [43]. Hybrid instruments like QToF ([4], [50], [51], [53], [57]–[59]) and LTQ-Orbitrap or Q-Orbitrap ([26], [46], [49], [54]–[56]), which permit the screening of the acquired data for a great number of compounds not only with target but also with suspect and non-target approaches are frequently used. Triple quadrupole is typically used for a limited number of compounds or for quantitative purposes ([10], [47], [48], [57]). Despite its limited use in aqueous matrices compared to LC-HRMS, GC-HRMS has been also used for wide-scope target analysis of ECs as well as for their qualitative detection in water samples. In GC-HRMS, APCI and EI

are the sources of choice and QToF or ToF are the mass analyzers commonly used [57], [59].

While the abovementioned sample preparation methods and analytical techniques refer to the implementation of a target screening approach for the determination of 'known' compounds (selected ECs and known TPs) in aqueous samples, other data treatment approaches have been reported for the 'unknown' ones. Briefly, for 'known unknown' DBPs and TPs (known name and structure), lists of suspect compounds are created and a suspect screening data treatment approach is adopted. For 'unknown unknown' ECs, TPs and DBPs (no previous knowledge is available) non-target workflows, which require the use of advanced data treatment tools, need to be implemented [43], [44].

Richardson has been investigating the occurrence of DBPs in DW for decades. In her recent article entitled *'Tackling unknown disinfection by-products: Lessons learned'*, the current status of knowledge is underlined and lessons learned from her year-long experience on investigating 'unknown' DBPs are addressed [22]. The investigation of DBPs in DW has also concerned *Kimura et al.* who proposed a method that enables the simultaneous determination of priority DBPs and the identification of the 'unknowns'. According to it, analytes are extracted by an LLE protocol and extracts are analyzed with GC-EI-TOF-MS [17]. However, *Richardson* suggests the use of both GC-HRMS and LC-HRMS techniques to characterize comprehensively the status of the sample analyzed [22]. Moreover, research on DBPs produced by different disinfection reagents at DWTPs has been reported in literature. A representative example is the investigation conducted by *Zhong et al.* who examined the seasonal variation of carbonyl compounds as DBPs at two DWTPs using different disinfectants (chlorination, ozonation) for one year [15].

Paszkiewicz et al. and *Menger et al.* in their review articles describe in detail the advances in suspect and non-target screening of polar ECs in environmental monitoring and highlight the current state and trends of LC-HRMS strategies for wide-scope screening of polar ECs in water samples respectively [43], [44]. In both reviews, the sampling strategies, sample preparation methods, analytical techniques and data treatment workflows that

are currently used are critically assessed. In general, the most common sampling strategy for water LC-HRMS analysis is grab sampling [43], [44]. While offline SPE is currently the sample preparation method of choice, both online SPE and direct injection are being increasingly used [43]. The applied sample preparation protocol needs to achieve a balance between sample purification to limit matrix effects and preserve the greater number of analytes with heterogeneous properties with good sensitivity. The use of hyphenated chromatographic techniques with HRMS instruments operating with generic settings are widely used. At the same time, the application of complementary chromatographic techniques is recommended [43], [44]. For example, *Been et al*. and *Castro at al*. implemented different chromatographic techniques (RPLC, HILIC, MMLC, SFC) during suspect screening for the identification of a great number compounds in surface water samples (river and estuary water) [31], [61]. Data processing for suspect and non-target screening require the use of sophisticated tools and software, the advances of whom will enable and facilitate the development of big-data treatment approaches [43], [44].

To conclude, the application of a generic sample preparation protocol followed by analysis with complementary HRMS analytical techniques produce data the treatment of which can provide a holistic characterization of the samples when the three conceptual data treatment approaches are applied.

CHAPTER 4
SCOPE AND OBJECTIVES

The vital role of DW in maintaining life and Nature's equilibrations makes it one of the most precious resources on Earth. Therefore, the quality both of the DW and the sources used for its production need to be safeguarded [9]. Regarding that scope, over the years the EU has established Policies concerning the protection of WB and the safety of DW [20], [21]. However, the regulated chemicals are only a small fraction of the chemicals that are used nowadays. Hence, numerous and multi-class chemicals called ECs are the main threat that WB and DW are facing [19].

In addition, at DWTPs the applied treatment processes can contribute to the formation of TPs of ECs. Chlorination, the most widely applied water disinfection procedure worldwide, aims at deactivating pathogens in water [15]. But, chlorination reagents can react with ECs and NOM producing a variety of DBPs and TPs that may have even greater toxicity than the parent compound. Therefore, the effect of chlorination reagents in the formation of TPs and DBPs needs to be studied [15], [19].

The detection of ECs and their TPs is a challenge for analytical chemistry. Their low concentrations in combination with their wide range of physicochemical characteristics require the development of multi-residue methods and the use of complementary analytical techniques [5], [19]. Advances in HRMS have contributed to the development of wide-scope target screening methods. Validation of these methods is also challenging since standardized criteria and harmonized guidance for the identification and quantification of the analytes are not clearly established [5]. Apart from target screening, suspect and non-target screening workflows should be also applied in order to achieve a holistic investigation approach [19].

The aim of this study was to validate a generic sample preparation protocol based on SPE in order to prove its efficiency for wide-scope target analysis of ECs in surface water as well as to assess the LODs obtained from a direct injection analysis of the same target analytes. Additionally, the scope of this

study included the investigation of the occurrence of ECs in water samples collected at the entrance of a DWTP through the application of a wide-scope target screening approach. The possible detection of compounds can indicate which ones could be possibly removed during the treatment process or transformed by chlorination that takes place at the DWTP.

EXPERIMENTAL PART

CHAPTER 5
EXPERIMENTAL PROCEDURE

5.1 Chemicals and reagents

Regarding the sample preparation, glass microfiber filters (WHATMAN 934-AH, diameter 47 mm, pore size 0.7 μm) were obtained from Cytiva (USA). For the SPE procedure, empty polyethylene tubes (6 mL) and frits were purchased from Restek (USA). The sorbent materials Oasis HLB, Strata-X-AW and Strata-X-CW were purchased from Phenomenex (Torrance, USA), while the Isolute ENV+ sorbent material was purchased from Biotage (Ystrad Mynach, UK). Regenerated cellulose (RC) syringe filters (diameter 15 mm, pore size 0.2 μm) were obtained from MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany).

The reagents and solvents used during sample preparation (sulfuric acid analytical reagent grade, ammonium hydroxide, methanol HPLC grade, ethyl acetate HPLC grade) were purchased from Fischer Scientific (Loughborough, UK). Formic Acid 98-100% for analysis was purchased from Carlo Erba Reagents S.A.S. (Barcelona, Spain), and ammonia hydroxide solution 25% for analysis was purchased from Chem-Lab NV (Zedelgem, Belgium). For the chromatographic analysis, methanol for LC-MS was obtained from Merck (Darmstadt, Germany). The eluent additives ammonium formate, ammonium acetate and formic acid 99% were purchased from Sigma Aldrich (Steinheim, Germany). Ultrapure water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA).

All reference standards used for method validation and analysis were purchased from Sigma Aldrich (Steinheim, Germany), Toronto Research Chemicals (Ontario, Canada), LGC (Mercatorstrass, Germany), Acros Organics (Morris Plains, NJ) and Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). A stock solution at concentration 1 mg/L in MeOH was prepared by mixing 8 solutions consisting of all analytes of interest. This stock solution was used for the preparation of standard working solutions in methanol/water

(50/50) as well as for the spiking of analytes in spiked samples. Concerning the Internal standards (IS) used, Amisulpride-d^5, Bisphenol A (BPA)-d^{16}, Diuron-d^6, Diazepam-d^5, rac-methamphetamine-d^5, Fenbendazole-d^3, Flunixin-d^3, Meloxicam-d^3, Morphine-d^3 and Ranitidine-d^6 were obtained from Sigma Aldrich (Steinheim, Germany), Sulfadiazine-d^4, Mefenamic Acid-d^3, Diethyl Phthalate-d^4 were obtained from Toronto Research Chemicals (Ontario, Canada) and Sulfamerazine-d^4 was purchased from Target Analysis S.A. (Athens, Greece). A solution consisting of all IS was prepared at concentration 1 mg/L in methanol.

5.2 Sampling

The DWTP located in Aspropyrgos, Attika, Greece (thereafter referred as Aspropyrgos DWTP) was chosen as the sampling location. This DWTP began operating in 1997 and it is located directly below the Mornos aqueduct. It has an altitude of approximately 232 m and its total nominal daily water treatment capacity is 200,000 m^3. It receives water solely from Mornos and it has only one store tank for finished DW [62].

The treatment process applied in this DWTP in order to convert raw water into DW consists of the following five stages:

1. Disinfection/ pre-chlorination: chlorine is added in order to deactivate the microorganisms that exist in raw water and facilitate the subsequent treatment.
2. Flocculation: aluminum sulfate is added to help particulate solids to bind and settle to the bottom.
3. Sedimentation: after flocculation, the aggregated solids settle to the bottom of the tank. In this way the water is purified up to 80%.
4. Filtration: the very light particles (20%) that were not removed in the previous treatment step are now retained in special sand filters. The water coming out from these filters is ready for consumption.
5. Post-chlorination: if pre-chlorination is not sufficient, an additional dose of chlorine is added when the water enters the closed storage tanks and before

it enters the water distribution system. Finished DW will be transferred through a pipeline system so that to be available for consumer's use [62].

The collection of the samples was conducted by the personnel of the Athens Water Supply and Sewerage Company (EYDAP S.A). Twenty-four-hour composite samples of three consecutive days of raw (untreated water) were collected from Aspropyrgos DWTP. In particular, every three hours approximately 1 L of sample was collected at determined sampling points at the entrance of the DWTP. After the collection of 8 L that were representative of 24 h, samples were mixed at the laboratory of EYDAP located in Galatsi, Attika, Greece. A procedural blank sample created in the lab consisting of Milli-Q water was also analyzed. In addition, blank samples from the sampling procedure were obtained using ultra-pure water. Samples were stored in amber glass bottles until analysis. In the following table (**Table 2**) the coding of the received samples is presented.

Table 2 Samples' coding.

Sampling day 1 (13/02/2023)	Sampling day 2 (14/02/2023)	Sampling day 3 (15/02/2023)
Untreated_day1	Untreated_day2	Untreated_day3
Procedural blank_day1	Blank_sampling_day2	Blank_sampling_day3

5.3 Sample preparation

5.3.1 SPE procedure

A generic sample preparation protocol described by *Gago-Ferrero et al.* with slight modifications was performed [5]. The sample preparation protocol is depicted in *Figure 17*. Briefly, 1L of sample was filtered through a glass fiber filter (WHATMAN 934-AH GFF, pore size 0.7 μm) using a pressurized filtration apparatus (*Figure 34* in *Annex I* depicts the filters after sample filtration). The pH of the samples was adjusted to 6.5 (±0.2) with a few drops of H_2SO_4 and/or NH_4OH. 150 μL of an Internal Standards (IS) mix (**Table 14** in *Annex I*) were spiked in each sample while 150 μL of a mix of standards were added to spiked samples (C_{final} = 300 ppb). Sample clean-up and pre-concentration were realized by SPE. Multilayered in-house made cartridges (*Figure 17*) were used

containing 350 mg of a mixed phase consisting of 100 mg of Strata-X-AW, 100 mg of Strata-X-CW and 150 mg of Isolute ENV+ at the bottom and 200 mg of Oasis HLB on the top. The conditioning of the mixed-bed cartridges was performed with 6 mL of methanol HPLC grade and 6 mL of Milli-Q water. The loading of the samples was performed overnight using polytetrafluoroethylene (PTFE) tubes with screw-fitted SPE tube adapters on the end to connect with the cartridges. Afterwards, the cartridges were dried by passing air through for 30 min. The elution of the analytes from the sorbent material was performed by a basic solution (6 mL of ethyl acetate/methanol (50/50 v/v) containing 2% ammonia hydroxide (v/v)) followed by an acidic solution (4 mL of ethyl acetate/methanol (50/50 v/v) containing 1.7% formic acid (v/v)). The extract was evaporated to dryness under a gentle nitrogen stream at 45 °C and reconstituted to a final volume of 500 µL (methanol LC-MS grade/Milli-Q water, 50/50 v/v, 2000 times pre-concentration). The extract was filtered directly into a 2 mL vial using a syringe fitted with a 0.22 µm RC membrane filter and was ready for RP-LC-HRMS analysis. Vials were stored at -20 °C until analysis.

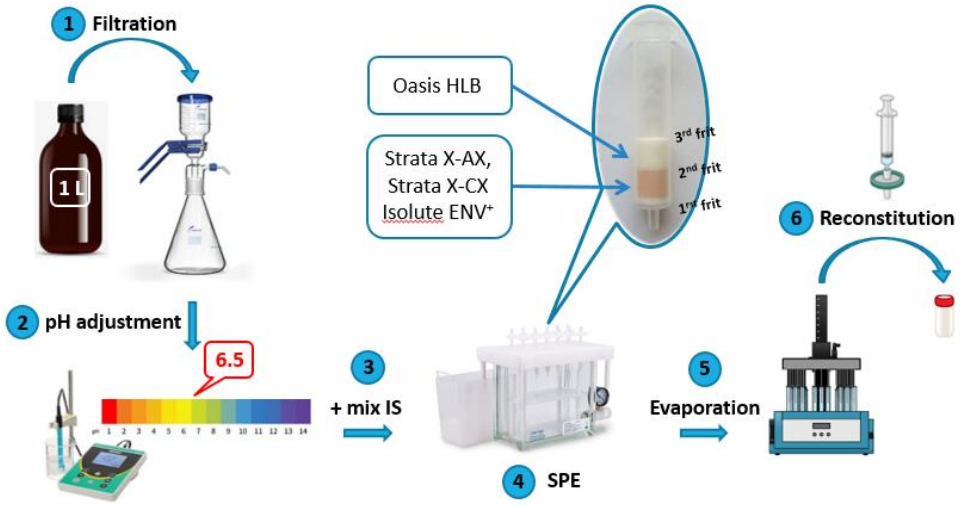


Figure 17 Schematic illustration of the SPE procedure.

5.3.2 Direct injection

As an alternative sample preparation approach, direct injection of the samples with minimum pretreatment was tested. The scope of these experiments was to test the sensitivity of the direct method, compare it with the solid phase extraction clean-up process and assess whether it is applicable for drinking

water analysis. The sensitivity of the direct method was evaluated with the determination of the recovery of specific compounds after spiking. In more detail, 150 µL of an IS mix (C_{final} = 300 ppb, **Table 7** in *Annex I*) and 150 µL of a mix of standards for spiked samples (C_{final} = 300 ppb) were transferred into testing tubes and evaporated to dryness under a gentle nitrogen stream at 45 °C. Afterwards, 500 µL of sample that has been filtered using a syringe fitted with a 0.22 µm RC membrane filter were added and shaken using a Vortex device. The sample was transferred into a vial and stored at -20 °C until analysis.

5.4 Instrumentation

A UHPLC system with an HPG-3400 pump (Dionex Ultimate 3000 RSLC, Thermo Fischer Scientific, Dreieich, Germany) coupled to a QToF mass spectrometer (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for samples' analyses (*Figure 18*).

Figure 18 UHPLC-QToF-MS, Maxis Impact, Bruker Daltonics [63]**.**

Chromatographic separation was performed using an Acclaim RSLC C_{18} column (2.1 × 100 mm, 2.2 µm, Thermo Fischer Scientific, Dreieich, Germany), preceded by a guard column of the same packaging material, thermostated at 30 °C. For positive ionization mode (PI) the mobile phases consisted of water/methanol 90/10 (solvent A) and methanol (solvent B), both containing 5

mM ammonium formate and 0.01% formic acid. For negative ionization mode (NI), the mobile phases consisted of water/methanol 90/10 (solvent A) and methanol (solvent B), both acidified with 5 mM ammonium acetate. The gradient elution program applied in both ionization modes is presented in **Table 13** in *Annex I*. It started with 1.0% of organic phase (flow rate 0.200 mL/min) for 1 min, increasing to 39.0% by 3 min (flow rate 0.200 mL/min), and then to 99.9% (flow rate 0.400 mL/min) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.480 mL/min) and then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.200 mL/min for the last minute. The injection volume was 5 µL.

The QToF-MS system was equipped with an ESI interface, operating in positive and negative mode, with the following operation parameters: capillary voltage 2,500 V for PI and 3,500 V for NI, end plate offset 500 V, nebulizer pressure 2.0 bar, drying gas (N_2) 8.0 L min^{-1} and gas temperature 200 °C. Data were acquired through both DIA (termed as broad-band Collision Induced Dissociation (bbCID)) and DDA (autoMS) mode. Spectra were recorded over the range of m/z 50–1,000 with a scan rate of 4 Hz. A QToF-MS external calibration was performed before analysis with the manufacturer's solution (sodium formate). The calibrant solution consisted of 10 mM sodium formate clusters in a mixture of water: isopropanol 1:1. At the beginning of each run and in particular in the segment from 0.05 to 0.25 min the calibrant solution was automatically injected so that to be used for internal calibration. The instrument provided a typical resolving power (full width at half maximum, FWHM) between 36,000 and 40,000 during calibration.

5.5 Method smart validation

Regarding the SPE sample preparation protocol, before the analysis of the samples, validation of the method was performed. Tap water was used to conduct all necessary experiments and the approach of method smart validation approach proposed by *Gago-Ferrero et al.* was applied [5]. A representative validation dataset of around 90 compounds from different classes of ECs (pharmaceuticals and their TPs, PPPs and their TPs, artificial

sweeteners, stimulants, DoA, industrial chemicals) was used in order to evaluate linearity, accuracy, precision, matrix effect and detectability of the screening method. The compounds of the validation dataset along with their CAS number, calculated m/z of the precursor ion, molecular formula, retention time, ionization mode and fragments are shown in **Table 3**. These selected compounds represented the different classes of ECs included in the database and have a wide range of physicochemical properties, so they elute throughout the chromatogram.

Linearity was studied for each compound by analyzing spiked samples (standard addition curve) at 7 different concentration levels ranging from 10 μg/L to 300 μg/L, as well as standard solutions (standard curve) at the same concentration levels. The calibration curves were constructed using the least squares linear regression analysis. A coefficient of determination (R^2) of > 0.9 was considered acceptable for a generic wide-scope target screening method. Standard addition curves were used to calculate the limits of detection (LOD) and limits of quantification (LOQ) both of the instrument and the method.

Method LOD and LOQ for each compound were calculated using the signal to noise ratio of the spiked sample (*eq. 12, eq. 13*).

$$LOD\ \left(\frac{ng}{L}\right) = \left(\frac{3.3 \times \frac{C_{spike}}{S/N_{spike}}}{2{,}000}\right) \times 1{,}000 \quad \text{(eq. 12)}$$

$$LOQ\ \left(\frac{ng}{L}\right) = 3\ \times LOD \quad \text{(eq. 13)}$$

Accuracy was assessed by calculating recoveries at three concentration levels (25, 75 and 150 μg/L in test solutions corresponding to 12.5, 37.5 and 75 ng/L in samples). Samples were analyzed for the determination of the compounds of the validation dataset and if the sample already contained the analyte, its peak area was subtracted from the peak area of the spiked sample and the peak area of the matrix matched sample before any calculations.

Precision was expressed as method repeatability, and therefore relative standard deviation (%RSD) was calculated for 6 spiked samples at each concentration level (75 μg/L and 150 μg/L in test solutions corresponding to 37.5 ng/L and 75 ng/L in samples).

Matrix effect was determined by comparing the response of the compounds in matrix matched samples and their response in standard solutions at three concentration levels (25, 75 and 150 μg/L L in test solutions corresponding to 12.5, 37.5 and 75 ng/L in samples). Matrix matched samples were prepared by adding in testing tubes a determined volume of the stock solution containing all compounds of interest (the stock solution preparation is described in *Paragraph 5.1*) as well as a determined volume of the IS mixture. After their evaporation under a gentle N_2 stream at 45 °C, reconstitution was performed using sample matrix. Sample matrix was obtained by analyzing a sample of tap water applying the procedure described in *Paragraph 5.3.1* without the spiking of the IS. The % matrix effect and the % factor of matrix effect (%FME) were calculated.

