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RAQUEL WIELENS BECKER

Analytical strategies for the screening of microcontaminants and transformation products in aquatic environments

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Porto Alegre, September 2021

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Analytical strategies for the screening of microcontaminants and transformation products in aquatic environments

Thesis presented as a partial requirement to
obtain the title of Doctor in Chemistry

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Porto Alegre, September 2021

Sejamos incontroláveis então… e que a gente não desista porque ninguém acredita!

Machado de Assis

I dedicate this work to all those who really
believe in science, humanity, and love.
Especially to my family, my
inexhaustible source of strength

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ABBREVIATIONS

AOPs - advanced oxidation process

DBE - double-bond equivalency

DLLME - dispersive liquid-liquid microextraction

DZP - diazepam

LC-HRMS - liquid chromatography coupled with high resolution mass spectrometry

MCDA - multi-criteria decision making analysis

MCDM - multi-criteria decision-making

PBT - persistence, bioaccumulation and toxicity

PEC - predicted environmental concentrations

PNEC - predicted no effect concentration

RHW - raw hospital wastewater

RQ - risk quotient

SMART simple multi-attribute rating technique

SPE - solid phase extraction

SPFP - solar photo-Fenton process

SW - simulate wastewater

TOPSIS - technique for order of preference by similarity to ideal solution

ToxPi - toxicological priority index

TPs - transformation products

UPW - ultrapure water

WWTPs - wastewater treatment plants

ABSTRACT

The characterization of anthropogenic contamination and understanding of the associated risks for humans and the environment is a challenge, since tens of thousands of compounds are constantly discharged into different environmental compartments. The hydrosphere has a very powerful potential to disseminate contaminants of emerging concern (CECs), which can then reach other compartments such as soil, plants, and sediments, so evaluation of its contamination is essential. The identification of CECs in aquatic systems is analytically difficult, since there is a need to achieve increasingly low detection limits (μg L^{-1} and ng L^{-1}) and cover the widest possible range of compounds. Expanding knowledge about aquatic contamination requires the use of sensitive methods that allow unequivocal identification of CECs, which may be achieved by methods using liquid or gas chromatography coupled with high resolution mass spectrometry. In addition, sensitive analytical methods should be associated with *in silico* prediction by (quantitative) structure-activity relationship ((Q)SAR) tools and multi-criteria decision analysis ranking methods, in order to not only obtain conclusions about contaminants present in the environment, but also to identify those of most concern. Considering these issues, the present thesis is divided into three chapters.

Chapter 1 describes an adapted analytical method for the identification of pharmaceuticals and metabolites in raw hospital wastewater, using three different identification strategies: i) for confirmed compounds (when analytical standards are available); ii) for suspect compounds (when analytical standards are not available); and iii) for metabolites by common fragmentation profile. The method employed a custom database containing up to 1380 compounds. Six samples collected monthly were analyzed by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QTOF MS). A total of 35 metabolites and 43 pharmaceuticals were identified. Risk assessment of the identified compounds was performed using *in silico* (Q)SAR prediction methods.

Chapter 2 presents a study of the degradation of diazepam (DZP), a pharmaceutical identified in all the samples analyzed, as described in Chapter 1, by solar photo-Fenton treatment, which is an advanced oxidation process (AOP). The identification of previously reported and new transformation products (TPs) formed during DZP degradation was performed by LC-QTOF MS analysis. In addition, a method

for the preconcentration of DZP and its TPs was developed, based on dispersive liquid-liquid microextraction (DLLME). The extraction method was fast, cheap, easy, and efficient. In the absence of this preconcentration step, it was not possible to identify one of the TPs formed during the solar photo-Fenton process. In this study, (Q)SAR tools were also used to predict some of the toxicological parameters of DZP and its TPs. These predictions showed mutagenicity alerts for two TPs, reflecting their higher toxicity, compared to DZP itself.