For samples that were tested in the direct injection approach, compounds consisting the smart validation dataset were used to calculate LODs and LOQs as well as to assess recovery and repeatability at 300 μg/L.

Table 3 Validation dataset

Compound	CAS number	Molecular Formula	Calculated m/z precursor ion	Retention time (min)	ESI mode	Fragment 1	Fragment 2	Fragment 3
2-Amino-Benzothiazole	(136-95-8)	$C_7H_6N_2S$	151.0324	5.8	(+)	124.0215	118.0525	92.0495
Acesulfame	(33665-90-6)	$C_4H_5NO_4S$	161.9867	2.3	(-)	82.0298	77.9655	
Amitriptyline	(50-48-6)	$C_{20}H_{23}N$	278.1903	8.2	(+)	233.1325	91.0452	105.0699
Amphetamine	(300-62-9)	$C_9H_{13}N$	136.1121	4.2	(+)	119.0855	91.0542	65.0386
Atenolol	(29122-68-7)	$C_{14}H_{22}N_2O_3$	267.1703	3.1	(+)	190.0863	225.1234	145.0648
Atrazine-desethyl	(6190-65-4)	$C_6H_{10}ClN_5$	188.0697	5.7	(+)	146.0228	104.0010	110.0461
Caffeine	(58-08-2)	$C_8H_{10}N_4O_2$	195.0877	4.2	(+)	138.0662	110.0713	83.0604
Cetirizine	(83881-51-0)	$C_{21}H_{25}N_2O_3Cl$	389.1626	8.8	(+)	201.0466	166.0777	165.0699
Chloramphenicol	(56-75-7)	$C_{11}H_{12}Cl_2N_2O_5$	321.0051	5.7	(-)	257.0335	152.0358	176.0358
Chlorpromazine	(50-53-3)	$C_{17}H_{19}ClN_2S$	319.1030	8.9	(+)	246.0136		
Citalopram	(59729-33-8)	$C_{20}H_{21}FN_2O$	325.1711	6.6	(+)	262.1028	109.0454	
Clarithromycin	(81103-11-9)	$C_{38}H_{69}NO_{13}$	748.4842	9.2	(+)	590.3899	158.1176	
Clazuril	(101831-36-1)	$C_{17}H_{10}Cl_2N_4O_2$	371.0108	9.4	(-)	300.0101	265.0174	
Clofibric acid	(882-09-7)	$C_{10}H_{11}ClO_3$	213.0324	6.5	(-)	126.9956	85.0295	
Clopidol	(2971-90-6)	$C_7H_7Cl_2NO$	191.9977	4.2	(+)	101.0153	86.9996	157.0289
Closantel	(145149-50-4)	$C_{22}H_{14}Cl_2I_2N_2O_2$	660.8449	12.2	(-)	no fragmentation		
Colchicine	(64-86-8)	$C_{22}H_{25}NO_6$	400.1755	6.4	(+)	358.1649	341.1384	382.1649
Coumaphos	(56-72-4)	$C_{14}H_{16}ClO_5PS$	363.0217	11.2	(+)	306.9591	334.9904	226.9923

Compound	CAS number	Molecular Formula	Calculated m/z precursor ion	Retention time (min)	ESI mode	Fragment 1	Fragment 2	Fragment 3
Dapsone	(80-08-0)	$C_{12}H_{12}N_2O_2S$	249.0692	4.1	(+)	156.0114	108.0444	92.0495
Decoquinate	(18507-89-6)	$C_{24}H_{35}NO_5$	418.2588	13.4	(+)	no fragmentation		
Diaveridine	(5355-16-8)	$C_{13}H_{16}N_4O_2$	261.1346	3.9	(+)	245.1033	123.0665	217.1084
Diclazuril	(10320-42-0)	$C_{17}H_9Cl_3N_4O_2$	404.9718	10.3	(-)	333.9711	335.9672	
Diclofenac	(15307-86-5)	$C_{14}H_{11}Cl_2NO_2$	294.0094	9.2	(-)	214.0429	250.0185	130.9780
Dimethoate	(60-51-5)	$C_5H_{12}NO_3PS_2$	230.0069	5.2	(+)	198.9647	170.9698	124.9821
Dimetridazole	(551-92-8)	$C_5H_7N_3O_2$	142.0611	4.3	(+)	95.0604	81.0447	
Diuron	(330-54-1)	$C_9H_{10}Cl_2N_2O$	233.0243	8.6	(+)	72.04439	105.0340	
			231.0097	8.5	(-)	185.9519	149.9752	159.9726
Doxepine	(1668-19-5)	$C_{19}H_{21}NO$	280.1696	7.0	(+)	220.0883	235.1117	107.0491
Ephedrine	(299-42-3)	$C_{10}H_{15}NO$	166.1226	3.8	(+)	148.1121	117.0699	133.0886
Ethopabate	(59-06-3)	$C_{12}H_{15}NO_4$	238.1074	6.6	(+)	206.0812	164.0706	136.0393
Fenbendazole	(43210-67-9)	$C_{15}H_{13}N_3O_2S$	300.0801	10.3	(+)	268.0539	159.0427	
Fenoxycarb	(79127-80-3)	$C_{17}H_{19}NO_4$	302.1387	10.6	(+)	88.03931	256.0968	116.0706
Florfenicol	(73231-34-2)	$C_{12}H_{14}Cl_2FNO_4S$	355.9932	4.7	(-)	185.0278	335.9870	151.9675
Flubendazole	(31430-15-6)	$C_{16}H_{12}FN_3O_3$	314.0935	8.7	(+)	282.0673	229.0772	
Flunixin	(38677-85-9)	$C_{14}H_{11}F_3N_2O_2$	295.0700	8.3	(-)	251.0802	231.0751	211.0688
Furosemide	(54-31-9)	$C_{12}H_{11}ClN_2O_5S$	329.0004	5.1	(-)	285.0106	204.9844	
Gemfibrozil	(25812-30-0)	$C_{15}H_{22}O_3$	249.1496	10.9	(-)	121.0659		
Hydrochlorthiazide	(58-93-5)	$C_7H_8ClN_3O_4S_2$	295.9572	3.4	(-)	268.9463		

Compound	CAS number	Molecular Formula	Calculated m/z precursor ion	Retention time (min)	ESI mode	Fragment 1	Fragment 2	Fragment 3
Imidacloprid	(138261-41-3)	$C_9H_{10}ClN_5O_2$	256.0596	4.8	(+)	175.0978	209.0589	84.0808
Imipramine	(50-49-7)	$C_{19}H_{24}N_2$	281.2012	8.1	(+)	236.1434	208.1121	86.0964
Irgarol	(28159-98-0)	$C_{11}H_{19}N_5S$	254.1434	10.5	(+)	198.0808	125.0822	91.0324
Ketoprofen	(22071-15-4)	$C_{16}H_{14}O_3$	255.1016	8.5	(+)	105.0335	209.0961	
			253.0870	6.8	(-)	169.0659	209.0972	197.0608
Lamotrigine	(84057-84-1)	$C_9H_7Cl_2N_5$	256.0151	5.3	(+)	210.9824	166.0292	186.9824
Levamisol	(14769-73-4)	$C_{11}H_{12}N_2S$	205.0794	3.7	(+)	178.0685	146.0964	123.0263
Levetiracetam	(102767-28-2)	$C_8H_{14}N_2O_2$	171.1128	3.7	(+)	154.0863	126.0913	69.0335
Lidocaine	(137-58-6)	$C_{14}H_{22}N_2O$	235.1805	4.5	(+)	86.0964		
Lincomycin	(154-21-2)	$C_{18}H_{34}N_2O_6S$	407.221	4.1	(+)	359.2177	317.2071	126.1277
Lorazepam	(846-49-1)	$C_{15}H_{10}Cl_2N_2O_2$	321.0192	8.4	(+)	275.0137	229.0527	303.0086
Lufenuron	(103055-07-8)	$C_{17}H_8Cl_2F_8N_2O_3$	508.9711	12.4	(-)	488.9649	325.958	338.9732
Mebendazole	(31431-39-7)	$C_{16}H_{13}N_3O_3$	296.1030	8.3	(+)	264.0768		
Mefenamic acid	(61-68-7)	$C_{15}H_{15}NO_2$	240.1030	9.2	(-)	196.1132	91.0189	180.0819
Meloxicam	(71125-38-7)	$C_{14}H_{13}N_3O_4S_2$	350.0275	6.4	(-)	146.0611	113.0179	192.0125
Metformin	(657-24-9)	$C_4H_{11}N_5$	130.1087	1.4	(+)	71.0604	60.0570	85.0509
Metronidazole	(443-48-1)	$C_6H_9N_3O_3$	172.0717	3.6	(+)	128.0455	111.0441	82.0525
Morantel	(20574-50-9)	$C_{12}H_{16}N_2S$	221.1107	4.4	(+)	111.0263	123.0263	164.0528
Niflumic acid	(4394-00-7)	$C_{13}H_9F_3N_2O_2$	281.0543	8.3	(-)	237.0645	217.0583	197.0521
Omeprazole	(73590-58-6)	$C_{17}H_{19}N_3O_3S$	346.1220	7.5	(+)	198.0583	151.0992	218.0144

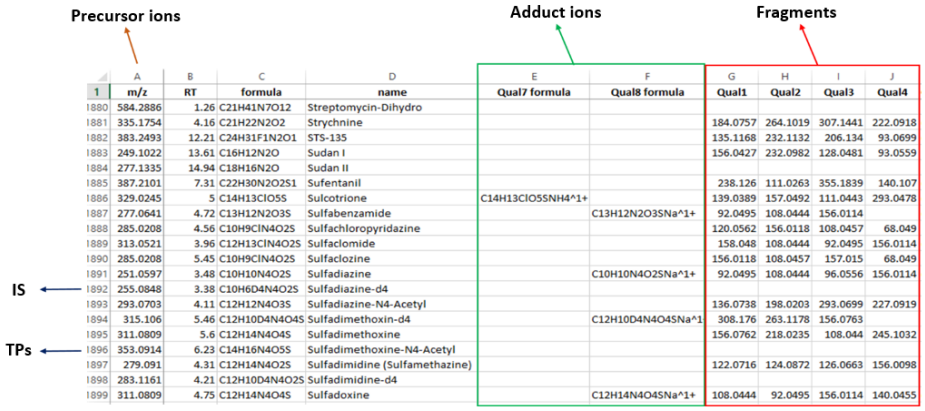
Compound	CAS number	Molecular Formula	Calculated m/z precursor ion	Retention time (min)	ESI mode	Fragment 1	Fragment 2	Fragment 3
Oxfendazole	(53716-50-0)	$C_{15}H_{13}N_3O_3S$	316.0750	6.7	(+)	278.0594	191.0689	284.0488
Paracetamol	(103-90-2)	$C_8H_9NO_2$	152.0706	3.5	(+)	110.0600	92.0495	65.0386
Paroxetine	(61869-08-7)	$C_{19}H_{20}FNO_3$	330.1500	7.9	(+)	192.1183	70.0651	
Primidone	(125-33-7)	$C_{12}H_{14}N_2O_2$	219.1128	5.3	(+)	162.0956		
Propranolol	(525-66-6)	$C_{16}H_{21}NO_2$	260.1645	6.6	(+)	183.0804	116.107	157.0648
Rafoxanide	(22662-39-1)	$C_{19}H_{11}Cl_2I_2NO_3$	623.8133	12.4	(-)	344.8279		
Ronidazole	(7681-76-7)	$C_6H_8N_4O_4$	201.0618	3.6	(+)	140.0455	55.0417	
Saccharine	(6381-61-9)	$C_7H_5NO_3S$	181.9917	3.1	(-)	no fragmentation		
Salicylic acid	(69-72-7)	$C_7H_6O_3$	137.0244	3.6	(-)	93.0346	65.0397	
Simvastatin	(79902-63-9)	$C_{25}H_{38}O_5$	419.2792	12.5	(+)	199.1481	285.1850	225.1638
Sucralose	(56038-13-2)	$C_{12}H_{19}Cl_3O_8$	395.0073	4.7	(-)	no fragmentation		
Sulfachloropyridazine	(23282-55-5)	$C_{10}H_9ClN_4O_2S$	285.0208	4.6	(+)	156.0114	108.0444	92.0495
Sulfaclozine	(102-65-8)	$C_{10}H_9ClN_4O_2S$	285.0208	5.5	(+)	219.0432	130.018	94.0651
Sulfadiazine	(68-35-9)	$C_{10}H_{10}N_4O_2S$	251.0597	3.5	(+)	156.0114	108.0444	96.0556
Sulfadimethoxine	(122-11-2)	$C_{12}H_{14}N_4O_4S$	311.0809	5.6	(+)	156.0114	108.0444	92.0495
Sulfadimidine	(57-68-1)	$C_{12}H_{14}N_4O_2S$	279.0910	4.3	(+)	156.0114	108.0444	124.0869
Sulfadoxine	(2447-57-6)	$C_{12}H_{14}N_4O_4S$	311.0809	4.8	(+)	156.0114	108.0444	140.0455
Sulfamerazine	(127-79-7)	$C_{11}H_{12}N_4O_2S$	265.0754	4.0	(+)	156.0114	108.0444	92.0495
Sulfamethizole	(144-82-1)	$C_9H_{10}N_4O_2S_2$	271.0318	4.2	(+)	156.0114	108.0444	92.0495

Compound	CAS number	Molecular Formula	Calculated m/z precursor ion	Retention time (min)	ESI mode	Fragment 1	Fragment 2	Fragment 3
Sulfamethoxazole	(723-46-6)	$C_{10}H_{11}N_3O_3S$	254.0594	4.6	(+)	156.0114	108.0444	92.0495
Sulfamethoxypyridazine	(80-35-3)	$C_{11}H_{12}N_4O_3S$	281.0703	4.4	(+)	156.0114	108.0444	92.0495
Sulfamonomethoxine	(1220-83-3)	$C_{11}H_{12}N_4O_3S$	281.0703	4.8	(+)	156.0114	108.0444	92.0495
Sulfapyridine	(144-83-2)	$C_{11}H_{11}N_3O_2S$	250.0645	3.4	(+)	156.0114	108.0444	184.0869
Sulfaquinoxaline	(59-40-5)	$C_{14}H_{12}N_4O_2S$	301.0754	5.8	(+)	156.0114	108.0444	92.0495
Sulfathiazole	(72-14-0)	$C_9H_9N_3O_2S_2$	256.0209	3.6	(+)	156.0114	108.0444	92.0495
Sulfisoxazole	(127-69-5)	$C_{11}H_{13}N_3O_3S$	268.0750	4.7	(+)	156.0114	108.0444	92.0495
Ternidazole	(1077-93-6)	$C_7H_{11}N_3O_3$	186.0873	4.2	(+)	128.0455	82.0525	56.0369
Theophylline	(58-55-9)	$C_7H_8N_4O_2$	181.0720	3.9	(+)	124.0505	96.0556	69.0447
Thiabendazole	(148-79-8)	$C_{10}H_7N_3S$	202.0433	6.2	(+)	175.0324	131.0604	92.0495
Thiamphenicol	(847-25-6)	$C_{12}H_{15}Cl_2NO_5S$	353.9975	4.1	(-)	290.0259	185.0283	240.0336
Toltrazuril	(69004-03-1)	$C_{18}H_{14}F_3N_3O_4S$	424.0584	11.0	(-)	no fragmentation		
Tramadol	(27203-92-5)	$C_{16}H_{25}NO_2$	264.1958	4.9	(+)	58.0651		
Triamterene	(396-01-0)	$C_{12}H_{11}N_7$	254.1149	4.9	(+)	237.0883		
Triclabendazole	(68786-66-3)	$C_{14}H_9Cl_3N_2OS$	358.9574	12	(+)	343.9339		
Trimethoprim	(738-70-5)	$C_{14}H_{18}N_4O_3$	291.1452	4.1	(+)	261.0982	230.1176	

5.6 Data treatment

5.6.1 Identification procedure

Raw data were processed with the software tools Data Analysis 5.1 and TASQ CLIENT 2.1 (Bruker Daltonics, Germany). The acquired data were screened applying a wide-scope target screening approach for more than 2,400 compounds according to the target lists of the National and Kapodistrian University of Athens (NKUA) database. This database is available as S21 UATHTARGETS on the Suspect lists Exchange Platform (https://www.norman-network.com/nds/SLE/, DOI: 10.5281/zenodo.3723478). of NORMAN. It includes multi-class compounds such as pharmaceuticals, personal care products, illicit drugs, plant protection products, stimulants, industrial chemicals, sweeteners, plasticizers, drugs of abuse, surfactants, flame retardants, phthalates and their metabolites and TPs, and is constantly enriched with new compounds. Apart from the compounds, it includes isotopically labeled compounds used as IS. The database has been developed throughout the years by analyzing a reference standard of each compound using the UHPLC-ESI-QToF system described in detail in *Paragraph 4.4*. It contains information regarding the name, the molecular formula, the classification and the m/z of the precursor ion, the fragmentation profile of the compound (fragments, adduct ions, in-source fragments) and its retention time along with identifiers (CAS number, InChi) for every compound. The structure of the database is presented in *Figure 19*.



	A	B	C	D	E	F	G	H	I	J
1	m/z	RT	formula	name	Qual7 formula	Qual8 formula	Qual1	Qual2	Qual3	Qual4
1880	584.2886	1.26	C21H41N7O12	Streptomycin-Dihydro						
1881	335.1754	4.16	C21H22N2O2	Strychnine			184.0757	264.1019	307.1441	222.0918
1882	383.2493	12.21	C24H31F1N2O1	STS-135			135.1168	232.1132	206.134	93.0699
1883	249.1022	13.61	C16H12N2O	Sudan I			156.0427	232.0982	128.0481	93.0559
1884	277.1335	14.94	C18H16N2O	Sudan II						
1885	387.2101	7.31	C22H30N2O2S1	Sufentanil			238.126	111.0263	355.1839	140.107
1886	329.0245	5	C14H13ClO5S	Sulcotrione	C14H13ClO5SNH4^1+		139.0389	157.0492	111.0443	293.0478
1887	277.0641	4.72	C13H12N2O3S	Sulfabenzamide		C13H12N2O3SNa^1+	92.0495	108.0444	156.0114	
1888	285.0208	4.56	C10H9ClN4O2S	Sulfachloropyridazine			120.0562	156.0118	108.0457	68.049
1889	313.0521	3.96	C12H13ClN4O2S	Sulfaclomide			158.048	108.0444	92.0495	156.0114
1890	285.0208	5.45	C10H9ClN4O2S	Sulfaclozine			156.0118	108.0457	157.015	68.049
1891	251.0597	3.48	C10H10N4O2S	Sulfadiazine		C10H10N4O2SNa^1+	92.0495	108.0444	96.0556	156.0114
1892	255.0848	3.38	C10H6D4N4O2S	Sulfadiazine-d4						
1893	293.0703	4.11	C12H12N4O3S	Sulfadiazine-N4-Acetyl			136.0738	198.0203	293.0699	227.0919
1894	315.106	5.46	C12H10D4N4O4S	Sulfadimethoxin-d4		C12H10D4N4O4SNa^1	308.176	263.1178	156.0763	
1895	311.0809	5.6	C12H14N4O4S	Sulfadimethoxine			156.0762	218.0235	108.044	245.1032
1896	353.0914	6.23	C14H16N4O5S	Sulfadimethoxine-N4-Acetyl						
1897	279.091	4.31	C12H14N4O2S	Sulfadimidine (Sulfamethazine)			122.0716	124.0872	126.0663	156.0098
1898	283.1161	4.21	C12H10D4N4O2S	Sulfadimidine-d4						
1899	311.0809	4.75	C12H14N4O4S	Sulfadoxine		C12H14N4O4SNa^1+	108.0444	92.0495	156.0114	140.0455

Figure 19 Structure of the in-house developed NKUA Database.

TASQ CLIENT 2.1 (*Figure 20*, Bruker Daltonics, Germany) software permits the screening of numerous samples for numerous compounds by creating a processing method. Although samples' processing with the set method is conducted in an automated way, the critical evaluation of its results is obligatory. Hence, the data treatment is performed in a partially automated way. In brief, the workflow applied for the data treatment consist of the following steps:

1) Internal calibration

The calibrant solution injected at the beginning of each run is used to perform internal calibration by applying the high precision calibration (HPC) algorithm. The desired standard deviation is up to 1.

2) Extracted ion chromatogram

Extracted ion chromatogram (EIC) of each compound of the target list is created by the software depending on its m/z expected. EIC are also created for each fragment included in the target list. The EIC depicts the change of intensity of the determined m/z along with the chromatographic time. The mass error window applied was ± 5 mDa.

3) IS check

The EIC of the IS need to be inspected. The retention time and the areas of all IS used in the analysis is crucial to remain stable through the analysis of the whole batch.

4) Identification of target compounds

All hits acquired by the automatically processed data need to be visually inspected and evaluated in order to conclude if the compound is actually detected or if it is a false positive result. The screening parameters that were set to method were: area threshold of 1,000 counts and intensity threshold of 500 counts. Strict thresholds are set regarding the identification of the compounds. In particular, mass accuracy, which refers to the difference between the accurate mass (measured) and the exact mass (theoretical), had a threshold of 5 mDa (2 ppm). Retention time shift, expressing the difference

between the measured retention time and the one that is recorded in the database, is accepted to be up to ± 0.2 min. For compounds that contain Cl and/or Br and therefore have a determined isotopic profile, the mSigma value is evaluated. The mSigma refers to the correlation between the theoretical and the experimental isotopic pattern and lower mSigma value indicates better isotopic fitting. The mSigma threshold was set to 200. However, this value was only considered as a positive confirmation and not for rejecting peaks, because strong matrix effects combined with low concentration levels of analytes may affect the isotopic pattern results and give an unacceptable mSigma value, although the compound may be present. Finally, the fragmentation profile is evaluated by assessing the MS and the MS/MS spectra. The detection of qualifier ions (characteristic fragments, adduct ions, in-source fragments) is considered as an additional identification criterion of the evaluated compound.

5) Evaluation in batch mode

After evaluating all compounds in each sample for all samples, results are further evaluated in batch mode (one analyte in all samples) to ensure that the same peak is integrated properly in all samples, meeting all established criteria. Compounds with the same m/z are further evaluated to conclude if their separation and detection was achieved.

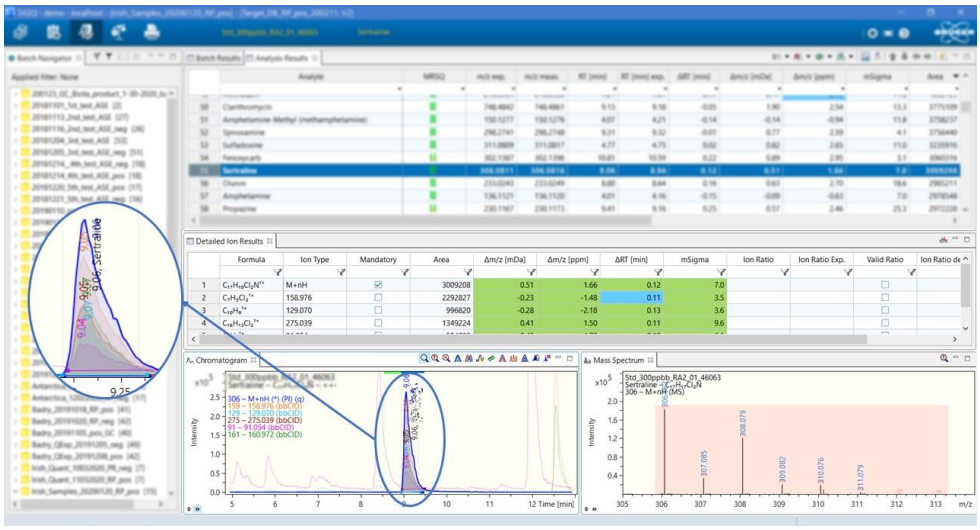


Figure 20 Data treatment interface of TASQ Client 2.1 (Bruker Daltonics)

5.6.2 Quantification procedure

In order to convert the area of the detected analytes into concentration in the sample, every analyte was assigned to an IS. The IS was chosen depending on the structural similarity and polarity of the analyte, so that they present similar chromatographic behavior. In cases in which the compound had also been detected in the procedural blank, quantification was performed only when the area of the analyte in samples exceeded the limit of area in the procedural blank plus 30%. One-point quantification using a spiked sample (C_{spike}= 300 ng/mL) was performed applying the following equation (*eq. 14*):

$$C\ \left(\frac{ng}{L}\right) = \left(\frac{\frac{Realtive\ area\ (sample)}{Relative\ area\ (spiked\ sample)}}{2{,}000}\ \times\ C_{spike}\right) \times 1{,}000 \quad \text{(eq. 14)}$$

In detail, the concentration of the detected analytes in the samples (C) was calculated by dividing analyte's relative peak area in sample with the corresponding relative peak area in the spiked sample. The abovementioned ratio was divided by the preconcentration factor (2,000 times) and multiplied by the concentration of the analyte in the spiked sample (ng/mL). The result was multiplied by 1,000 in order to be expressed in ng/L.

Analytes detected in blank samples from sampling were also quantified as abovementioned.

CHAPTER 6
RESULTS AND DISCUSSION

6.1 Validation results

Since the number of compounds included in the database does not permit the application of a classic validation approach due to the excessive amount of time needed, a smart validation approach was applied. The most crucial step in this procedure is the selection of the compounds. The number of the selected compounds should be adequate and manageable and at the same time compounds should represent the different categories and physicochemical properties of all compounds included in the database. The diagram of *Figure 21* depicts the distribution of the m/z of the validation dataset compounds in relation with their retention time. Compounds are distinguished by the ionization mode used for their detection. IS are also presented.

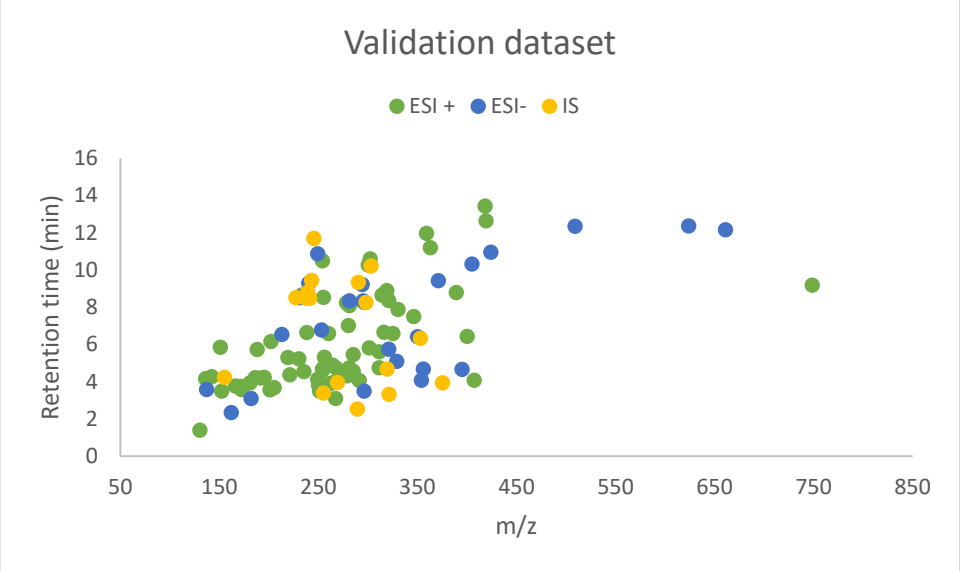


Figure 21 Distribution of the validation dataset compounds.

6.1.1 SPE results

5.1.1.1 Linearity

Linearity was studied for each compound by analyzing spiked samples at seven concentration levels (10, 25, 50, 75, 100, 150 and 300 μg/L in test solutions

corresponding to 5, 12.5, 25, 37.5, 50, 75 and 150 ng/L in samples) as well as standard solutions at the same concentration levels. The regression lines determined by the least squares method were of the form (*eq. 15*):

$$y = (a \pm S_a) * x + (b \pm S_b) \text{ (eq. 15)}$$

in which:

y: relative peak area of each compound defined as shown in *eq. 16*

a: slope

b: intercept (omitted for curves that it was not statistically significant)

x: concentration of compound in the sample (ng/L)

S_a: Standard deviation of slope

S_b: Standard deviation of intercept

The correlation of determination (R^2) was calculated for all curves. The abovementioned information regarding spiked samples is presented in **Table 4** while calibration curves for standard solutions are illustrated in **Table 15** in *Annex II*.

The IS method was applied to enhance the accuracy of quantification. Every compound was assigned to an IS depending on the structural similarity and physicochemical properties. Therefore, relative peak areas were used in calculations instead of absolute areas. Relative peak areas were determined according to the following equation (*eq. 16*):

$$Relative\ area = \frac{\text{Absolute area of compound}}{Area\ of\ IS} \ (eq. 16)$$

Calibration curves using absolute areas and relative areas were constructed and corresponding R^2 were compared. In general, it was observed that R^2 was improved when using relative peak areas. *Figure 22* presents the number of compounds for which relative areas improved the value of R^2 comparing to the curves with absolute area (>1 represents enhancement of R^2 while <1 implies that no improvement was observed when using relative areas). Moreover,

except for few compounds, the values of R^2 obtained were above 0.9 which is considered satisfactory for a generic wide-scope target screening method.

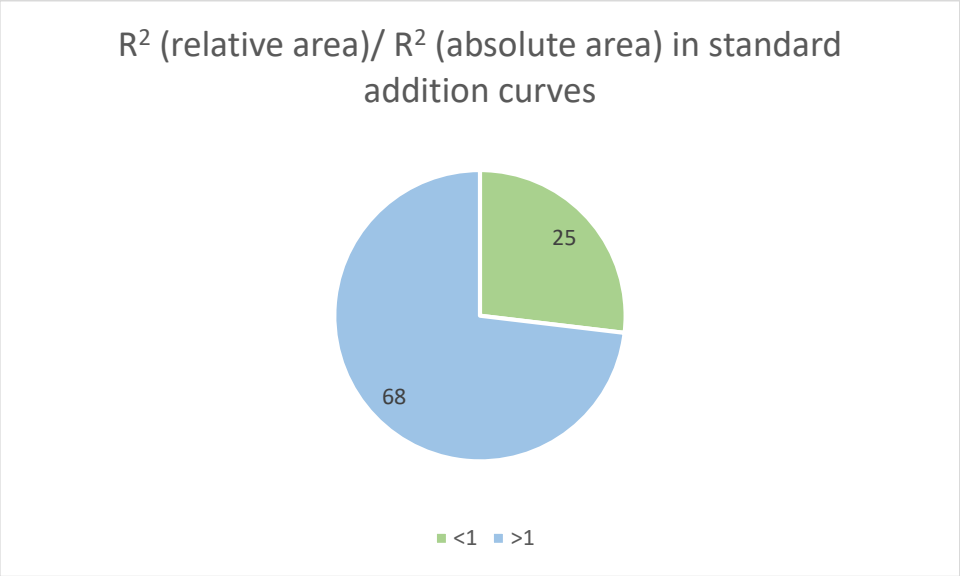


Figure 22 Enhancement of R^2 using relative areas in standard addition curves.

In case of an example, in *Figure 23* the standard addition curve of niflumic acid constructed using absolute areas is presented and in *Figure 24* the same information is illustrated using relative areas. The value of R^2 is significantly improved when using relative peak areas calculated by dividing absolute peak areas with the corresponding areas of flunixin-d3.

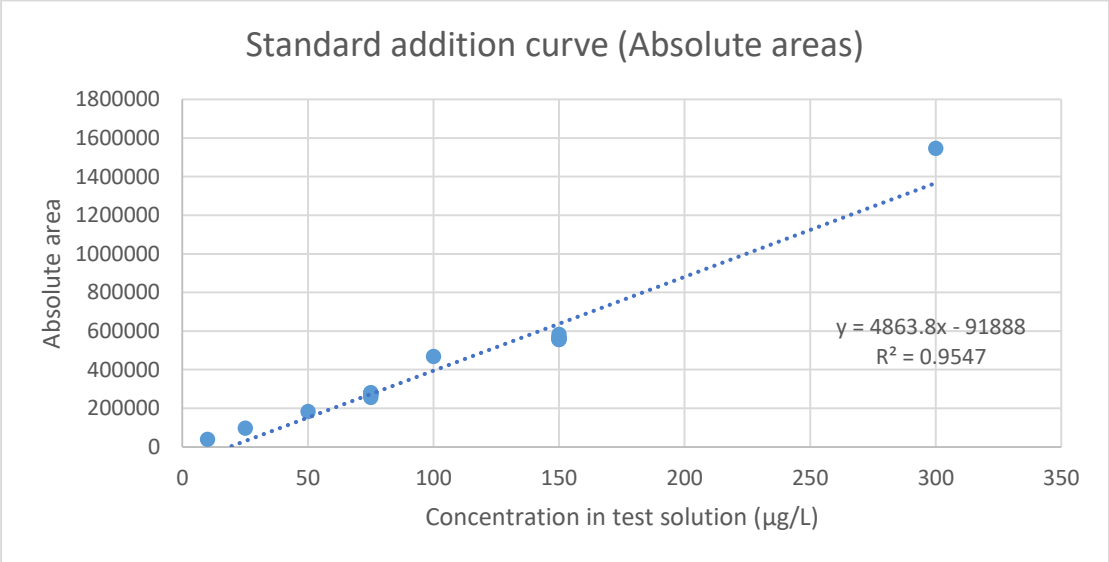


Figure 23 Standard addition curve of niflumic acid using absolute areas.

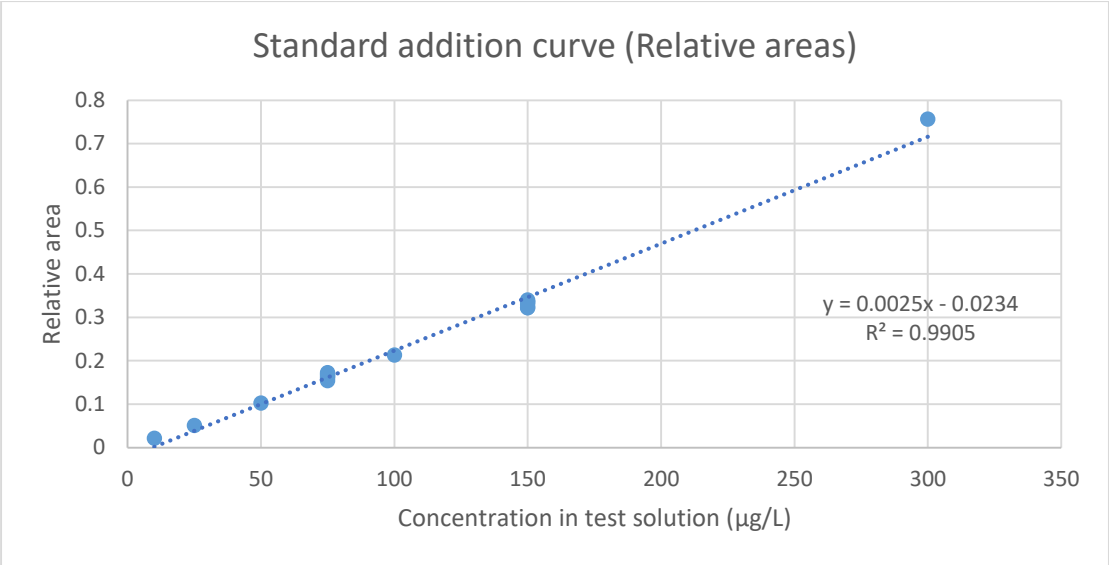


Figure 24 Standard addition curve of niflumic acid using relative areas.

Table 4 Standard addition curves and detectability of the method.

Compound	Concentration range (ng/L)	Standard addition curve (with IS)	R^2	LOD (ng/L)	LOQ (ng/L)
2-Amino-Benzothiazole	18-150	$y=(257*10^{-5} \pm 7.33*10^{-5})*x$	0.988	5.6	18
Acesulfame	38-150	$y=(165*10^{-6} \pm 9.74*10^{-6})*x$	0.95	12	38
Amitriptyline	31-150	$y=(33.1*10^{-4} \pm 1.62*10^{-4})*x - (68.01*10^{-3} \pm 20.50*10^{-3})$	0.97	10	31
Amphetamine	18-75	$y=(129*10^{-5} \pm 4.29*10^{-5})*x$	0.985	5.8	18
Atenolol	21-150	$y=(432.2*10^{-5} \pm 14.17*10^{-5})*x$	0.98	6.8	21
Atrazine-desethyl	20-150	$y=(61.6*10^{-5} \pm 1.92*10^{-5})*x$	0.986	6.5	20
Caffeine	8-150	$y=(27.4*10^{-5} \pm 2.04*10^{-5})*x - (12.7*10^{-3} \pm 2.85*10^{-3})$	0.94	2.8	85
Cetirizine	21-150	$y=(92.7*10^{-5} \pm 2.98*10^{-5})*x$	0.986	6.9	21
Chloramphenicol	13-75	$y=(187*10^{-5} \pm 4.61*10^{-5})*x$	0.992	4.4	13
Chlorpromazine	75-150	$y=(19.4*10^{-4} \pm 2.32*10^{-4})*x - (81.18*10^{-3} \pm 29.34*10^{-3})$	0.82	25	76
Citalopram	14-150	$y=(237*10^{-5} \pm 5.14*10^{-5})*x$	0.993	4.5	14
Clarithromycin	26-150	$y=(151*10^{-5} \pm 6.16*10^{-5})*x$	0.98	8.5	26
Clazuril	20-75	$y=(226*10^{-6} \pm 8.29*10^{-6})*x$	0.98	6.5	20
Clofibric acid	31-75	$y=(146*10^{-6} \pm 8.34*10^{-6})*x$	0.96	10	31
Clopidol	23-150	$y=(208*10^{-5} \pm 7.31*10^{-5})*x + (17.7*10^{-3} \pm 9.36*10^{-3})$	0.98	7.4	23
Closantel	36-75	$y=(75.2*10^{-5} \pm 5.06*10^{-5})*x$	0.96	12	36
Colchicine	17-150	$y=(72.2*10^{-5} \pm 1.98*10^{-5})*x$	0.990	5.7	17
Coumaphos	24-150	$y=(27.7*10^{-4} \pm 1.05*10^{-4})*x$	0.98	8.0	24
Dapsone	21-150	$y=(44.9*10^{-4} \pm 1.45*10^{-4})*x + (80.80*10^{-3} \pm 18.53*10^{-3})$	0.986	6.8	21
Decoquinate	44-150	$y=(62.9*10^{-4} \pm 5.40*10^{-4})*x$	0.93	14	44
Diaveridine	13-150	$y=(215*10^{-5} \pm 4.29*10^{-5})*x$	0.994	4.2	13
Diclazuril	44-75	$y=(20.3*10^{-5} \pm 1.36*10^{-5})*x$	0.96	14	44
Diclofenac	39-150	$y=(81.2*10^{-6} \pm 4.59*10^{-6})*x$	0.96	13	39
Dimethoate	16-150	$y=(131*10^{-5} \pm 3.32*10^{-5})*x$	0.990	5.3	16