Chapter 3 describes a more embracing approach. Surface water analysis was carried out by LC-QTOF MS, with application of a screening methodology using a database containing information about 3250 compounds belonging to different CEC classes. After LC-QTOF MS screening analyses of 27 river samples, it was possible to identify 150 compounds (133 compounds as suspects, and 17 compounds as confirmed). *In silico* predictions for the identified compounds were performed using (Q)SAR tools, providing information about eight different selected endpoints. The great number of compounds and predicted endpoints hindered the general evaluation of toxicity. Therefore, in order to obtain a better understanding of the risk of each identified compound, two different multi-criteria decision analysis ranking methods (toxicological priority index (ToxPi) and technique for order of preference by similarity to ideal solution (TOPSIS)) were used, considering a different weight for each endpoint. After ranking, the ToxPi and TOPSIS results were evaluated and showed similarity for the first 20 priority compounds. TOPSIS showed high robustness in sensitivity tests, indicating its suitability as an appropriate tool for use in association with screening results, which could support quantitative analytical methods performed subsequently.

Throughout the different studies, it was possible to propose strategies for identification, degradation, extraction, toxicity evaluation, and ranking of microcontaminants present in aquatic environments. It was possible to obtain new results never previously reported, highlighting the contribution and importance of the study for research concerning contamination of the aquatic environment and possible treatment methods.

Keywords: Screening analysis, high resolution mass spectrometry, transformation products, *in silico* (Q)SAR predictions, multi-criteria decision-making methods.

RESUMO

A caracterização e compreensão da contaminação antropogênica e dos seus riscos para o homem e o meio ambiente é um desafio, uma vez que dezenas de milhares de compostos são constantemente despejados em diferentes compartimentos ambientais. A hidrosfera tem potencial muito poderoso para disseminar contaminantes de preocupação emergente (CECs), os quais podem atingir outros compartimentos como solo, plantas e sedimentos. Portanto, a avaliação de sua contaminação é essencial. A identificação de CECs em sistemas aquáticos é analiticamente complexa, sendo necessário atingir limites de detecção cada vez mais baixos (μg L^{-1} e ng L^{-1}) e abranger a maior gama possível de compostos. Tal necessidade requer o uso de métodos sensíveis que permitem a identificação inequívoca de CECs e, para isso, uma possibilidade é o uso da cromatografia líquida ou a gás associada a espectrometria de massa de alta resolução. Além disso, os métodos analíticos podem ser associados a predições *in silico* por métodos de relações quantitativas entre a estrutura e atividade ((Q)SAR) e métodos de tomada de decisão multicritério, a fim de não apenas obter conclusões sobre os contaminantes presentes no ambiente, mas também para identificar aqueles que merecem maior atenção. Considerando essas questões, a presente tese está dividida em três capítulos.

O Capítulo 1 descreve um método analítico para a identificação de fármacos e metabólitos em efluente hospitalar bruto, usando três estratégias de identificação: i) compostos confirmados (quando padrões analíticos estão disponíveis); ii) para compostos suspeitos (quando padrões analíticos não estão disponíveis); e iii) para metabólitos com perfil de fragmentação comum. O método empregou uma base de dados personalizada contendo 1380 compostos. Seis amostras coletadas mensalmente foram analisadas por cromatografia líquida acoplada à espectrometria de massa por tempo de vôo (LC-QTOF MS). Um total de 35 metabólitos e 43 fármacos foram identificados. A avaliação de risco dos compostos identificados foi realizada usando métodos de predição *in silico* (Q)SAR.

O Capítulo 2 apresenta um estudo da degradação do diazepam (DZP), fármaco identificado em todas as amostras analisadas no Capítulo 1, através do processo de foto-Fenton solar, que é um processo avançado de oxidação (AOP). A identificação de produtos de transformação (TPs) formados durante a degradação do DZP foi realizada pela análise em um sistema LC-QTOF MS. Além disso, um método para a pré-

concentração de DZP e seus TPs foi desenvolvido, baseado em microextração líquido-líquido dispersiva (DLLME). O método de extração proposto é rápido, barato, fácil e eficiente. Na ausência desta etapa de pré-concentração, não foi possível identificar um dos TPs formados durante o processo de foto-Fenton solar. Neste estudo, métodos (Q)SAR também foram usados para predizer alguns dos parâmetros toxicológicos do DZP e seus TPs. Essas predições mostraram alertas de mutagenicidade para dois TPs, refletindo sua maior toxicidade, em comparação com o próprio DZP.