Compound	Concentration range (ng/L)	Standard addition curve (with IS)	R^2	LOD (ng/L)	LOQ (ng/L)
Dimetridazole	113-150	$y=(23.5*10^{-5} \pm 4.06*10^{-5})*x$	0.71	37	113
Diuron (ESI-)	13-150	$y=(70.3*10^{-5} \pm 1.40*10^{-5})*x$	0.990	4.2	13
Diuron (ESI+)	16-150	$y=(69.7*10^{-5} \pm 1.76*10^{-5})*x - (6.91*10^{-3} \pm 2.23*10^{-3})$	0.994	5.3	16
Doxepine	15-75	$y=(201*10^{-5} \pm 5.80*10^{-5})*x - (16.3*10^{-3} \pm 6.18*10^{-3})$	0.988	5.0	15
Ephedrine	16-75	$y=(45.7*10^{-4} \pm 1.37*10^{-4})*x + (35.40*10^{-3} \pm 14.68*10^{-3})$	0.988	5.3	16
Ethopabate	24-150	$y=(151*10^{-5} \pm 5.69*10^{-5})*x$	0.98	7.8	24
Fenbendazole	37-150	$y=(24.3*10^{-4} \pm 1.22*10^{-4})*x - (124.8*10^{-3} \pm 17.71*10^{-3})$	0.98	12	37
Fenoxycarb	17-150	$y=(388.7*10^{-5} \pm 10.45*10^{-5})*x$	0.990	5.7	17
Florfenicol	5-75	$y=(168*10^{-5} \pm 3.60*10^{-5})*x$	0.994	12	3.8
Flubendazole	21-150	$y=(279*10^{-6} \pm 9.11*10^{-6})*x - (4.40*10^{-3} \pm 1.20*10^{-3})$	0.986	7.1	21
Flunixine	10-150	$y=(292*10^{-5} \pm 4.45*10^{-5})*x - (12.3*10^{-3} \pm 5.63*10^{-3})$	0.997	3.2	9.6
Furosemide	23-150	$y=(203*10^{-6} \pm 6.60*10^{-6})*x$	0.990	7.4	23
Gemfibrozil	15-150	$y=(89.6*10^{-5} \pm 2.11*10^{-5})*x$	0.994	5.0	15
Hydrochlorothiazide	62-150	$y=(98.1*10^{-5} \pm 8.89*10^{-5})*x - (29.95*10^{-3} \pm 12.10*10^{-3})$	0.93	20	62
Imidacloprid	26-150	$y=(40.8*10^{-5} \pm 1.69*10^{-5})*x + (12.2*10^{-3} \pm 2.13*10^{-3})$	0.97	8.6	26
Imipramine	27-75	$y=(201*10^{-5} \pm 9.83*10^{-5})*x + (33.07*10^{-3} \pm 10.91*10^{-3})$	0.97	8.9	27
Irgarol	17-150	$y=(7226.7*10^{-5} \pm 191.02*10^{-5})*x - (0.57 \pm 0.24)$	0.990	5.5	17
Ketoprofen (ESI+)	19-150	$y=(38.8*10^{-5} \pm 1.16*10^{-5})*x$	0.986	6.3	19
Ketoprofen (ESI-)	103-150	$y=(33.5*10^{-5} \pm 6.32*10^{-5})*x$	0.74	34	103
Lamotrigine	16-150	$y=(88.4*10^{-5} \pm 2.28*10^{-5})*x$	0.990	5.4	16
Levamisol	20-150	$y=(161*10^{-5} \pm 5.12*10^{-5})*x$	0.985	6.6	20
Levetiracetam	7-150	$y=(35.9*10^{-5} \pm 1.15*10^{-5})*x + (9.15*10^{-3} \pm 1.46*10^{-3})$	0.985	20	6.7
Lidocaine	17-150	$y=(181*10^{-4} \pm 4.81*10^{-4})*x$	0.990	5.6	17
Lincomycin	26-150	$y=(52.6*10^{-5} \pm 2.10*10^{-5})*x$	0.98	8.7	26
Lorazepam	38-150	$y=(82.9*10^{-6} \pm 4.60*10^{-6})*x + (22.6*10^{-4} \pm 6.28*10^{-4})$	0.96	13	38

Compound	Concentration range (ng/L)	Standard addition curve (with IS)	R^2	LOD (ng/L)	LOQ (ng/L)
Lufenuron	10-75	$y=(49.2*10^{-5} \pm 1.80*10^{-5})*x$	0.988	3.2	9.5
Mebendazole	17-150	$y=(148*10^{-5} \pm 3.94*10^{-5})*x$	0.990	5.6	17
Mefenamic acid	20-150	$y=(111*10^{-5} \pm 3.32*10^{-5})*x + (15.3*10^{-3} \pm 4.33*10^{-3})$	0.988	6.5	20
Meloxicam	15-150	$y=(87.2*10^{-5} \pm 2.11*10^{-5})*x$	0.991	5.1	15
Metformin	23-150	$y=(206*10^{-5} \pm 8.25*10^{-5})*x - (46.08*10^{-3} \pm 12.00*10^{-3})$	0.98	7.5	23
Metronidazole	12 -150	$y=(411*10^{-6} \pm 7.65*10^{-6})*x$	0.995	3.9	12
Morantel	22-150	$y=(64.5*10^{-5} \pm 2.23*10^{-5})*x$	0.98	7.2	22
Niflumic acid	16-150	$y=(246*10^{-5} \pm 6.25*10^{-5})*x - (23.4*10^{-3} \pm 7.90*10^{-3})$	0.990	5.3	16
Omeprazole	36-150	$y=(86.0*10^{-5} \pm 4.03*10^{-5})*x$	0.987	12	36
Oxfendazole	21-150	$y=(124*10^{-5} \pm 4.16*10^{-5})*x$	0.98	7.1	21
Paracetamol	23-150	$y=(121*10^{-5} \pm 4.55*10^{-5})*x$	0.98	7.7	23
Paroxetine	70-150	$y=(45.8*10^{-5} \pm 4.75*10^{-5})*x$	0.90	23	70
Primidone	25-150	$y=(29.1*10^{-5} \pm 1.26*10^{-5})*x$	0.985	8.4	25
Propranolol	7-75	$y=(189*10^{-5} \pm 2.31*10^{-5})*x$	0.998	2.2	6.6
Rafoxanide	68-150	$y=(28.6*10^{-5} \pm 2.64*10^{-5})*x - (10.2*10^{-3} \pm 3.88*10^{-3})$	0.94	22	68
Ronidazole	21-150	$y=(201*10^{-5} \pm 6.50*10^{-5})*x$	0.986	6.8	21
Saccharine	51-150	$y=(50.0*10^{-5} \pm 3.68*10^{-5})*x$	0.95	17	51
Salicylic acid	32-150	$y=(366.3*10^{-5} \pm 17.70*10^{-5})*x$	0.98	11	32
Simvastatin	47-150	$y=(32.2*10^{-5} \pm 2.32*10^{-5})*x + (7.92*10^{-3} \pm 3.00*10^{-3})$	0.95	15	47
Sucralose	85-150	$y=(24.0*10^{-5} \pm 2.97*10^{-5})*x$	0.878	28	85
Sulfachloropyridazine	23-75	$y=(73.7*10^{-5} \pm 3.18*10^{-5})*x$	0.98	7.5	23
Sulfaclozine	24-150	$y=(52.5*10^{-5} \pm 1.90*10^{-5})*x$	0.98	7.8	24
Sulfadiazine	15-150	$y=(312*10^{-5} \pm 7.35*10^{-5})*x$	0.992	4.9	15
Sulfadimethoxine	19-150	$y=(253*10^{-5} \pm 7.65*10^{-5})*x$	0.986	6.3	19
Sulfadimidine	13-150	$y=(350*10^{-5} \pm 7.05*10^{-5})*x$	0.994	4.2	13

Compound	Concentration range (ng/L)	Standard addition curve (with IS)	R^2	LOD (ng/L)	LOQ (ng/L)
Sulfadoxine	9-150	$y=(422*10^{-5} \pm 5.91*10^{-5})*x + (30.7*10^{-3} \pm 7.47*10^{-3})$	0.997	2.9	8.8
Sulfamerazine	12-150	$y=(289*10^{-5} \pm 5.68*10^{-5})*x - (19.1*10^{-3} \pm 7.19*10^{-3})$	0.994	4.1	12
Sulfamethizole	18-150	$y=(94.0*10^{-5} \pm 2.71*10^{-5})*x$	0.988	6.0	18
Sulfamethoxazole	16-150	$y=(266*10^{-5} \pm 6.61*10^{-5})*x$	0.991	5.2	16
Sulfamethoxypyridazine	13-150	$y=(296*10^{-5} \pm 5.93*10^{-5})*x$	0.994	4.2	13
Sulfamonomethoxine	20-150	$y=(113*10^{-5} \pm 3.61*10^{-5})*x + (21.3*10^{-3} \pm 4.57*10^{-3})$	0.985	6.7	20
Sulfapyridine	16-75	$y=(39.4*10^{-4} \pm 1.18*10^{-4})*x + (4.30*10^{-2} \pm 1.27*10^{-2})$	0.990	5.3	16
Sulfaquinoxaline	21-150	$y=(80.4*10^{-5} \pm 2.60*10^{-5})*x - (9.68*10^{-3} \pm 3.38*10^{-3})$	0.98	6.9	21
Sulfathiazole	21-150	$y=(144*10^{-5} \pm 4.71*10^{-5})*x - (17.9*10^{-3} \pm 6.03*10^{-3})$	0.985	6.9	21
Sulfisoxazole	39-150	$y=(82.8*10^{-5} \pm 5.14*10^{-5})*x$	0.96	123	39
Ternidazole	21-150	$y=(62.8*10^{-5} \pm 1.93*10^{-5})*x + (10.8*10^{-3} \pm 2.58*10^{-3})$	0.990	6.8	21
Theophylline	5-75	$y=(61.6*10^{-5} \pm 2.51*10^{-5})*x + (13.9*10^{-3} \pm 3.21*10^{-3})$	0.98	0.55	1.6
Thiabendazole	23-150	$y=(34.0*10^{-4} \pm 1.24*10^{-4})*x$	0.98	7.7	23
Thiamphenicol	70-150	$y=(13.8*10^{-4} \pm 1.50*10^{-4})*x$	0.90	23	70
Toltrazuril	16-75	$y=(40.0*10^{-5} \pm 1.25*10^{-5})*x$	0.990	5.3	16
Tramadol	21-150	$y=(98.0*10^{-4} \pm 3.24*10^{-4})*x + (43.64*10^{-3} \pm 40.95*10^{-3})$	0.98	6.9	21
Triamterene	23-150	$y=(239*10^{-5} \pm 8.60*10^{-5})*x + (35.39*10^{-3} \pm 11.00*10^{-3})$	0.98	7.6	23
Triclabendazole	72-100	$y=(54.5*10^{-5} \pm 7.12*10^{-5})*x$	0.83	24	72
Trimethoprim	16-150	$y=(293*10^{-5} \pm 7.21*10^{-5})*x$	0.991	5.1	16

5.1.1.2 Detectability

The matrix-instrument LODs and LOQs were calculated by multiplying the standard error (S_b) by 3.3 or 10 respectively and dividing it by the slope (a) of the standard addition curve (*eq.17, eq.18*). The overall method LODs and LOQs taking into consideration the preconcentration were calculated by multiplying the standard error (S_b) by 3.3 or 10 respectively and dividing it by the slope (a) and the preconcentration factor (*eq.19, eq.20*).

$$Matrix - Instrument\ LOD = \frac{3.3 \times S_b}{a}\ (eq.17)$$

$$Matrix - Instrument\ LOQ = \frac{10 \times S_b}{a}\ (eq.18)$$

$$Method\ LOD = \frac{\frac{3.3 \times S_b}{a}}{preconcentration\ factor}\ (eq.19)$$

$$Method\ LOQ = \frac{\frac{10 \times S_b}{a}}{preconcentration\ factor}\ (eq.20)$$

Method LODs and LOQs for each compound are presented in **Table 4**. *Figure 25* presents the distribution of the compounds at different LOD levels. The majority of the compounds can be detected at concentrations below 10 ng/L.

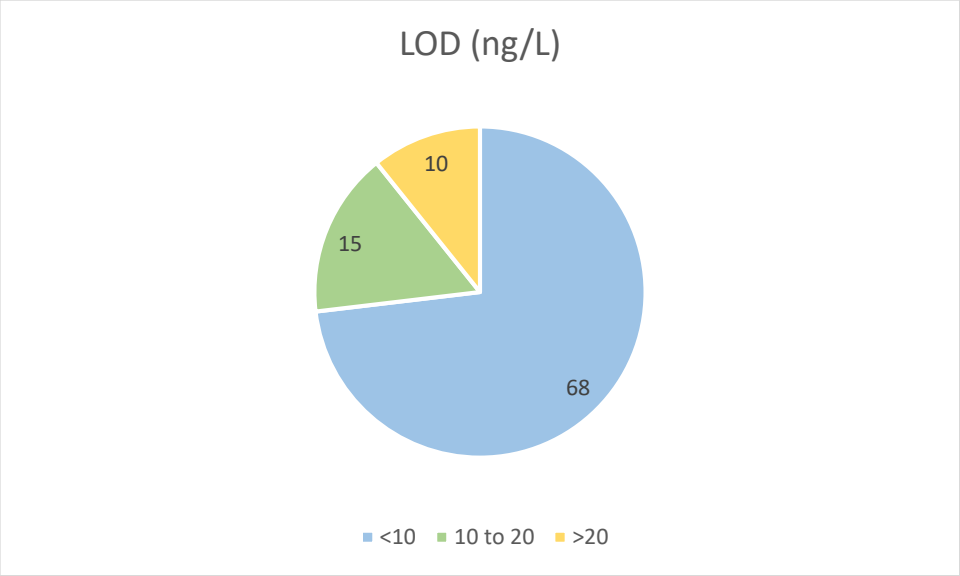


Figure 25 Distribution of LODs of validation dataset compounds.

5.1.1.3 Accuracy

Accuracy was assessed with recovery experiments. Method recovery was calculated according to *equation 21* by dividing the relative peak area of the spiked samples by the relative peak area of the matrix-matched samples at 25, 75 and 150 μg/L in test solutions (corresponding to 12.5, 37.5 and 75 ng/L in samples). In cases in which a compound of interest was also detected in procedural blank or matrix blank sample, its peak area was subtracted from the peak area of the spiked sample and the peak area of the matrix-matched sample before calculations.

$$\%\ Recovery = \left(\frac{Rel.area_{spiked\ sample} - Rel.area_{matrix\ blank}}{Rel.area_{matrix\ matched} - Rel.area_{matrix\ blank}}\right) \times 100\ (eq.21)$$

The recoveries obtained for each compound in every concentration level are presented in **Table 5** and summarized in *Figure 26*. The majority of the compounds presented satisfactory recoveries in all concentration levels and almost half of the compounds had recoveries within the range of 80-120%.

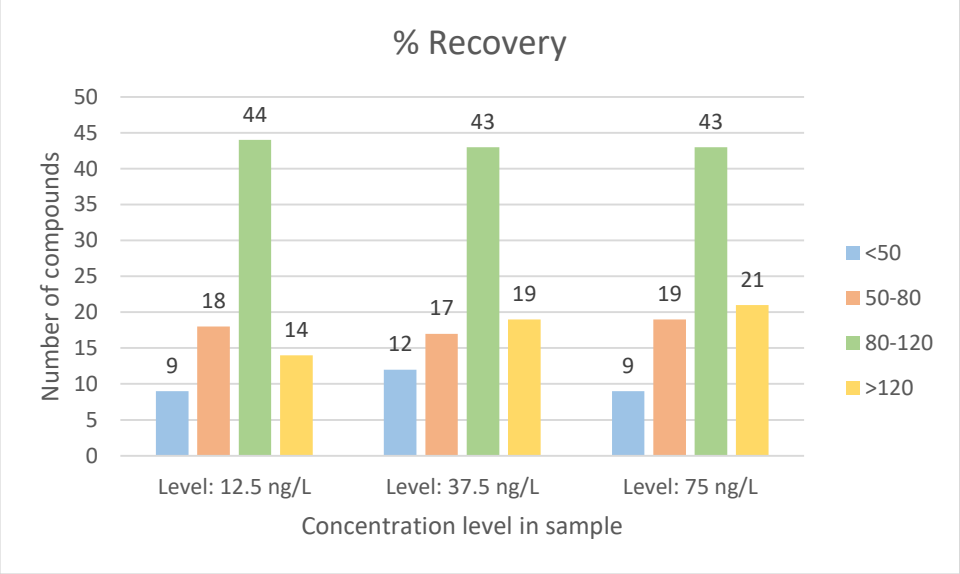


Figure 26 Distribution of % recoveries of compounds at 12.5, 37.5 and 75 ng/L (concentration levels in sample).

Table 5 % Recoveries of validation dataset compounds at three concentration levels.

Compound	Level: 12.5 ng/L	Level: 37.5 ng/L	Level: 75 ng/L
2-Amino-Benzothiazole	127	131	120
Acesulfame	24.9	27.3	23.8

Compound	Level: 12.5 ng/L	Level: 37.5 ng/L	Level: 75 ng/L
Amitriptyline	52.6	50.5	55.1
Amphetamine	58.1	57.3	55.2
Atenolol	106	110	118
Atrazine-desethyl	139	139	139
Caffeine	n.a.	n.a.	237
Cetirizine	80.2	82.5	96.8
Chloramphenicol	111	102	105
Chlorpromazine	37.9	12.1	16.3
Citalopram	70.4	66.6	73.8
Clarithromycin	63.4	52.6	59.8
Clazuril	96.7	87.7	85.8
Clofibric acid	99.8	91.6	80.7
Clopidol	137	148	154
Closantel	68.3	42.5	44.5
Colchicine	82.4	87.1	91.3
Coumaphos	118	104	89.2
Dapsone	94.4	97.2	93.8
Decoquinate	46.8	35.6	31.1
Diaveridine	114	97.3	99.4
Diclazuril	58.2	73.2	75.2
Diclofenac	108	75.5	74.2
Dimethoate	111	115	104
Dimetridazole	n.a.	n.a.	284
Diuron (ESI-)	95.8	91.8	94.2
Diuron (ESI+)	114	89.6	96.2
Doxepine	58.1	52.4	58.5
Ephedrine	74.7	91.8	90.2
Ethopabate	128	149	139
Fenbendazole	n.a.	41.8	75.3
Fenoxycarb	130	115	104
Florfenicol	120	101	104
Flubendazole	75.3	84.1	83.5
Flunixine	98.5	90.8	91.6
Furosemide	48.7	71.9	71.3
Gemfibrozil	91.4	91.7	102
Hydrochlorothiazide	104	185	211
Imidacloprid	119	103	110
Imipramine	57.2	40.7	44.7
Irgarol	121	136	137
Ketoprofen (ESI+)	96.9	85.7	87.7
Ketoprofen (ESI-)	73.3	136	123
Lamotrigine	88.7	85.9	89.9
Levamisol	47.6	60.4	63.9
Levetiracetam	126	115	110
Lidocaine	114	123	123

Compound	Level: 12.5 ng/L	Level: 37.5 ng/L	Level: 75 ng/L
Lincomycin	89.9	54.2	72.4
Lorazepam	n.a.	88.7	91.4
Lufenuron	204	166	153
Mebendazole	122	125	118
Mefenamic acid	145	108	106
Meloxicam	97.7	90.6	94.2
Metformin	n.a.	360	246
Metronidazole	135	130	121
Morantel	221	189	199
Niflumic acid	107	32.1	72.9
Oxfendazole	139	142	135
Paracetamol	77.4	76.9	96.2
Paroxetine	41.5	21.8	20.3
Primidone	114	118	145
Propranolol	73.3	67.8	69.8
Rafoxanide	13.1	37.8	28.2
Ronidazole	106	114	112
Saccharine	93.3	93.7	123
Salicylic acid	51.2	75.6	75.2
Simvastatin	25.1	27.8	40.2
Sucralose	100	151	162
Sulfachloropyridazine	87.8	94.5	78.1
Sulfaclozine	n.a.	84.1	76.5
Sulfadiazine	110	94.8	91.7
Sulfadimethoxine	82.1	83.9	86.5
Sulfadimidine	97.1	88.4	89.7
Sulfadoxine	95.5	87.3	89.4
Sulfamerazine	101	86.7	91.7
Sulfamethizole	60.4	67.6	72.2
Sulfamethoxazole	96.1	92.6	95.4
Sulfamethoxypyridazine	89.3	86.5	88.3
Sulfamonomethoxine	90.4	87.6	94.8
Sulfapyridine	99.8	90.9	82.3
Sulfaquinoxaline	68.6	75.2	82.8
Sulfathiazole	55.8	59.7	61.9
Sulfisoxazole	53.2	38.5	38.3
Ternidazole	129	146	157
Theophylline	106	113	103
Thiabendazole	113	107	105
Thiamphenicol	110	153	160
Toltrazuril	103	88.8	99.3
Tramadol	116	120	118
Triamterene	105	123	121
Triclabendazole	43.2	51.4	50.7
Trimethoprim	112	103	103

5.1.1.4 Precision

Precision was expressed as method repeatability in terms of relative standard deviation (%RSD) of 6 spiked samples at 75 μg/L and 150 μg/L in test solutions (corresponding to 37.5 ng/L and 75 ng/L in samples). The calculated values (*eq. 22*) are presented in **Table 6**. The repeatability of the method was below 20% for the vast majority of the compounds and most of them present values below 10% as it is illustrated in *Figure 27*.

$$\%RSD = \left(\frac{Standard\ deviation_{Rel.areas}}{Average_{Rel.areas}}\right) \times 100\ (eq.22)$$

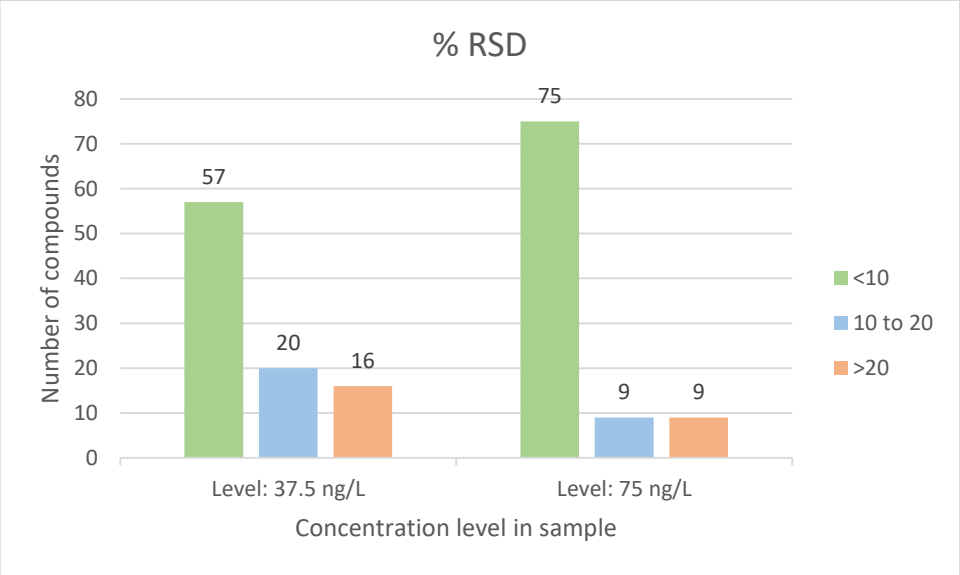


Figure 27 (%) RSD of validation dataset compounds at 37.5 ng/L and 75 ng/L (concentration levels in sample).