O Capítulo 3 descreve uma abordagem mais abrangente. Análise de águas superficiais, realizada por LC-QTOF MS, com aplicação de uma metodologia de *screening* utilizando bases de dados contendo informações sobre 3250 compostos pertencentes a diferentes classes de CEC. Após análise *screening* de 27 amostras de rios, foi possível identificar 150 compostos (133 compostos suspeitos e 17 compostos confirmados). As predições *in silico* dos compostos identificados foram realizadas usando métodos (Q)SAR, para oito variáveis selecionadas. O grande número de compostos e as diferentes variáreis preditas dificultaram a avaliação geral da toxicidade. Portanto, a fim de obter uma melhor compreensão do risco de cada composto identificado, foram utilizados dois métodos de tomada de decisão multicritério (*toxicological priority index* (ToxPi) e *technique for order of preference by similarity to ideal solution* (TOPSIS)), considerando diferentes pesos para cada uma das variáveis. Após a classificação, os resultados de ToxPi e TOPSIS foram avaliados e mostraram similaridade para os 20 compostos mais preocupantes. O TOPSIS mostrou alta robustez em testes de sensibilidade, indicando ser uma ferramenta apropriada para uso em associação com resultados de análise *screening*, o que pode apoiar e direcionar o desenvolvimento de métodos analíticos quantitativos como segunda etapa.

Ao longo dos diferentes estudos, foi possível propor estratégias de identificação, degradação, extração, avaliação de toxicidade e classificação de microcontaminantes presentes em ambientes aquáticos. Foi possível obter novos resultados nunca antes reportados, evidenciando a contribuição e importância do estudo para a pesquisa sobre contaminação do meio aquático e possíveis métodos de tratamento.

Palavras-chave: Análise *screening*, espectrometria de massa de alta resolução, produtos de transformação, predições *in silico* (Q)SAR, métodos de tomada de decisão multicritério.

INTRODUCTION

The world's population has grown significantly in recent decades, from 2.5 billion inhabitants in 1950 to 7.8 billion in April 2021, with projections of 9 billion in 2050, according to the United States Census Bureau [1]. Concomitantly, the high demands of mostly urbanized modern society are driving significant increases in environmental stress, associated with the indiscriminate exploitation of natural resources. This exploitation generates massive amounts of waste and is responsible for the release of thousands of anthropogenic substances into the environment [2,3].

Considering natural resources, water is indispensable for the existence and maintenance of life, so action to ensure its quality is essential. The main way to assess water quality is to investigate the presence of a wide range of contaminants in the aquatic environment, including study of the occurrence of micropollutants in different aqueous matrices. Many microcontaminants, known as contaminants of emerging concern (CECs), are compounds that are widely used, but are mostly not covered by legislation. They can be found in aqueous matrices and represent still unknown risks to humans and to the environment [4]. CECs belong to different classes of compounds, such as pharmaceuticals, pesticides, plasticizers, flame retardants, illicit drugs, and hormones, in addition to metabolites and transformation products of these substances [5].

Considering the different classes of CECs, it is very important to define two classifications that can sometimes lead to misunderstanding: "metabolites" and "transformation products". "Metabolite" is a definition that should be used for compounds transformed within or on the human body, animal bodies, and plants. Compounds generated by modifications occurring outside these organisms, such as those produced by bacteria and fungi in the environment, by chemical reactions in sewage and drinking water treatment plants, and by photodegradation, hydrolysis, or oxidation, should be defined as "transformation products" (TPs) [6]. A scheme illustrating the transformations and the proper definitions is presented in Figure 1.

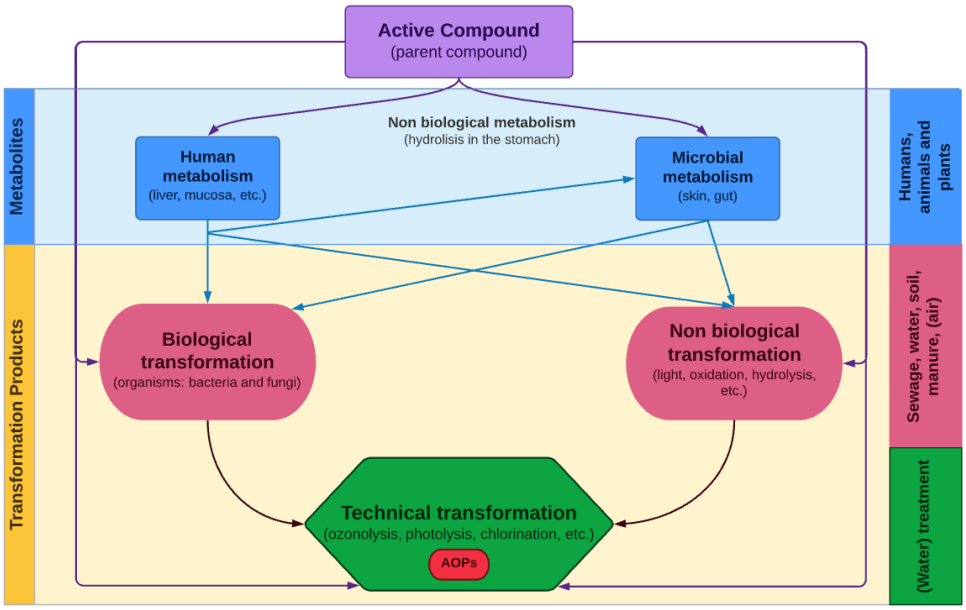

Figure 1. Differences between metabolites and transformation products. Adapted from Kümmerer, 2009 [6].

Different CECs are present in the aquatic environment at trace and ultra-trace levels (µg L^{-1} and ng L^{-1}) [7], originating from a variety of sources including agricultural and livestock activities, industrial effluents, leachates from landfills, and hospital and domestic wastewaters [8]. In order to address this problem, the implementation of environmental monitoring programs has been considered imperative in recent years, representing the first step of actions aiming at the control of environmental pollution. The scientific literature contains many studies carried out to identify aquatic contamination and to try to understand its dynamics and the different routes by which microcontaminants can reach aqueous media including drinking water [9–12], surface water [13–17], groundwater [18–20], and effluents [4,5,13,16].

To carry out the monitoring of microcontaminants, the development of appropriate methods for multi-residue analysis is essential. In order to achieve adequate sensitivity, an efficient first step of extraction/preconcentration is necessary, such as using solid phase extraction (SPE). SPE is a technique that can provide high analyte recovery rates, high robustness, and high sample concentration factors, as well as the availability of a wide range of commercially available sorbents [21–24]. Besides SPE, extraction methodologies based on dispersive liquid-liquid microextraction (DLLME) offer the advantages of being extremely fast and simple, using only a few microliters of solvents. They are less expensive than SPE and can be applied to different organic compounds in

a wide variety of matrices [25]. However, DLLME is less sensitive than SPE [26], achieving lower sample concentration factors.

After the extraction/preconcentration step, the samples must be analyzed using specific and sensitive analytical techniques capable of detecting the analytes of interest (the microcontaminants), in the presence of many impurities. With technological advances, it has become possible to monitor microcontaminants present at very low concentrations. The use of liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) allows the separation, detection, identification, and quantification of a wide range of pollutants at very low concentration levels [27,28]. In this case, the detection and identification of CECs is achieved using information contained in mass spectrum data, such as the exact masses of the molecular ion and characteristic fragments, isotopic pattern, and double-bond equivalency (DBE) [29,30]. This type of methodology enables target and nontarget analyses, as well as the screening of suspect compounds, without the need for analytical standards. Such strategies have been applied in several studies [31,32] and are very well accepted by the scientific community. Another advantage is the possibility of retrospective data processing, without any need for additional analyses [33].