Table 6 Repeatability (%RSD) of validation dataset compounds at two concentration levels.

Compound	Level: 37.5 ng/L	Level: 75 ng/L
2-Amino-Benzothiazole	5.7	5.4
Acesulfame	16	12
Amitriptyline	13	7.9
Amphetamine	3.8	5.0
Atenolol	9.9	5.3
Atrazine-desethyl	8.3	6.9
Caffeine	61	9.8
Cetirizine	11	6.3
Chloramphenicol	3.6	4.4
Chlorpromazine	74	15

Compound	Level: 37.5 ng/L	Level: 75 ng/L
Citalopram	3.9	3.9
Clarithromycin	8.3	7.0
Clazuril	7.1	6.3
Clofibric acid	11	7.3
Clopidol	9.6	6.3
Closantel	16	10
Colchicine	4.8	7.0
Coumaphos	9.0	5.1
Dapsone	4.0	4.0
Decoquinate	22	25
Diaveridine	4.5	1.9
Diclazuril	18	13
Diclofenac	9.8	9.2
Dimethoate	5.9	3.5
Dimetridazole	234	29
Diuron (ESI-)	4.6	2.5
Diuron (ESI+)	2.6	3.1
Doxepine	7.2	4.0
Ephedrine	5.8	1.7
Ethopabate	13	4.6
Fenbendazole	75	7.0
Fenoxycarb	6.7	4.6
Florfenicol	5.0	3.5
Flubendazole	7.4	4.0
Flunixine	4.6	3.1
Furosemide	13	7.4
Gemfibrozil	5.5	7.3
Hydrochlorothiazide	35	31
Imidacloprid	6.8	3.5
Imipramine	16	7.2
Irgarol	4.8	2.7
Ketoprofen (ESI+)	3.8	4.6
Ketoprofen (ESI-)	21	18
Lamotrigine	1.9	5.5
Levamisol	8.2	2.0
Levetiracetam	8.5	3.6
Lidocaine	6.0	5.1
Lincomycin	20	3.9
Lorazepam	13	8.5
Lufenuron	14	21
Mebendazole	7.2	5.0
Mefenamic acid	9.1	3.3
Meloxicam	6.4	4.3
Metformin	26	4.3
Metronidazole	6.2	1.8

Compound	Level: 37.5 ng/L	Level: 75 ng/L
Morantel	6.2	2.8
Niflumic acid	4.1	2.3
Omeprazole	8.6	9.5
Oxfendazole	10	5.8
Paracetamol	6.6	10
Paroxetine	22	15
Primidone	11	5.3
Propranolol	3.7	1.7
Rafoxanide	162	77
Ronidazole	6.5	4.6
Saccharine	23	23
Salicylic acid	27	32
Simvastatin	12	18
Sucralose	26	23
Sulfachloropyridazine	11	2.5
Sulfaclozine	11	4.5
Sulfadiazine	5.3	3.6
Sulfadimethoxine	3.3	1.6
Sulfadimidine	3.3	3.6
Sulfadoxine	3.4	2.0
Sulfamerazine	3.1	1.8
Sulfamethizole	6.7	7.4
Sulfamethoxazole	8.8	2.8
Sulfamethoxypyridazine	1.3	3.1
Sulfamonomethoxine	8.9	5.3
Sulfapyridine	5.9	2.8
Sulfaquinoxaline	6.8	6.1
Sulfathiazole	13	3.5
Sulfisoxazole	23	9.4
Ternidazole	13	5.1
Theophylline	7.8	2.6
Thiabendazole	11	7.8
Thiamphenicol	21	21
Toltrazuril	7.9	4.1
Tramadol	7.0	5.8
Triamterene	6.2	4.3
Triclabendazole	30	15
Trimethoprim	4.6	3.0

5.1.1.5 Matrix effect

The % factor of matrix effect (%FME) was calculated by dividing the relative peak area of matrix matched samples by the peak area of the standard

solutions multiplying with 100 (*eq. 23*) at three concentration levels (25, 75 and 150 µg/L in test solutions corresponding to 12.5, 37.5 and 75 ng/L in samples). % Matrix effect was calculated according to *equation 24*.

$$\%\ FME = \left(\frac{Rel.area_{matrix\ matched} - Rel.area_{matrix\ blank}}{Rel.area_{standard} - Rel.area_{solvent}}\right) \times 100\ (eq.23)$$

$$\%\ Matrix\ Effect = (\mathrm{FME} - 1) \times 100\ (eq.24)$$

As it is illustrated in *Figure 28*, around 50% of the compounds of the validation dataset showed an % percentage of matrix effect below 20. In **Table 7** the calculated %FME are presented.

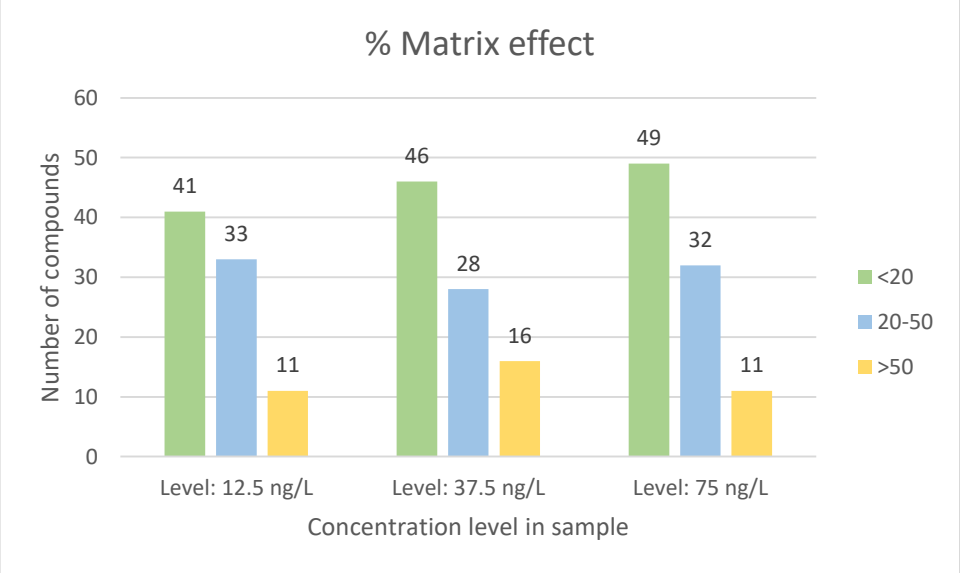


Figure 28 Distribution of % matrix effect of validation dataset compounds at 12.5, 37.5, 75 ng/L (concentration levels in sample).

Table 7 %FME of validation dataset compounds at three different concentration levels.

Compound	Level: 12.5 ng/L	Level: 37.5 ng/L	Level: 75 ng/L
2-Amino-Benzothiazole	70.7	77.0	85.3
Acesulfame	110	121	121
Amitriptyline	95.7	107	101
Amphetamine	113	150	145
Atenolol	136	124	109
Atrazine-desethyl	72.4	64.6	70.3
Caffeine	n.a.	n.a.	17.9
Cetirizine	134	133	99.6
Chloramphenicol	50.9	84.8	86.1
Chlorpromazine	71.2	96.5	99.8
Citalopram	88.7	93.5	85.9

Compound	Level: 12.5 ng/L	Level: 37.5 ng/L	Level: 75 ng/L
Clarithromycin	125	143	129
Clazuril	101	111	110
Clofibric acid	112	318	243
Clopidol	92.9	95.4	81.8
Closantel	24.6	29.2	29.3
Colchicine	72.1	80.5	78.8
Coumaphos	111	125	123
Dapsone	78.7	88.5	84.4
Decoquinate	48.6	74.5	79.2
Diaveridine	78.1	101	90.5
Diclazuril	104	104	102
Diclofenac	108	125	117
Dimethoate	45.8	59.7	64.1
Dimetridazole	n.a.	n.a.	32.5
Diuron (ESI-)	89.4	105	98.2
Diuron (ESI+)	90.1	103	93.9
Doxepine	72.8	88.1	81.7
Ephedrine	108	107	106
Ethopabate	78.7	70.5	69.6
Fenbendazole	n.a.	144	111
Fenoxycarb	113	126	117
Florfenicol	64.8	80.5	78.6
Flubendazole	99.3	114	111
Flunixine	108	123	111
Furosemide	69.7	83.8	79.2
Gemfibrozil	235	202	148
Hydrochlorothiazide	150	172	156
Imidacloprid	87.2	130	120
Imipramine	82.7	95.8	92.9
Irgarol	54.5	68.3	66.4
Ketoprofen (ESI+)	137	137	136
Ketoprofen (ESI-)	498	388	311
Lamotrigine	63.1	73.3	71.2
Levamisol	80.7	102	103
Levetiracetam	111	102	95.3
Lidocaine	103	109	111
Lincomycin	124	161	141
Lorazepam	n.a.	134	187
Lufenuron	13.7	20.6	22.4
Mebendazole	90.9	102	101
Mefenamic acid	86.1	112	104
Meloxicam	104	118	103
Metformin	n.a.	21.6	38.6
Metronidazole	69.2	98.4	100
Morantel	90.3	87.4	87.2

Compound	Level: 12.5 ng/L	Level: 37.5 ng/L	Level: 75 ng/L
Niflumic acid	n.a.	25.9	67.2
Oxfendazole	71.8	86.1	83.5
Paracetamol	104	97.8	82.2
Paroxetine	70.3	80.6	74.3
Primidone	147	117	85.8
Propranolol	67.3	86.1	84.1
Rafoxanide	14.5	18.8	16.6
Ronidazole	80.2	83.8	75.8
Saccharine	131	159	154
Salicylic acid	133	82.3	77.2
Simvastatin	426	281	138
Sucralose	133	153	148
Sulfachloropyridazine	123	125	128
Sulfaclozine	n.a.	97.4	99.6
Sulfadiazine	113	117	115
Sulfadimethoxine	81.2	84.6	84.3
Sulfadimidine	108	119	114
Sulfadoxine	90.1	106	101
Sulfamerazine	110	123	109
Sulfamethizole	128	151	130
Sulfamethoxazole	111	125	109
Sulfamethoxypyridazine	89.3	107	100
Sulfamonomethoxine	89.2	115	114
Sulfapyridine	125	131	124
Sulfaquinoxaline	107	89.2	90.1
Sulfathiazole	120	135	125
Sulfisoxazole	108	126	122
Ternidazole	161	137	124
Theophylline	54.7	73.4	75.6
Thiabendazole	56.6	70.9	70.5
Thiamphenicol	82.0	97.3	94.9
Toltrazuril	99.7	108	109
Tramadol	109	108	105
Triamterene	98.2	90.3	80.7
Triclabendazole	167	153	139
Trimethoprim	72.9	90.7	86.4

6.1.2 Direct injection results

5.1.2.1 Detectability

In order to evaluate the direct injection method, three spiked samples were analyzed. They were prepared to the concentration level of 300 µg/L, a relatively high level to ensure that a satisfactory response will be obtained in this trial. For spiked samples that underwent direct injection analysis, LODs and LOQs were calculated for each compound in every spiked sample according to the *equations 25* and *26*.

$$LOD\ (\frac{\mu g}{L}) = \frac{3.3 \times C_{spike}}{S/N_{spike}}\ (eq.25)$$

$$LOQ\ (\frac{\mu g}{L}) = \frac{10 \times C_{spike}}{S/N_{spike}}\ (eq.26)$$

The calculated values are presented in **Table 8** and the distribution of the average LOD calculated from the three spiked samples is shown in *Figure 29*.

Table 8 LODs and LOQs calculated from direct injection analysis (µg/L).

Compound	Spike_1		Spike_2		Spike_3		Average	
	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD
2-Amino-Benzothiazole	6.83	2.26	7.37	2.43	4.72	2.39	6.31	2.36
Acesulfame	21.3	7.09	24.0	8.00	21.3	7.09	22.2	7.39
Amitriptyline	10.6	3.50	9.58	3.16	2.18	3.57	7.45	3.41
Amphetamine	6.82	2.25	6.02	1.99	4.57	1.98	5.81	2.07
Atenolol	2.69	0.887	2.65	0.873	2.99	0.863	2.77	0.874
Atrazine-desethyl	25.9	8.53	25.9	8.53	15.8	8.32	22.5	8.46
Caffeine	21.7	7.17	21.7	7.17	21.7	7.02	21.7	7.12
Cetirizine	12.6	4.16	12.7	4.18	12.7	4.13	12.6	4.15
Chloramphenicol	8.88	2.96	12.1	4.03	9.58	3.19	10.2	3.40
Chlorpromazine	25.2	8.32	17.2	5.69	1.85	7.33	14.8	7.11
Citalopram	5.88	1.94	6.33	2.09	3.16	2.06	5.12	2.03
Clazuril	81.1	27.0	111	37.0	78.9	26.3	90.4	30.1
Clofibric acid	167	55.6	250	83.3	188	62.5	201	67.1
Clopidol	7.39	2.44	7.43	2.45	5.33	2.35	6.71	2.41
Colchicine	6.36	2.10	7.30	2.41	4.16	2.21	5.94	2.24
Coumaphos	111	36.7	88.2	29.1	5.65	31.9	68.3	32.6
Dapsone	11.4	3.75	11.0	3.63	7.73	3.13	10.0	3.50
Diaveridine	5.11	1.69	5.08	1.68	3.50	1.55	4.56	1.64
Diclazuril	69.8	23.3	71.4	23.8	78.9	26.3	73.4	24.5
Diclofenac	150	50.0	231	76.9	214	71.4	198	66.1

Compound	Spike_1		Spike_2		Spike_3		Average	
	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD
Dimethoate	5.39	1.78	4.96	1.64	3.29	1.71	4.55	1.71
Dimetridazole	51.7	17.1	50.8	16.8	21.4	19.4	41.3	17.8
Diuron	8.47	2.80	9.46	3.12	6.33	2.93	8.09	2.95
Diuron	10.5	3.50	15.9	5.29	12.4	4.13	12.9	4.31
Doxepine	9.49	3.13	9.32	3.07	3.40	3.02	7.40	3.08
Ephedrine	2.09	0.691	1.96	0.645	1.56	0.672	1.87	0.67
Ethopabate	9.38	3.09	9.49	3.13	6.71	3.27	8.53	3.16
Fenbendazole	34.1	11.3	33.7	11.1	6.10	11.6	24.6	11.3
Fenoxycarb	15.2	5.00	18.6	6.15	3.97	6.15	12.6	5.77
Florfenicol	6.98	2.33	8.40	2.80	7.06	2.35	7.48	2.49
Flubendazole	35.3	11.6	36.1	11.9	14.4	12.7	28.6	12.1
Flunixine	7.87	2.62	10.5	3.51	8.70	2.90	9.03	3.01
Furosemide	57.7	19.2	75.0	25.0	83.3	27.8	72.0	24.0
Gemfibrozil	93.8	31.3	136	45.5	120	40.0	117	38.9
Hydrochlorothiazide	85.7	28.6	150	50.0	103	34.5	113	37.7
Imidacloprid	26.3	8.68	25.4	8.39	26.1	7.12	25.9	8.07
Imipramine	8.04	2.65	7.50	2.48	2.44	2.59	5.99	2.57
Irgarol	4.13	1.36	4.74	1.56	1.69	1.42	3.52	1.45
Ketoprofen	18.0	5.93	21.3	7.02	12.8	6.69	17.3	6.55
Ketoprofen	250	83.3	375	125	333	111	319	106
Lamotrigine	5.42	1.79	5.71	1.89	3.35	1.77	4.83	1.82
Levamisol	4.69	1.55	4.71	1.55	2.74	1.54	4.05	1.55
Levetiracetam	16.7	5.50	14.4	4.74	14.6	4.85	15.2	5.03
Lidocaine	1.01	0.335	0.950	0.313	0.858	0.301	0.941	0.316
Lincomycin	11.0	3.64	11.9	3.91	12.5	3.78	11.8	3.78
Lorazepam	231	76.2	273	90.0	130	99.0	211	88.4
Mebendazole	26.8	8.84	34.5	11.4	12.2	10.0	24.5	10.1
Mefenamic acid	167	55.0	176	58.2	93.8	52.1	146	55.1
Mefenamic acid	63.8	21.3	103	34.5	83.3	27.8	83.5	27.8
Meloxicam	21.0	6.99	32.6	10.9	29.1	9.71	27.6	9.19
Metformin	8.17	2.70	8.43	2.78	7.39	2.88	8.00	2.79
Metronidazole	16.8	5.53	18.6	6.15	17.3	5.16	17.6	5.61
Morantel	18.1	5.96	18.6	6.15	13.7	5.47	16.8	5.86
Niflumic acid	13.8	4.54	17.5	5.79	8.67	4.93	13.3	5.09
Niflumic acid	8.29	2.76	13.1	4.37	11.4	3.80	10.9	3.64
Omeprazole	15.1	4.97	17.2	5.69	78.9	5.05	37.1	5.24
Oxfendazole	25.4	8.39	25.9	8.53	14.6	9.52	21.9	8.81
Paracetamol	10.3	3.41	9.06	2.99	9.80	3.03	9.74	3.14
Paroxetine	42.3	13.9	34.5	11.4	5.85	14.6	27.5	13.3
Primidone	53.6	17.7	54.5	18.0	40.0	14.1	49.4	16.6
Propranolol	7.25	2.39	7.32	2.41	4.24	2.34	6.27	2.38
Ronidazole	17.4	5.76	17.4	5.76	15.8	5.27	16.9	5.59
Saccharine	38.5	12.8	38.5	12.8	41.1	13.7	39.3	13.1