In the monitoring and analysis of microcontaminants, the use of analytical standards can assist in identification of the analyte, since in addition to the fragmentation profile of the compound (using at least two characteristic fragments), information regarding its retention time can assist in confirming its identify, when detected in a sample [23]. However, in many cases, analytical standards may be difficult to obtain or be unavailable (especially for TPs). In addition, the development of quantitative methodologies is expensive and time-consuming, especially if qualitative analyses (screening) have not been performed previously. Therefore, it is necessary to develop new strategies capable of circumventing these limitations. One strategy that can be used is to perform initial qualitative analysis, using HRMS combined with customized databases that usually contain information for a few thousand compounds [34–38]. From the results obtained with the qualitative method, it is possible to develop a quantitative method that is more focused, saving time and economic resources. A wide-ranging custom database can be constructed by collecting information about the chemical formulas and exact masses of compounds and their characteristic fragments, available in free access databases and in the scientific literature. It should be noted that a customized database is

a dynamic tool that must be constantly updated whenever information about new compounds and their fragments becomes available [33].

Many microcontaminants present in effluents pass through treatment plants, where they are not completely removed by conventional biological processes [39], so they are consequently released into the environment. Hence, there is a need for additional treatment processes capable of maximizing the removal of recalcitrant microcontaminants such as CECs, before the treated effluent is discharged to the environment. The development of methodologies based on AOPs to degrade many organic compounds is becoming increasingly common [40]. The efficiency of AOPs is due to the formation of hydroxyl radicals (HO•), which have high oxidizing power at atmospheric pressure and ambient temperature, but do not have selectivity [41]. Various AOPs have been applied for the degradation of CECs [42]. In a more environmentally friendly approach, the solar photo-Fenton process (SPFP) offers a very efficient alternative, because it uses solar radiation (UV) to form hydroxyl radicals, instead of the artificial radiation used in other processes [41,43]. However, when AOPs are used to degrade CECs, besides the original compound, it is necessary to identify and monitor the TPs generated during the treatment process, since the TPs can sometimes be more toxic and persistent than the parent compound [44,45].

After screening analyses, the use of *in silico* prediction provides a valuable tool to evaluate the toxicological parameters of the screened compounds and TPs, guiding the development of quantitative methodologies and improving risk assessment of the quantified compounds. Undertaking experimental studies to assess the risk of each compound requires a lot of time, considerable financial resources, and a large number of animal tests. An approach that circumvents all of these issues is *in silico* prediction based on (quantitative) structure-activity relationship ((Q)SAR) models [46]. These are computational mathematical modeling methods that are robust, economical, and applicable for use in risk assessment and toxicity prediction [47]. The predictions are based on the assumption that the chemical structure of a molecule contains characteristics responsible for its physical, chemical, and biological properties. Consequently, the activity of a compound can be determined using numerical descriptors, and mathematical predictions can be used for the activities of structurally similar compounds [47–49].

The results obtained with (Q)SAR model predictions can be very useful. However, when dozens of compounds are evaluated and many endpoints are predicted, the amount of data produced can make it difficult to identify the compounds that could present the

greatest risk to the environment. In this situation, the use of multi-criteria decision-making analysis (MCDA) can provide safer evaluation, considering the different endpoints predicted by the (Q)SAR tools [50]. Examples of MCDA ranking methods are ToxPi and TOPSIS. These two different techniques are able to rank the compounds considering different endpoints, which can have different weights, in order to determine the environmental risk scores. ToxPi was proposed by Reif et al. [51] as a dimensionless score index that allows the integration of several endpoints to provide a classification, considering the weighted average among all endpoints. TOPSIS is a ranking method based on the Euclidean distance between a "positive ideal" solution and a "negative ideal" solution, with the best alternative being the one presenting the closest proximity to the ideal positive alternative and the greatest possible distance from the ideal negative alternative [50].

Chapter 1

Investigation of pharmaceuticals and their metabolites in Brazilian hospital wastewater by LC-QTOF MS screening combined with a preliminary exposure and *in silico* risk assessment

Introduction

Pharmaceuticals are biologically active compounds that after administration follow different routes in the human body, until being excreted either as metabolites or in unchanged form. Until a few decades ago, very little attention was given to pharmaceuticals discharged into the environment, and even less to their metabolites [52]. For example, the first published study concerning the harm caused by pharmaceuticals in the environment dates from 2004 [53]. Pharmaceuticals are a very important class of compounds that contribute significantly to human health and life, but the damage caused by them and their metabolites in the environment is still not completely understood [54].

A large number of studies have focused on quantitative screening of pharmaceuticals in different aquatic environmental matrices [55–60], but fewer works have investigated the presence of their metabolites in the same matrices [61–64]. Studies concerning the presence of these compounds are essential as a first step, prior to the introduction of appropriate legislation. As an example, in the European Union, there is a "watch list" of priority substances, implemented to facilitate assessment of the risks presented by chemicals found in surface waters. In this program, the member states should monitor the substances present in the list, at least once per year, for up to four years, in order to improve the information available for identifying the substances of greatest concern. In the last update by the European Commission, in 2020, besides pharmaceuticals, the metabolite o-desmethylvenlafaxine [65] was included in the "watch list". This was a very important indication that not only the active compounds, but also their metabolites, require attention concerning the possible harm caused by them to humans and the environment.

Objectives

This study concerns a screening analysis of pharmaceuticals and their metabolites by LC-QTOF MS, using different strategies for identification of the compounds: i) compounds identified as "confirmed", when analytical standards were available; ii) compounds screening as "suspect", when analytical standards were not available, but there was identification of the molecular ion and at least two characteristic fragments with

m/z errors below 5 ppm; and iii) evaluation of the presence of non-expected/non-related metabolites, using a "common fragmentation" strategy.

Besides screening, this study evaluates the risk assessment of pharmaceuticals and metabolites using *in silico* predictions employing various (Q)SAR models.

Main results

The results showed that the applied screening strategy was very helpful for understanding the composition of raw hospital wastewater (RHW). The extended database, which included 1111 pharmaceuticals and 272 metabolites, enabled the identification of 43 pharmaceuticals and 31 metabolites in the samples analyzed. In addition, 4 metabolites were identified using the "common fragmentation" method. The strategy adopted, consisting of an initial qualitative analysis with broad scope, was important as a first step, prior to performing quantitative analysis in a more efficient way [66].

Due to their physicochemical properties, pharmaceuticals and their metabolites are incompletely removed in conventional wastewater treatment plants [67], resulting in their release into the environment. This is of great concern, especially in countries such as Brazil, where aggravating factors are that only 43% of the population has access to sewage collection and treatment [68], and RHW may often be discharged into the environment without any prior treatment. In the case of the RHW studied here, it did not receive any treatment before being discharged together with domestic effluent. In the study city, only 80% of wastewater is treated [69], so it is possible for untreated RHW to enter the environment, increasing the potential for contamination by a very large number of pharmaceuticals and metabolites. This situation provides further justification of the need to study the composition of this effluent and perform risk assessment. Even when effluents are treated, the removal of pharmaceuticals and metabolites in conventional wastewater treatment plants depends on the physicochemical properties of the compounds, as well as the treatment conditions (considering the wastewater composition, temperature, and solids retention time, among other factors), which can influence the efficiency of the process [70].

Metabolites can represent a very important part of pharmacological contamination in effluents and other matrices of environmental relevance, for several reasons: i) Some

pharmaceuticals may not be found in the effluents or in the environment in their original forms, because during the metabolization process they are transformed into more stable forms (metabolites), which should therefore be the structures monitored [71]; ii) Some studies have reported an increase in the average concentration of pharmaceuticals in effluents (output), relative to influents (input). This can be explained by the fact that some compounds may be excreted conjugated with glucuronic acid or other polar groups, with the conjugated metabolite subsequently being cleaved by microorganisms, consequently returning to the unchanged (active) form of the pharmaceutical [71]; iii) There are some pharmaceuticals that only become bioactive after transformation of the parent compounds into metabolites [72]. For these three reasons, it is clear that screening of metabolites needs to be carried out concomitantly with screening of drugs in environmental matrices, because both the pharmaceuticals and the metabolites can present substantial risks to human health and the environment.