Compound	Spike_1		Spike_2		Spike_3		Average	
	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD
Salicylic acid	9.43	3.14	12.9	4.31	12.2	4.07	11.5	3.84
Sucralose	107	35.7	143	47.6	125	41.7	125	41.7
Sulfachloropyridazine	54.5	18.0	68.2	22.5	41.1	21.5	54.6	20.7
Sulfaclozine	93.8	30.9	90.9	30.0	58.8	34.1	81.2	31.7
Sulfadiazine	15.6	5.16	16.9	5.59	14.7	5.00	15.8	5.25
Sulfadimethoxine	17.6	5.82	18.6	6.15	9.74	5.69	15.3	5.89
Sulfadimidine	11.0	3.64	12.3	4.06	8.29	3.91	10.5	3.87
Sulfadoxine	9.3	3.08	10.2	3.36	6.74	3.05	8.75	3.16
Sulfamerazine	11.2	3.69	12.8	4.21	8.65	3.91	10.9	3.94
Sulfamethizole	42.9	14.1	46.9	15.5	3.49	12.4	31.1	14.0
Sulfamethoxazole	19.7	6.51	20.3	6.69	15.0	6.69	18.3	6.63
Sulfamethoxypyridazine	11.7	3.87	12.7	4.19	8.09	3.60	10.8	3.89
Sulfamonomethoxine	40.0	13.2	40.0	13.2	30.6	12.4	36.9	12.9
Sulfapyridine	10.1	3.34	10.1	3.33	8.26	3.20	9.50	3.29
Sulfaquinoxaline	68.2	22.5	69.8	23.0	36.1	19.0	58.0	21.5
Sulfathiazole	24.8	8.18	24.2	7.98	20.1	7.98	23.0	8.05
Sulfisoxazole	31.3	10.3	28.8	9.52	28.0	10.3	29.4	10.0
Ternidazole	18.1	5.96	21.6	7.12	22.9	6.43	20.9	6.50
Theophylline	32.3	10.6	32.3	10.6	36.6	11.1	33.7	10.8
Thiabendazole	8.55	2.82	9.58	3.16	4.21	2.97	7.45	2.99
Thiamphenicol	11.6	3.86	14.0	4.67	12.6	4.18	12.7	4.24
Toltrazuril	71.4	23.8	107	35.7	85.7	28.6	88.1	29.4
Tramadol	1.92	0.634	1.94	0.640	1.40	0.608	1.75	0.627
Triamterene	10.7	3.54	9.97	3.29	6.47	3.27	9.05	3.36
Trimethoprim	2.89	0.953	2.93	0.967	2.15	1.00	2.65	0.974

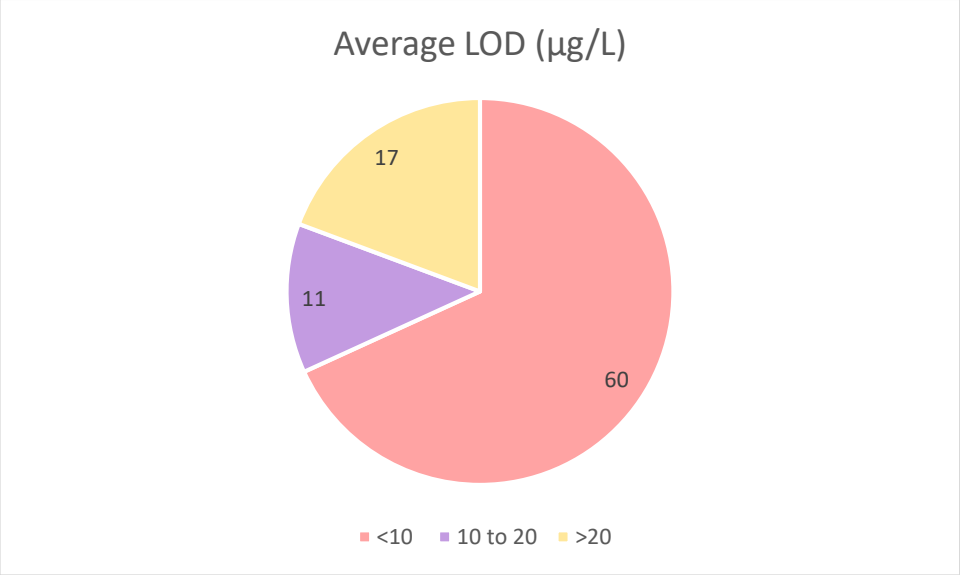


Figure 29 Distribution of average LODs (µg/L) calculated from direct analysis.

5.1.2.2 Accuracy

The % recoveries at 300 µg/L were calculated according to the *equation 27*. Results are presented in **Table 9** and depicted schematically in *Figure 30*. For the majority of compounds satisfactory % recoveries were achieved.

$$\%\ Recovery = \left(\frac{Rel.area_{spiked\ sample} - Rel.area_{sample}}{Rel.area_{standard} - Rel.area_{solvent}}\right) \times 100\ (eq.27)$$

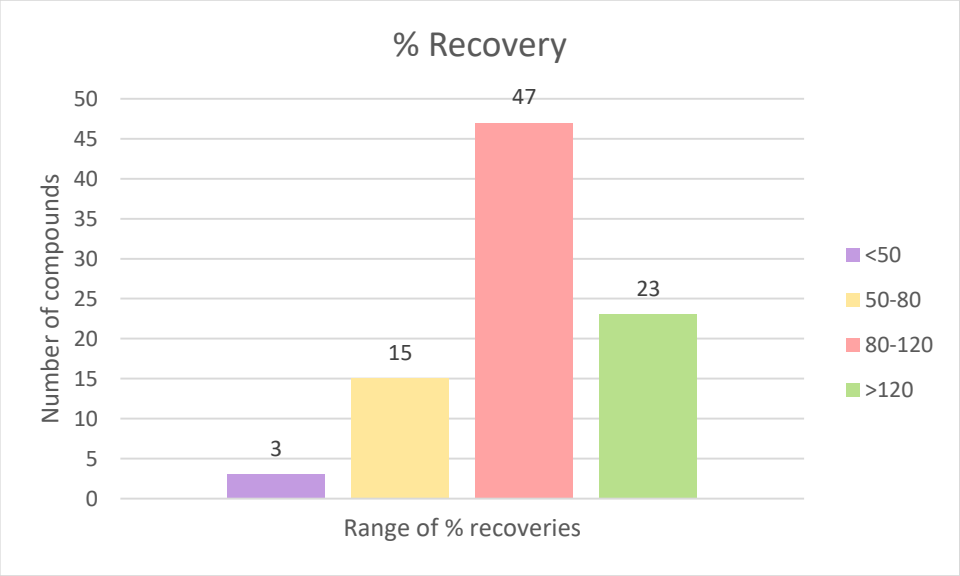


Figure 30 Distribution of % recoveries calculated at 300 µg/L.

5.1.2.3 Precision

% RSD was calculated for 3 spiked samples at 300 μg/L. For the vast majority of compounds % RSD below 10 was achieved as it is shown in *Figure 31*. All calculated % RSD are presented in detail in **Table 9**.

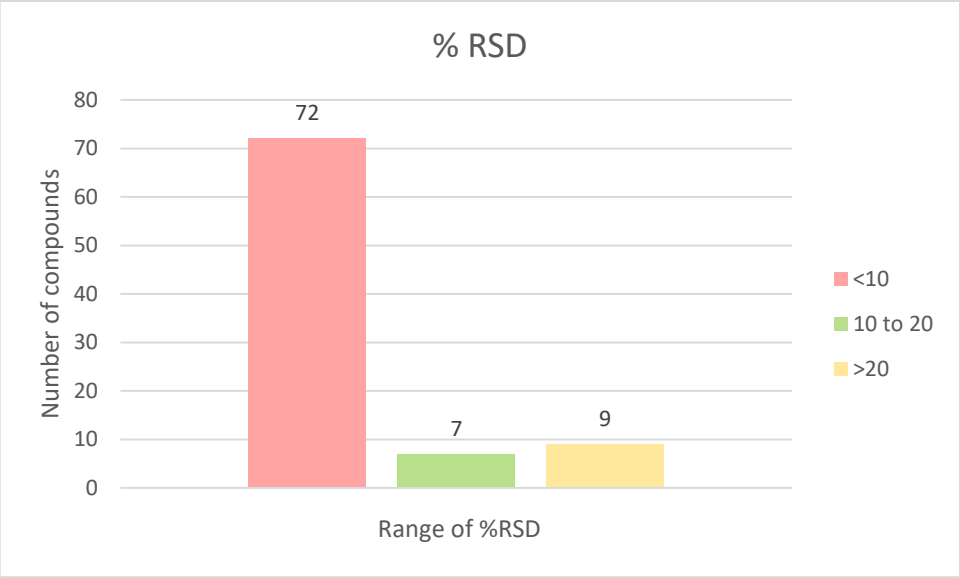


Figure 31 Repeatability (%RSD) at 300 μg/L.

Table 9 % Recovery and % repeatability calculated at 300 μg/L.

Compound	% Recovery (300 μg/L)				% Repeatability
	Spike_1	Spike_2	Spike_3	Average	
2-Amino-Benzothiazole	103	93.9	103	100	5.3
Acesulfame	92.7	112	102	102	9.5
Amitriptyline	46.4	62.6	49.5	52.8	16
Amphetamine	103	103	108	105	2.7
Atenolol	118	122	125	122	3.1
Atrazine-desethyl	106	100	101	102	2.8
Caffeine	146	149	149	148	1.2
Cetirizine	161	171	154	162	5.4
Chloramphenicol	102	114	104	107	7.5
Chlorpromazine	15.4	26.3	19.3	20.3	27
Citalopram	108	123	112	114	6.5
Clazuril	89.9	107	104	100	8.7
Clofibric acid	117	129	129	125	13
Clopidol	102	99.0	101	101	1.6
Colchicine	103	111	102	106	4.7
Coumaphos	40.3	44.8	38.2	41.1	8.2
Dapsone	90.6	97.3	92.5	93.5	3.7

Compound	% Recovery (300 µg/L)				% Repeatability
	Spike_1	Spike_2	Spike_3	Average	
Diaveridine	111	115	119	115	3.2
Diclazuril	59.4	79.8	70.5	69.9	26
Diclofenac	82.6	87.7	89.5	86.6	9.9
Dimethoate	99.9	108	98.7	102	5.0
Dimetridazole	59.9	61.9	60.0	60.6	1.8
Diuron (ESI+)	124	124	122	123	1.1
Diuron (ESI-)	98.8	99.1	101	99.6	0.63
Doxepine	85.4	98.3	92.8	92.2	7.0
Ephedrine	112	119	123	118	4.9
Ethopabate	112	102	110	108	4.6
Fenbendazole	109	106	111	109	2.2
Fenoxycarb	163	130	137	143	12
Florfenicol	94.8	110	100	102	7.7
Flubendazole	68.8	72.0	66.8	69.2	3.8
Flunixine	104	109	110	108	4.5
Furosemide	98.9	102.1	95.0	98.6	1.6
Gemfibrozil	136	156	143	145	22
Hydrochlorothiazide	80.5	77.1	78.7	78.8	14
Imidacloprid	144	140	143	143	1.8
Imipramine	64.9	81.6	70.2	72.2	12
Irgarol	77.1	77.8	79.6	78.2	1.7
Ketoprofen (ESI+)	116	122	110	116	4.9
Ketoprofen (ESI-)	77.9	61.5	63.6	67.7	24
Lamotrigine	107	120	108	112	6.4
Levamisol	105	108	111	108	2.8
Levetiracetam	150	161	158	156	3.9
Lidocaine	118	119	126	121	3.8
Lincomycin	178	184	185	182	2.2
Lorazepam	84.1	85.0	89.7	86.3	3.5
Mebendazole	72.7	75.8	73.4	74.0	13
Mefenamic acid (ESI+)	122	116	119	119	2.6
Mefenamic acid (ESI-)	112	127	112	117	12
Meloxicam	105	98.6	104	102	3.2
Metformin	303	357	313	324	8.8
Metronidazole	135	137	143	138	3.1
Morantel	121	126	131	126	3.8
Niflumic acid	92.1	23.9	93.9	69.9	1.5
Niflumic acid	103	103	106	104	1.9
Omeprazole	635	607	613	618	2.3
Oxfendazole	94.1	26.5	91.3	70.6	5.3
Paracetamol	142	141	149	144	2.9
Paroxetine	29.4	44.0	31.9	35.1	22
Primidone	120	111	120	117	4.6

Compound	% Recovery (300 µg/L)				% Repeatability
	Spike_1	Spike_2	Spike_3	Average	
Propranolol	128	147	135	137	6.9
Ronidazole	110	118	114	114	3.5
Saccharine	147	170	168	162	26
Salicylic acid	119	117	103	113	9.4
Sucralose	166	185	183	178	30
Sulfachloropyridazine	91.2	91.9	79.9	87.7	7.7
Sulfaclozine	81.9	78.2	72.9	77.7	5.8
Sulfadiazine	106	110	108	108	2.2
Sulfadimethoxine	80.4	73.9	76.0	76.8	4.3
Sulfadimidine	102	97.7	102.3	101	2.6
Sulfadoxine	103	101	104	103	1.3
Sulfamerazine	108	108	110	109	0.84
Sulfamethizole	92.4	95.6	94.3	94.1	1.7
Sulfamethoxazole	100	103	95.7	99.5	3.7
Sulfamethoxypyridazine	98.5	99.9	102	100	1.9
Sulfamonomethoxine	99.0	105	101	102	2.9
Sulfapyridine	98.8	107	99.7	102	4.3
Sulfaquinoxaline	82.5	76.6	79.9	79.7	3.8
Sulfathiazole	97.2	104	99.6	100	3.6
Sulfisoxazole	99.9	105	97.9	101	3.8
Ternidazole	163	166	169	166	1.7
Theophylline	159	158	155	158	1.3
Thiabendazole	89.4	95.9	89.2	91.5	4.2
Thiamphenicol	163	186	187	179	30
Toltrazuril	64.9	57.8	63.6	62.1	30
Tramadol	116	114	120	117	2.7
Triamterene	103	104	108	105	2.6
Trimethoprim	125	129	124	126	1.8

6.1.3 Comparison of SPE and direct injection analysis

LODs calculated from SPE and direct analysis experiments are illustrated in *Figure 32*. LODs calculated from direct injection are around 1,000 times higher than the ones calculated from SPE. With SPE most compounds can be detected up to 20 ng/L while LODs from direct injection are in general above 1,000 ng/L. The better detectability of SPE in comparison to direct injection is attributed to the high preconcentration and the extensive clean-up that are taking place during SPE pretreatment. Based on the generated results direct injection analysis would be suitable for analytes that are present in

concentrations of μg/L. The comparison of the two procedures shows that SPE clean-up and preconcentration is necessary for the detection of analytes of ECs that may be present at the low concentrations of ng/L. However, direct injection can be useful for suspect and non-target screening approaches when combined with HRMS. It can also eliminate common contamination sources from sample treatment for target analytes while it is a fast, cheap and eco-friendly sample treatment approach [64]. Optimization of the chromatographic parameters and especially careful column selection can lead to the detection of ECs at environmentally relevant concentrations in water matrices. In addition, large volume injection can be also applied to achieve lower limits of detection in target analysis while being efficient also for suspect and non-target screening approaches [28], [64].

Calculated recoveries and repeatability were satisfactory for the majority of compounds at concentration levels studied in each experimental approach.

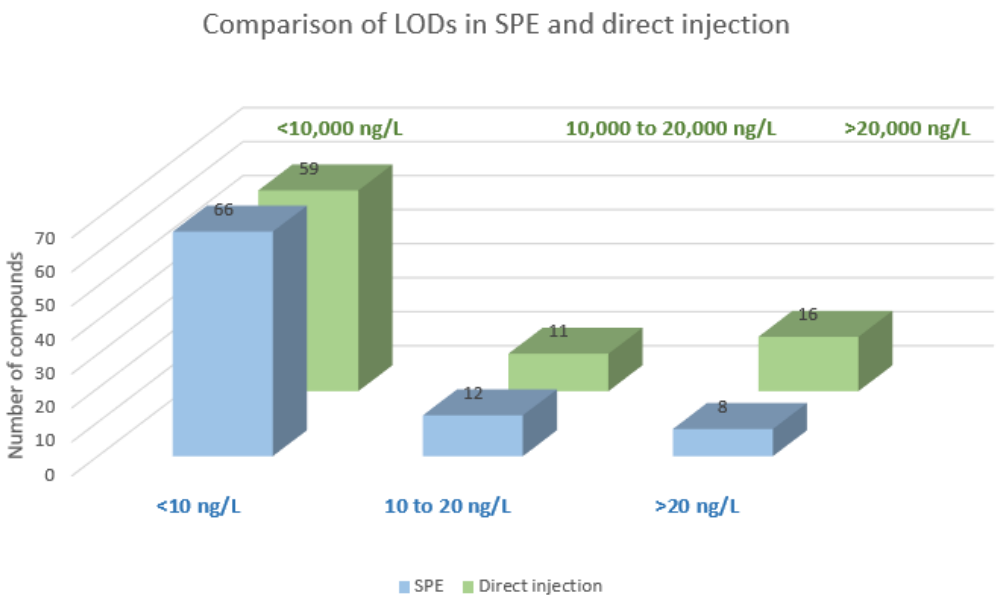


Figure 32 Comparison of LODs calculated from SPE and direct analysis experiments.

6.2 Target analysis results

6.2.1 Results from Aspropyrgos DWTP samples

In **Table 10** the results of target analysis applying the SPE sample preparation protocol in water samples collected at the entrance of Aspropyrgos DWTP are

summarized. In total, 10 compounds were detected belonging to the classes of pharmaceuticals (1), PPPs (3) and industrial chemicals (6). The detected concentrations range from sub ng/L levels to 12 ng/L.

The antidiabetic agent metformin was detected (<LOQ) in all untreated water samples. Moreover, the insect grow regulator lufenuron as well as the most common and effective active ingredient in commercial insect repellents, diethyltoluamide (DEET) were detected only once in the analyzed water samples. The TP of the herbicide dimethachlor, dimethachlor-ESA, was detected only in untreated_day1 sample below the LOQ level (1.1 ng/L).

The industrial chemical 2,4 dinitrophenol (DNP) used in the manufacture of dyes and in wood preservatives was also detected in all samples. The EIC of DNP in untreated_day3 and in untreated_spike_day3 samples are shown in *Figure 33.*

Table 10 Target analysis results.

Class	Compound	Untreated				
		Concentration (ng/L)			LOD	LOQ
		Day1	Day2	Day3		
Pharmaceuticals	**Metformin**	<LOQ	<LOQ	<LOQ	7.5	23
Plant Protection Products	**Lufenuron**	<LOD	11	<LOD	3.2	9.5
	Diethyltoluamide	1.5	<LOD	<LOD	0.26	0.78
Plant Protection Products TPs	**Dimethachlor-ESA**	<LOQ	<LOD	<LOD	0.36	1.1
Industrial Chemicals	**2,4-Dinitrophenol**	6.5	5.5	12	0.42	1.3
Industrial Chemicals (PFAS)	**PFHxA**	4.4	<LOD	<LOQ	0.64	1.9
	PFOA	<LOQ	<LOD	<LOD	0.15	0.45
	PFHpA	<LOQ	<LOD	<LOD	0.10	0.31
	PFPeA	<LOQ	0.48	<LOD	0.16	0.47
	ADONA	<LOQ	<LOD	<LOD	0.53	1.6

Concerning PFAS, perfluorooctanoic acid (PFOA), perfluoroheptanoic acid (PFHpA) and 4,8-dioxa-3H-perfluorononanoate (ADONA) were detected only in untreated_day1 sample at levels below LOQ. Perfluoropentanoic acid (PFPeA) was detected shlightly above LOQ in untreated_day2 sample and perfluorohexanoic acid (PFHxA) was determined only in untreated_day1 sample.

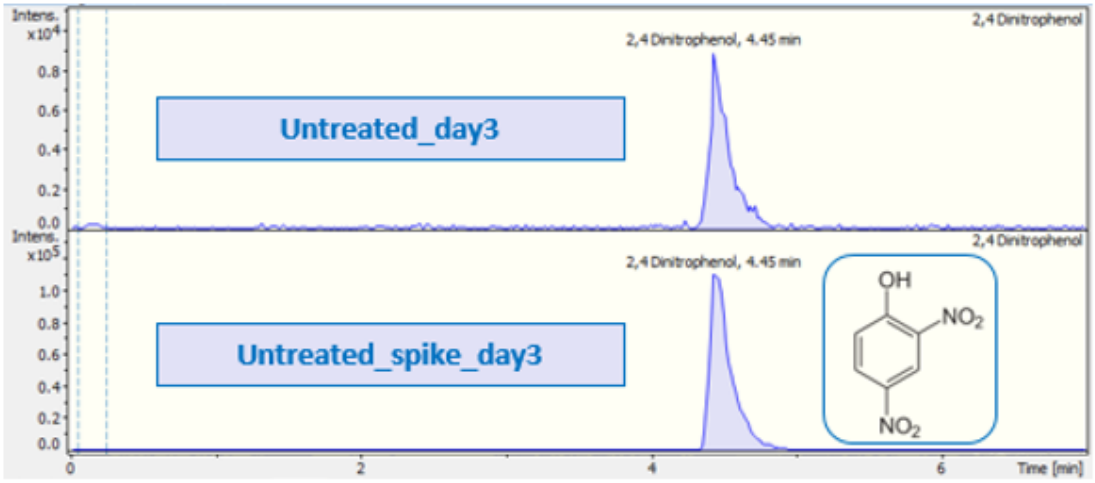


Figure 33 EICs of 2,4 dinitrophenol in untreated water sample_day3 and in spiked sample.

6.2.2 Discussion

In the following paragraphs, the results of the present study are compared with the ones obtained from a similar study conducted for Mornos water reservoir.

In 2018, the Laboratory of Analytical Chemistry of the National and Kapodistrian University of Athens in association with EYDAP conducted a study with the aim to investigate the occurrence of ECs and their TPs in water reservoirs used for DW production in Attika, Greece. Mornos water reservoir, which is the source of raw water for Aspropyrgos DWTP, was included in that study [65]. Two sampling campaigns were organized to represent two different seasons (first sampling campaign: summer, second sampling campaign: winter) and twenty-four-hour mixed water samples for three consecutive days were collected in each sampling campaign. Samples were analyzed using RPLC-(+/-)ESI-QToF-MS and HILIC-(+/-)ESI-QToF-MS. Through the application of wide-scope target, suspect and non-target screening data treatment approaches, 30 compounds were detected in water samples in both sampling campaigns belonging to the classes of industrial chemicals, pharmaceuticals, plant protection products and coffee and tobacco related compounds. Concerning industrial chemicals, benzothiazoles and benzotriazoles and PFHxA (PFAS) were mainly detected in the samples of the first sampling campaign. The same detection pattern was followed for alcohol ethoxysulfates surfactants while

linear alkylbenzene sulfonate surfactants were also detected in samples collected in the second sampling campaign at concentrations up to almost 2.5 μg/L. The pharmaceutical prolinamide was detected at concentrations below 206 ng/L and metformin was detected in all samples at concentrations up to 26.6 ng/L. Sulfadiazine was detected only in the samples of first sampling campaign (maximum concentration: 206 ng/L) while allethrin and bioallethrin were detected exclusively in the samples of the second sampling campaign (maximum concentration: 5.11 ng/L). DEET was detected only in two samples in both sampling campaigns and dinoterb, thiamethoxam and thiodicarb were only detected in samples from the second sampling campaign. Concerning coffee and tobacco related ECs, caffeine and its metabolites theobromine and theophylline were exclusively detected in the samples of the second sampling campaign at concentration levels that did not exceed 294, 65 and 66 ng/L respectively. Nicotine was detected in all samples and presented the highest detected concentration levels in the samples of the second sampling campaign (maximum concentration: 390 ng/L) [65].

Comparing the abovementioned results with the ones obtained from the present study, only three compounds were detected in both studies. In detail, metformin presented the same frequency of detection and was detected at similar concentration levels in both studies. PFHxA and DEET were detected at significantly lower concentrations at the samples collected at the entrance of Aspropyrgos DWTP. Moreover, in the present study four more PFAS were detected and 2,4 dinitrophenol, lufenuron and dimethachlor-ESA that were not detected in 2018 in Mornos water reservoir were detected in the present study [65]. The difference in the number of compounds detected in both studies can be attributed to the different sampling locations, to the double number of samples included in the study regarding water reservoirs, the seasonal variation of the occurrence of compounds and in possible changes in the quality of water that may have occurred during the five years period between the two studies. In addition, the use of complementary chromatographic techniques, the difference in sample preconcentration (10,000 times in the previous study versus 2,000 times in the present study) as well as the application of a holistic

data treatment approach have contributed to the detection of more compounds in the study regarding Mornos water reservoirs.

In the following paragraphs, details regarding the detected compounds in the present study are provided as well as data of their detection in water matrices worldwide are presented.

Zhang et al. studied the presence of metformin in surface and drinking water [66]. Metformin is one of the most prescribed medications in the world not only for the treatment of diabetes type 2 but also for its benefits in cancer prevention, women's infertility and lifespan expansion. It is known that metformin is not metabolized by human and, hence, it is excreted unmodified at almost 100%. Metformin has been found with the largest amount by weight among all drugs in the aquatic environment. Worldwide, it has been detected at concentrations within the range of 8.4-34,000 ng/L in surface water including sources used for DW production. In DW metformin has been detected at concentrations ranging from 5.1 to 1,204 ng/L in various countries [66]. In our study, metformin was detected in all untreated water samples at concentrations that did not exceed the LOQ of 23 ng/L. From ecotoxicity studies, it is proven that metformin affects the behavior of aquatic organisms. In addition, it is known that in DW systems metformin is inevitably transformed due to the chlorination process. But, whether metformin's chlorination by-products (Y: $C_4H_6ClN_5$ and C: $C_4H_6ClN_3$) exist in DW, remains unknown. Initial experiments conducted by *Zhang et al.* revealed the detection of metformin's TP C in tap water lying in the range of 1.3 to 9.7 ng/L while TP Y was not detected (possibly due to its relatively high LOD 72.6 ng/L). From toxicity experiments it was proven that both TPs are markedly toxic to live animals and human cells. Although the current concentrations of metformin present in DW may not directly cause safety concerns to humans, the potential threats of its chlorination byproducts cannot be neglected. Boiling water as well as adsorption on activate carbon are proposed as effective ways to remove the known TPs from DW, while bank filtration and biodegradation by certain species of bacteria are suggested to remove metformin from aqueous systems [66].

The insect repellent DEET is among the most frequently detected ECs in water across a wide range of geographies around the world. The occurrence of DEET in aqueous environments involves multiple and potentially complex routes. In 2004 it was the first time that DEET was detected in DW in America, and five years later, it was detected in Europe's DW [67]. *Padhye et al.* have reported concentrations of DEET in surface water and DW at 124 ng/L and 12 ng/L respectively [46]. *Merel et al.* in their review regarding DEET in water matrices, mentioned 13 ng/L and 97 ng/L as the maximum detected concentrations in DW in Europe and in the USA correspondingly [67]. Concentrations determined for DEET for Aspropyrgos DWTP samples were much lower (up to 1.5 ng/L). The attenuation of DEET by oxidation in water treatment or photocatalysis and biodegradation in rivers results in the formation of multiple TPs for which only a limited number of studies exists. While the occurrence of DEET in DW should not be of concern to human health according to toxicological data available to date, further studies are still required to assess the impact of long term exposure to low doses along with a mixture of other trace organic contaminants [67].

Concerning dimethachlor-ESA, data for its detection in water samples in literature are scarce. This TP of dimethachlor has been detected in the Tisza River at 80 ng/L and in the Danube River at 10 ng/L [68]. In addition, the literature review conducted for this research did not reveal any published data regarding the detection of lufenuron in DW. In total, comparing with the parametric values established by the Directive (EU) 2020/2184 for PPPs (**Table 12** in *Annex I*), for all detected compounds the determined concentrations (up to 12 ng/L) are much lower than the limits both for individual compounds (0.1 μg/L) and for their summary (0.5 μg/L) [12].

With respect to the industrial chemical 2,4 dinitrophenol, it was detected in Marathonas water reservoir in the study conducted in 2018 about the occurrence of ECs in water reservoirs in Attika, Greece at concentrations within the range 64-161 ng/L. In that study 4-nitrophenol was detected in all water reservoirs (included Mornos reservoir) through non-target screening [65].

PFAS are fluorinated aliphatic substances with unique properties, such as being hydrophobic, lipophobic, and extremely stable due to the strength of the C-F bond [69], [70]. Due to these properties, PFAS are widely used as surface active agents in products like stain repellants and fire-fighting foams. Their unique properties make PFAS persistent in the environment and, hence, they are ubiquitously detected in a wide variety of samples. They also have bioaccumulative properties and they have been detected in the food chain and in living organisms. To date, the effect of the majority of PFAS on living organisms including humans has not been excessively studied and therefore, potential risks caused by PFAS still remain unknown [69], [70]. *Tröger et al.* detected in Sweden's DW PFOA, PFHxA and PFHpA at concentrations 0.832 ng/L, 0.768 ng/L and 0.434 ng/L respectively [4]. Similarly, *Zafeiraki et al.* reported the detection of PFOA, PFHxA and PFHpA in tap water collected from different locations in Greece and in the Netherlands. In Greece, PFHxA and PFHpA were below 0.6 ng/L, while PFOA was detected up to 0.9 ng/L. In the Netherlands, the detected concentrations of PFOA, PFHxA and PFHpA reached higher levels (11.1 ng/L, 4.9 ng/L, 3 ng/L correspondingly), and additionally, PFPeA was detected up to 19.8 ng/L [70]. *Crone et al.* investigated the occurrence of PFAS in DW in the USA. Among other PFAS, PFOA (up to 4,300 ng/L), PFHxA (0.52-0.62 ng/L), PFHpA (up to 34 ng/L) and PFPeA (up to 43 ng/L) were detected in DW samples [69]. Quantitative results concerning the detected PFAS in our study are generally lower compared to the concentrations mentioned in the abovementioned studies, while they are slightly above the LOQ of the applied method. Only PFHxA was detected at a concentration around two times higher than the LOQ but still, it is within the range of detected concentrations in the Netherlands. Concerning ADONA, which was manufactured to replace PFOA, it presents a shorter lifetime in human fluids and is less toxic and bioaccumulative than PFOA. But, since it has not been included neither in environmental monitoring nor in biomonitoring studies, not much data regarding its occurrence and toxicity are available. It has been reported that ADONA was detected at concentrations that do not exceed 1.5 ng/L in surface water. In aqueous environments, it has been detected only in

the Rhine River [71]. In our research, ADONA was detected only in one untreated water sample below the LOQ level (<1.6 ng/L). In total, comparing with the parametric values established by the Directive (EU) 2020/2184 for PFAS (**Table 12** in *Annex I*), for all detected compounds the determined concentrations (up to 4.4 ng/L) are around two orders of magnitude lower than the limits both for individual compounds (0.1 μg/L) and for their summary (0.5 μg/L) [12]. Removal of PFAS during DW production is a challenge. Due to their stability, conventional processes have proven to be inadequate. Advanced treatment technologies including granular active carbon filtration, nanofiltration and reverse osmosis are some of the currently proposed methods that still require further research [69], [70].

Apart from the abovementioned compounds detected in water samples collected at the entrance of Aspropyrgos DWTP, tobacco related substance nicotine as well as its metabolite, cotinine, were also detected in all three samples. However, these compounds were additionally detected in blank samples for the sampling procedure, indicating that they may be detected in untreated water samples due to contamination from the sampling collection and treatment. Consequently, it is highlighted that both careful sample collection and treatment are crucial to avoid any possible sources of sample contamination and to obtain trustworthy results.

Still it is noted that these compounds have been also detected in water samples collected from Mornos reservoir in the previous study [65]. Comparing with literature, *Tröger et al.* detected nicotine and cotinine in water samples collected at a DWTP at concentrations up to 0.9 ng/L and 2.1 ng/L respectively [4]. *Padhye et al.* have also detected cotinine in surface water (maximum concentration 2.7 ng/L) as well as in finished DW (maximum concentration 0.4 ng/L) [46]. In a relative study conducted in Spain, nicotine was detected in tap water samples at concentrations ranging from 1 to 141 ng/L respectively. Cotinine was detected within the range 0.3-9.8 ng/L [72].

CHAPTER 7
CONCLUSIONS

The present study showed that the investigation of the occurrence of ECs and their TPs in water samples (surface water) requires the application of a generic sample preparation protocol covering a wide range of compounds of different chemical classes, with a high preconcentration factor and efficient clean-up to reach low concentrations of ng/L. Although validation of such a protocol is demanding due to the great number of compounds in wide-scope target screening, a smart validation approach is necessary to be followed to ensure that the method fits for purpose. A representative validation dataset of compounds should be tested for method's qualitative characteristics, and it is expected to obtain results within a relatively broad range. The diversity of compounds can explain values that in a classic validation approach would not be accepted. Moreover, the applied method should be able to achieve low LODs. Therefore, since great preconcentration of sample is required, the SPE protocol was adequate, while direct injection analysis presenting significantly higher LODs could not reveal the occurrence of ECs and their TPs in water samples. However, direct injection analysis may have provided valuable data for suspect and non-target screening approaches. Additionally, HRMS techniques providing high quality data are essential in such studies.

Wide-scope target screening analysis of untreated water samples collected at Aspropyrgos DWTP revealed the occurrence of 10 ECs. They belong to classes of pharmaceuticals, PPPs and industrial chemicals (especially PFAS), and in total, they were detected at concentrations ranging from sub ng/L levels to 12 ng/L which are much lower than the available regulatory limits. Some of the obtained results followed the same pattern (both regarding their occurrence and concentration level) with the previous study conducted in water reservoirs. Comparison of the findings of the present study are in general accordance with similar studies found in the open literature.

During the present study it was shown that sampling is a critical step of the analysis in order to avoid false positive results and analysis of the sampling and

laboratory procedural blanks is obligatory. Strict application of the sampling protocol along with careful sample handling by the sampling personnel are required to eliminate sample's contamination and prevent such incidents in future sampling campaigns. It is also noted that the detected concentrations are close to the limit of detection and quantification of the method, rendering the uncertainty of the results higher. To conclude, further research under the conditions of careful sampling and samples' analysis will permit further evaluation of the obtained results.

CHAPTER 8
FUTURE PERSPECTIVES

Apart from RPLC, HILIC can be applied in order to investigate the occurrence of ECs and especially their TPs with higher polarity that may have not been detected by RPLC-ESI-QToF-MS analysis. In addition, the application of GC-APCI-QToF-MS analysis could cover compounds that are nonpolar, volatile and thermally stable. Screening of the acquired HRMS data according to suspect and non-target workflows would enable the investigation of compounds that have not been included in the wide-scope target screening approach and would provide a holistic characterization of water samples.

Moreover, sampling could extend to include water samples collected at the exit of the DWTP or between the different treatment stages of DW production. The analysis of these samples could reveal the possible removal and/or transformation of the detected ECs (especially the formation of disinfection by-products) that may take place in the DWTP. The monitoring of ECs and their TPs could be also applied to the other DWTPs located in Attiki, Greece. Furthermore, sampling campaigns at different seasons could be organized to evaluate the seasonal variation of the detected compounds. The evaluation of data collected from all locations and seasons could reveal compounds that due to their characteristics may need to be included in routine monitoring programs.

The investigation of the occurrence of ECs, TPs and DBPs could also expand to include the water distribution system. Water distribution system is characterized in literature as a huge reactor. Samples collected within the distribution system at different distances from DWTPs and at the consumer's houses could provide an insight into the fate of these compounds in the whole hydrological pathway of DW production and consumption. In this way, a holistic approach regarding the occurrence and transformation of ECs and their TPs as well as DBPs fate through the whole hydrological pathway could be achieved.

ABBREVIATIONS AND ACRONYMS

CAS	Chemical Abstract Database
ECHA	European Chemicals Agency
PPs	Priority Pollutants
POPs	Persistent Organic Pollutants
ECs	Emerging Contaminants
EPs	Emerging Pollutants
DW	Drinking Water
PPCPs	Pharmaceuticals and Personal Care Products
DoAs	Drugs of Abuse
EDCs	Endocrine Disrupting Compounds
PPPs	Plant Protection Products
PFAS	Poly- and Perfluoroalkyl Substances
WWTPs	Wastewater Treatment Plants
DWTPs	Drinking Water Treatment Plants
WB	Water Bodies
NORMAN	Network of reference laboratories, research centers and related organizations for monitoring of emerging environmental substances
TPs	Transformation Products
EU	European Union
WHO	World Health Organization
UV	Ultra Violet
GAC	Granular Activated Carbon
NOM	Natural Organic Matter
DBPs	Disinfection By-Products
THMs	Trihalomethanes
HAAs	Haloacetic acids
HANs	Haloacetonitriles
HNMs	Halonitromethanes
HAMs	Halogenated Acetamides
EQSs	Environmental Quality Standards
SPE	Solid Phase Extraction
LLE	Liquid-Liquid Extraction
HLB	Hydrophilic Lipophilic Balanced
WAX	Weak Anion Exchange
WCX	Weak Cation Exchange
MAX	Mixed-mode Anion Exchange
MCX	Mixed-mode Cation Exchange
VEC	Vacuum-assisted Evaporative Concentration
HRMS	High Resolution Mass Spectrometry

GC	Gas Chromatography
LC	Liquid Chromatography
MS	Mass Spectrometry
UHPLC	Ultra High Performance Liquid Chromatography
MMLC	Mixed-Mode Liquid Chromatography
RP	Reverse Phase
HILIC	Hydrophilic Interaction Liquid Chromatography
NPLC	Normal Phase Liquid Chromatography
SFC	Supercritical Fluid Chromatography
GSC	Gas-Solid Chromatography
GLC	Gas-Liquid Chromatography
EI	Electron Impact
APCI	Atmospheric Pressure Chemical Ionization
ESI	Electro Spray Ionization
LRMS	Low Resolution Mass Spectrometry
Q	Quadrupole
RF	Radio Frequency
AC	Alternating Current
DC	Direct Current
QqQ	Triple Quadrupole
SRM	Single Reaction Monitoring
MRM	Multiple Reaction Monitoring
IT	Ion Trap
ToF	Time of Flight
FT	Fourier Transform
FTICR	Fourier-Transform Ion Cyclotron Resonance
DDA	Data Dependent Acquisition
DIA	Data Independent Acquisition
bbcid	Broadband collision-induced dissociation
IPs	Identification Points
LVI	Large Volume Injection
HESI	Heated Electrospray Ionization
EYDAP S.A	Athens Water Supply and Sewerage Company
RC	Regenerated Cellulose
IS	Internal Standard(s)
PTFE	Polytetrafluoroethylene
PI	Positive Ionization
NI	Negative Ionization
bbcid	broad-band Collision Induced Dissociation
FWHM	Full Width at Half Maximum
NKUA	National and Kapodistrian University of Athens
HPC	High Precision Calibration

EIC	Extracted Ion Chromatogram
RSD	Relative Standard Deviation
FME	Factor of Matrix Effect
PFBA	Perfluorobutanoic acid
PFDoA	Perfluorododecanoic acid
PFUnA	Perfluoroundecanoic acid
DNP	2,4 dinitrophenol
DEET	Diethyltoluamide
BTR	Benzotriazole
PFOA	Perfluorooctanoic acid
ADONA	4,8-dioxa-3H-perfluorononanoate
PFPeA	Perfluoropentanoic acid
PFHxA	Perfluorohexanoic acid
PFHpA	Perfluoroheptanoic acid
BTHs	Benzothiazoles

ANNEX I

Minimum requirements for parametric values used to assess the quality of water intended for human consumption according to the Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption.

Table 11 Microbial parameters.

Parameter	Parametric value	Unit	Note
Intestinal enterococci	0	number/100 mL	For water put into bottles or containers, the unit is number/250 mL.
Escherichia coli (*E. coli*)	0	number/100 mL	For water put into bottles or containers, the unit is number/250 mL.

Table 12 Chemical parameters.

Parameter	Parametric value	Unit	Note
Acrylamide	0.10	µg/L	The parametric value of 0.10 µg/L refers to the residual monomer concentration in the water as calculated according to specifications of the maximum release from the corresponding polymer in contact with the water
Antimony	10	µg/L	
Arsenic	10	µg/L	
Benzene	1,0	µg/L	
Benzo(a)pyrene	0.010	µg/L	
Bisphenol A	2.5	µg/L	
Boron	1.5	mg/L	A parametric value of 2.4 mg/L shall be applied when desalinated water is the predominant water source of the supply system concerned or in regions where geological conditions could lead to high levels of boron in groundwater.
Bromate	10	µg/L	
Cadmium	5.0	µg/L	
Chlorate	0.25	mg/L	A parametric value of 0.70 mg/L shall be applied where a disinfection method that generates chlorate, in particular chlorine dioxide, is used for disinfection of water intended for human consumption. Where possible, without compromising disinfection, Member States shall strive for a lower value. This parameter shall be measured only if such disinfection methods are used.

Chlorite	0.25	mg/L	A parametric value of 0.70 mg/L shall be applied where a disinfection method that generates chlorite, in particular chlorine dioxide, is used for disinfection of water intended for human consumption. Where possible, without compromising disinfection, Member States shall strive for a lower value. This parameter shall be measured only if such disinfection methods are used
Chromium	25	µg/L	The parametric value of 25 µg/L shall be met, at the latest, by 12 January 2036. The parametric value for chromium until that date shall be 50 µg/L.
Copper	2,0	mg/L	
Cyanide	50	µg/L	
1,2-dichloroethane	3.0	µg/L	
Epichlorohydrin	0.10	µg/L	The parametric value of 0.10 µg/L refers to the residual monomer concentration in the water as calculated according to specifications of the maximum release from the corresponding polymer in contact with the water.
Fluoride	1.5	mg/L	
Haloacetic acids (HAAs)	60	µg/L	This parameter shall be measured only when disinfection methods that can generate HAAs are used for the disinfection of water intended for human consumption. It is the sum of the following five representative substances: monochloro-, dichloro-, and trichloro-acetic acid, and mono- and dibromo-acetic acid.
Lead	5	µg/L	The parametric value of 5 µg/L shall be met, at the latest, by 12 January 2036. The parametric value for lead until that date shall be 10 µg/L.
			After that date, the parametric value of 5 µg/L shall be met at least at the point of supply to the domestic distribution system. For the purposes of point (b) of the first subparagraph of Article 11(2), the parametric value of 5 µg/L at the tap shall apply.
Mercury	1.0	µg/L	
Microcystin-LR	1.0	µg/L	This parameter shall be measured only in the event of potential blooms in source water (increasing cyanobacterial cell density or bloom forming potential).
Nickel	20	µg/L	
Nitrate	50	mg/L	Member States shall ensure that the condition [nitrate]/50 + [nitrite]/3 ≤ 1, where the square brackets signify the concentrations in mg/L for nitrate (NO_3) and nitrite (NO_2), is complied with and that the parametric value of 0.10 mg/L for nitrites is complied with ex water treatment works.

Nitrite	0.50	mg/L	Member States shall ensure that the condition [nitrate]/50 + [nitrite]/3 ≤ 1, where the square brackets signify the concentrations in mg/L for nitrate (NO_3) and nitrite (NO_2), is complied with and that the parametric value of 0.10 mg/L for nitrites is complied with ex water treatment works.
Pesticides	0.10	µg/L	'Pesticides' means: - organic insecticides, - organic herbicides, - organic fungicides, - organic nematocides, - organic acaricides, - organic algicides, - organic rodenticides - organic slimicides, - related products (inter alia, growth regulators), and their metabolites as defined in point (32) of Article 3 of Regulation (EC) No 1107/2009 of the European Parliament and of the Council ([1]), that are considered relevant for water intended for human consumption. A pesticide metabolite shall be deemed relevant for water intended for human consumption if there is reason to consider that it has intrinsic properties comparable to those of the parent substance in terms of its pesticide target activity or that either itself or its transformation products generate a health risk for consumers
			The parametric value of 0.10 µg/L shall apply to each individual pesticide. In the case of aldrin, dieldrin, heptachlor and heptachlor epoxide, the parametric value shall be 0.030 µg/L.
			Member States shall define a guidance value to manage the presence of non-relevant metabolites of pesticides in water intended for human consumption. Only pesticides which are likely to be present in a given supply need to be monitored. Based on the data reported by Member States, the Commission may establish a database of pesticides and their relevant metabolites taking into account their possible presence in water intended for human consumption.
Pesticides Total	0.50	µg/L	'Pesticides Total' means the sum of all individual pesticides, as defined in the previous row, detected and quantified in the monitoring procedure.

PFAS Total	0.50	µg/L	'PFAS Total' means the totality of per- and polyfluoroalkyl substances. This parametric value shall only apply once technical guidelines for monitoring this parameter are developed in accordance with Article 13(7). Member States may then decide to use either one or both of the parameters 'PFAS Total' or 'Sum of PFAS'.
Sum of PFAS	0.10	µg/L	'Sum of PFAS' means the sum of per- and polyfluoroalkyl substances considered a concern as regards water intended for human consumption. This is a subset of 'PFAS Total' substances that contain a perfluoroalkyl moiety with three or more carbons (i.e. $–C_nF_{2n}–$, n ≥ 3) or a perfluoroalkylether moiety with two or more carbons (i. e. $–C_nF_{2n}OC_mF_{2m}–$, n and m ≥ 1).
Polycyclic aromatic hydrocarbons	0.10	µg/L	Sum of concentrations of the following specified compounds: benzo(b)fluoranthene, benzo(k)fluoranthene, benzo (ghi)perylene, and indeno(1,2,3-cd)pyrene.
Selenium	20	µg/L	A parametric value of 30 µg/L shall be applied for regions where geological conditions could lead to high levels of selenium in groundwater.
Tetrachloroethene and Trichloroethene	10	µg/L	The sum of concentrations of these two parameters.
Trihalomethanes Total	100	µg/L	Where possible, without compromising disinfection, Member States shall strive for a lower parametric value. It is the sum of concentrations of the following specified compounds: chloroform, bromoform, dibromochloromethane and bromodichloromethane.
Uranium	30	µg/L	
Vinyl chloride	0.50	µg/L	The parametric value of 0.50 µg/L refers to the residual monomer concentration in the water as calculated according to specifications of the maximum release from the corresponding polymer in contact with the water
(1) Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC (OJ L 309, 24.11.2009, p.1).			

Figure 34 Images of glass fiber filters after sample filtration (approximately 4 L in untreated and 2 L in blank samples).

Table 13 Gradient program of mobile phase in UHPLC.

Time (min)	Flow rate (mL/min)	% Aqueous solvent	% Organic solvent
0	0.2	99	1
1	0.2	99	1
3	0.2	61.0	39.0
14	0.4	0.1	99.9
16	0.48	0.1	99.9
16,1	0.48	99	1
19,1	0.2	99	1
20	0.2	99	1

Table 14 Compounds of the IS mix.

Amisulpride-d^5
Bisphenol A-d^{16}
Diazepam-d^5
Diethyl Phthalate-d^4
Diuron-d^6
Fenbendazole-d^3
Flunixin-d^3
Mefenamic acid-d^3
Meloxicam-d^3
Morphine-d^3
rac-Methamphetamine-d^5
Ranitidine-d^6
Sulfadiazine-d^4
Sulfamerazine-d^4

ANNEX II

Table 15 Calibration curves calculated from standard solutions analysis.

Compound	C_{range} (µg/L)	Calibration curve (Rel. areas)	R^2
2-Amino-Benzothiazole	10-300	$y=(214*10^{-5} \pm 6.97*10^{-5})*x$	0.991
Acesulfame	10-150	$y=(48.9*10^{-5} \pm 1.84*10^{-5})*x$	0.989
Amitriptyline	10-300	$y=(468*10^{-5} \pm 6.81*10^{-5})*x$	0.998
Amphetamine	10-300	$y=(154*10^{-5} \pm 2.60*10^{-5})*x + (13.1*10^{-3} \pm 3.40*10^{-3})$	0.997
Atenolol	10-150	$y=(325*10^{-5} \pm 3.65*10^{-5})*x$	0.9990
Atrazine-desethyl	10-150	$y=(58.7*10^{-5} \pm 3.93*10^{-5})*x$	0.97
Caffeine	10-150	$y=(316*10^{-6} \pm 9.56*10^{-6})*x + (60.8*10^{-4} \pm 9.44*10^{-4})$	0.993
Cetirizine	10-300	$y=(80.0*10^{-5} \pm 3.93*10^{-5})*x$	0.98
Chloramphenicol	10-150	$y=(187*10^{-5} \pm 6.58*10^{-5})*x + (26.8*10^{-3} \pm 6.50*10^{-3})$	0.990
Chlorpromazine	10-300	$y=(64.6*10^{-4} \pm 1.39*10^{-4})*x$	0.996
Citalopram	10-300	$y=(322*10^{-5} \pm 7.00*10^{-5})*x + (23.6*10^{-3} \pm 9.15*10^{-3})$	0.996
Clarithromycin	10-300	$y=(139*10^{-5} \pm 8.06*10^{-5})*x$	0.97
Clazuril	10-300	$y=(217*10^{-6} \pm 5.98*10^{-6})*x$	0.993
Clofibric acid	50-300	$y=(86.0*10^{-6} \pm 3.65*10^{-6})*x$	0.988
Clopidol	10-300	$y=(165*10^{-5} \pm 2.58*10^{-5})*x + (7.54*10^{-3} \pm 2.55*10^{-3})$	0.998
Closantel	10-150	$y=(55.7*10^{-4} \pm 1.23*10^{-4})*x$	0.996
Colchicine	10-300	$y=(81.5*10^{-5} \pm 2.39*10^{-5})*x + (9.43*10^{-3} \pm 3.12*10^{-3})$	0.992
Coumaphos	10-300	$y=(219*10^{-5} \pm 6.94*10^{-5})*x$	0.991
Dapsone	10-150	$y=(60.4*10^{-4} \pm 3.68*10^{-4})*x$	0.97
Decoquinate	10-150	$y=(207*10^{-4} \pm 4.38*10^{-4})*x + (14.6*10^{-2} \pm 5.72*10^{-2})$	0.996
Diaveridine	10-300	$y=(204*10^{-5} \pm 7.62*10^{-5})*x + (30.6*10^{-3} \pm 9.95*10^{-3})$	0.988
Diclazuril	10-300	$y=(291*10^{-6} \pm 7.06*10^{-6})*x$	0.995
Diclofenac	10-300	$y=(75.2*10^{-6} \pm 3.30*10^{-6})*x + (10.2*10^{-4} \pm 4.30*10^{-4})$	0.98
Dimethoate	10-150	$y=(187*10^{-5} \pm 6.98*10^{-5})*x + (21.8*10^{-3} \pm 6.89*10^{-3})$	0.989
Dimetridazole	10-300	$y=(265*10^{-6} \pm 9.77*10^{-6})*x + (3.69*10^{-3} \pm 1.28*10^{-3})$	0.988
Diuron (ESI-)	10-300	$y=(67.9*10^{-5} \pm 1.16*10^{-5})*x$	0.997
Diuron (ESI+)	10-300	$y=(701*10^{-6} \pm 8.02*10^{-6})*x$	0.9988
Doxepine	10-300	$y=(387*10^{-5} \pm 6.54*10^{-5})*x$	0.997
Ephedrine	10-150	$y=(476*10^{-5} \pm 9.48*10^{-5})*x + (35.0*10^{-3} \pm 9.36*10^{-3})$	0.997
Ethopabate	10-150	$y=(153*10^{-5} \pm 2\ .10*10^{-5})*x + (8.67*10^{-3} \pm 2.07*10^{-3})$	0.998
Fenbendazole	10-300	$y=(18.6*10^{-4} \pm 1.74*10^{-4})*x$	0.95
Fenoxycarb	10-300	$y=(315*10^{-5} \pm 3.25*10^{-5})*x$	0.9990
Florfenicol	10-300	$y=(201*10^{-5} \pm 6.51*10^{-5})*x$	0.992
Flubendazole	10-300	$y=(261*10^{-6} \pm 2.80*10^{-6})*x - (8.93*10^{-4} \pm 3.66*10^{-4})$	0.99896
Flunixine	10-300	$y=(276*10^{-5} \pm 4.30*10^{-5})*x$	0.998
Furosemide	10-300	$y=(335*10^{-6} \pm 7.29*10^{-6})*x$	0.996
Gemfibrozil	10-300	$y=(57.1*10^{-5} \pm 1.78*10^{-5})*x$	0.991
Hydrochlorothiazide	10-300	$y=(259*10^{-6} \pm 6.61*10^{-6})*x$	0.994
Imidacloprid	10-150	$y=(33.3*10^{-5} \pm 1.66*10^{-5})*x + (7.76*10^{-3} \pm 1.64*10^{-3})$	0.98

Compound	C_{range} (µg/L)	Calibration curve (Rel. areas)	R^2
Imipramine	10-300	$y=(472*10^{-5} \pm 8.05*10^{-5})*x$	0.997
Irgarol	10-300	$y=(70.8*10^{-3} \pm 1.78*10^{-3})*x$	0.994
Ketoprofen (ESI+)	10-300	$y=(291*10^{-6} \pm 8.35*10^{-6})*x + (3.89*10^{-3} \pm 1.09*10^{-3})$	0.993
Ketoprofen (ESI-)	25-300	$y=(105*10^{-6} \pm 2.98*10^{-6})*x$	0.994
Lamotrigine	10-300	$y=(121*10^{-5} \pm 2.80*10^{-5})*x + (10.8*10^{-3} \pm 3.65*10^{-3})$	0.995
Levamisol	10-300	$y=(231*10^{-5} \pm 3.04*10^{-5})*x + (18.5*10^{-3} \pm 3.96*10^{-3})$	0.998
Levetiracetam	10-150	$y=(37.1*10^{-5} \pm 1.42*10^{-5})*x + (4.04*10^{-3} \pm 1.41*10^{-3})$	0.988
Lidocaine	10-150	$y=(130*10^{-4} \pm 2.83*10^{-4})*x + (10.3*10^{-2} \pm 2.79*10^{-2})$	0.996
Lincomycin	10-150	$y=(499*10^{-6} \pm 8.83*10^{-6})*x + (31.7*10^{-4} \pm 8.72*10^{-4})$	0.998
Lorazepam	10-300	$y=(43.1*10^{-6} \pm 1.11*10^{-6})*x + (17.9*10^{-4} \pm 1.58*10^{-4})$	0.995
Lufenuron	10-150	$y=(117*10^{-5} \pm 3.50*10^{-5})*x + (9.03*10^{-3} \pm 3.45*10^{-3})$	0.993
Mebendazole	10-300	$y=(111*10^{-5} \pm 3.05*10^{-5})*x + (11.8*10^{-3} \pm 3.98*10^{-3})$	0.993
Mefenamic acid	25-300	$y=(91.6*10^{-5} \pm 4.21*10^{-5})*x + (18.7*10^{-3} \pm 5.76*10^{-3})$	0.98
Meloxicam	10-300	$y=(90.5*10^{-5} \pm 1.36*10^{-5})*x - (5.39*10^{-3} \pm 1.78*10^{-3})$	0.998
Metformin	10-300	$y=(183*10^{-5} \pm 4.04*10^{-5})*x$	0.996
Metronidazole	10-150	$y=(32.1*10^{-5} \pm 1.06*10^{-5})*x$	0.991
Morantel	10-300	$y=(343*10^{-6} \pm 8.22*10^{-6})*x + (2.78*10^{-3} \pm 1.07*10^{-3})$	0.995
Niflumic acid	10-300	$y=(32.1*10^{-5} \pm 5.66*10^{-5})*x$	0.995
Oxfendazole	10-150	$y=(114*10^{-5} \pm 2.45*10^{-5})*x$	0.996
Paracetamol	10-150	$y=(71.5*10^{-5} \pm 1.59*10^{-5})*x + (5.98*10^{-3} \pm 1.57*10^{-3})$	0.996
Paroxetine	10-300	$y=(219*10^{-5} \pm 4.63*10^{-5})*x$	0.996
Primidone	10-150	$y=(215*10^{-6} \pm 4.50*10^{-6})*x + (12.9*10^{-4} \pm 4.44*10^{-4})$	0.997
Propranolol	10-150	$y=(313*10^{-5} \pm 5.18*10^{-5})*x$	0.998
Rafoxanide	10-300	$y=(248*10^{-5} \pm 5.40*10^{-5})*x + (17.5*10^{-3} \pm 7.05*10^{-3})$	0.996
Ronidazole	10-300	$y=(211*10^{-5} \pm 4.10*10^{-5})*x$	0.997
Saccharine	10-300	$y=(258*10^{-6} \pm 7.15*10^{-6})*x + (23.2*10^{-4} \pm 9.34*10^{-4})$	0.993
Salicylic acid	10-300	$y=(421*10^{-5} \pm 9.07*10^{-5})*x$	0.996
Simvastatin	10-150	$y=(58.3*10^{-5} \pm 1.91*10^{-5})*x$	0.992
Sucralose	10-300	$y=(109*10^{-6} \pm 3.04*10^{-6})*x$	0.993
Sulfachloropyridazine	10-150	$y=(76.6*10^{-5} \pm 1.44*10^{-5})*x$	0.997
Sulfaclozine	10-300	$y=(64.2*10^{-5} \pm 1.63*10^{-5})*x$	0.994
Sulfadiazine	10-300	$y=(271*10^{-5} \pm 2.54*10^{-5})*x$	0.9992
Sulfadimethoxine	10-300	$y=(329*10^{-5} \pm 4.78*10^{-5})*x$	0.998
Sulfadimidine	10-300	$y=(308*10^{-5} \pm 4.89*10^{-5})*x + (24.2*10^{-3} \pm 6.39*10^{-3})$	0.998
Sulfadoxine	10-150	$y=(46.2*10^{-4} \pm 1.12*10^{-4})*x + (3.67*10^{-2} \pm 1.10*10^{-2})$	0.995
Sulfamerazine	10-300	$y=(265*10^{-5} \pm 3.58*10^{-5})*x$	0.998
Sulfamethizole	10-300	$y=(94.4*10^{-5} \pm 2.89*10^{-5})*x$	0.992
Sulfamethoxazole	10-150	$y=(248*10^{-5} \pm 5.49*10^{-5})*x$	0.996
Sulfamethoxypyridazine	10-300	$y=(295*10^{-5} \pm 7.68*10^{-5})*x$	0.994
Sulfamonomethoxine	10-100	$y=(136*10^{-5} \pm 7.43*10^{-5})*x$	0.985
Sulfapyridine	10-300	$y=(37.4*10^{-4} \pm 1.03*10^{-4})*x$	0.993
Sulfaquinoxaline	10-300	$y=(92.8*10^{-5} \pm 2.22*10^{-5})*x$	0.995
Sulfathiazole	10-300	$y=(172*10^{-5} \pm 3.03*10^{-5})*x - (10.0*10^{-3} \pm 3.96*10^{-3})$	0.997

Compound	C_{range} (µg/L)	Calibration curve (Rel. areas)	R^2
Sulfisoxazole	10-150	$y=(177*10^{-5} \pm 5.22*10^{-5})*x + (18.3*10^{-3} \pm 4.77*10^{-3})$	0.994
Ternidazole	10-150	$y=(34.4*10^{-5} \pm 1.63*10^{-5})*x + (4.25*10^{-3} \pm 1.61*10^{-3})$	0.98
Theophylline	10-150	$y=(155*10^{-6} \pm 3.68*10^{-6})*x + (21.0*10^{-4} \pm 3.63*10^{-4})$	0.996
Thiabendazole	10-300	$y=(42.7*10^{-4} \pm 1.23*10^{-4})*x$	0.993
Thiamphenicol	10-300	$y=(104*10^{-5} \pm 2.43*10^{-5})*x + (9.00*10^{-3} \pm 3.18*10^{-3})$	0.995
Toltrazuril	10-300	$y=(348*10^{-6} \pm 7.44*10^{-6})*x$	0.996
Tramadol	10-150	$y=(74.5*10^{-4} \pm 2.19*10^{-4})*x + (6.09*10^{-2} \pm 2.16*10^{-2})$	0.993
Triamterene	10-150	$y=(246*10^{-5} \pm 6.54*10^{-5})*x + (20.5*10^{-3} \pm 6.46*10^{-3})$	0.994
Triclabendazole	10-150	$y=(82.9*10^{-5} \pm 1.24*10^{-5})*x - (5.08*10^{-3} \pm 1.62*10^{-3})$	0.998
Trimethoprim	10-150	$y=(319*10^{-5} \pm 8.39*10^{-5})*x + (27.7*10^{-3} \pm 8.29*10^{-3})$	0.995

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