This study demonstrates the effectiveness of a metabolite monitoring strategy complemented by *in silico* (Q)SAR prediction, an excellent tool for assessment of the potential environmental risks of contaminants, especially in the aquatic environment. The results indicate the importance of developing studies of this type, in order to understand the composition of effluents and enable the implementation of appropriate control measures. The strategy enables identification of the most concerning compounds present in aquatic matrices, contributing to the development of robust and effective monitoring methods that consider both pharmaceuticals and their metabolites in effluents and other environmental matrices. This can then enable the proposal of efficient new advanced treatment processes for the removal of metabolites from effluents.

All the methods, results, and conclusions are presented in **Paper I** and **Supplementary Material I**.

PAPER I

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Investigation of pharmaceuticals and their metabolites in Brazilian hospital wastewater by LC-QTOF MS screening combined with a preliminary exposure and *in silico* risk assessment

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highlights

- Wide-scope screening for investigation of drugs and metabolites in hospital wastewater
- Home-made database with >1000 pharmaceuticals and 250 metabolites
- Additional metabolites found sharing common fragments with the parent compound.
- Identification of 43 pharmaceuticals and 31 metabolites in raw hospital wastewater
- Proactive risk assessment based on exposure and *in silico* QSAR tools.

graphical abstract

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abstract

This work evaluates the occurrence of pharmaceuticals, with special emphasis on their metabolites, in raw hospital wastewater (HWW) using wide-scope screening based on liquid chromatography coupled to high resolution mass spectrometry. The applied strategy uses an extended purpose-built database, containing >1000 pharmaceuticals and 250 metabolites. Raw HWW samples from a hospital located in south Brazil were collected over six months, with a monthly sampling frequency. Accurate-mass full-spectrum data provided by quadrupole-time of flight MS allowed the identification of 43 pharmaceuticals and up to 31 metabolites in the samples under study. Additionally, other four metabolites not included in the initial database could be identified using a complementary strategy based on the common fragmentation pathway between the parent compound and its metabolites. Nine metabolites derived from four pharmaceuticals were identified in the raw HWW samples, whereas their parent compounds were not found in these samples. The results of this work illustrate the importance of including not only parent pharmaceuticals but also their main metabolites in screening analysis. Besides, the inclusion of *in silico* QSAR predictions allowed assessing the environmental fate and effect of pharmaceuticals and metabolites in terms of biodegradability, as possible Persistent, Bioaccumulative and Toxic (PBT) compounds, and their potential hazard to the aquatic environment.

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1. Introduction

Nowadays, multiple studies have shown that the main route of entry of compounds with pharmacological activity into the environment occurs through urban and hospital wastewater (HWW) (Comber et al., 2018; Hernández et al., 2019a, 2019b; Nguyen et al., 2018). Hospitals, clinics and emergency rooms deserve spe- cial attention because the continuous disposal of pharmaceuticals derived from the innumerable activities carried out within these establishments (anaesthesia, cancer treatment, diagnosis, analgesia, *etc.*) (Frédéric and Yves, 2014).

Advances in analytical chemistry have allowed the monitoring of a large variety of emerging contaminants in water at very low concentrations (sub-ppb levels). As a result, pharmaceutical compounds such as analgesics, antibiotics, anti-inflammatories and contrast media have frequently been detected in the aquatic environment (Al-Qaim et al., 2014; Botero-Coy et al., 2018; Fatta-Kassinos et al., 2011; Gracia-Lor et al., 2012; Olalla et al., 2018; Pérez-Alvarez et al., 2018).

Pharmaceuticals are excreted through the faeces or urine (Gaso-Sokac et al., 2017), after total or partial metabolism, with the consequent impact on the aquatic environment (Han and Lee, 2017). A rapid search in the literature reveals many studies about pharmaceuticals screening in the aquatic environment in the last years around the world (Pérez-Alvarez et al., 2018; Singer et al., 2016; Tewari et al., 2013). However, the number of studies reporting the screening and identification of human metabolites in wastewateris less significant (Aceña et al., 2016; Asghar et al., 2018; Brown and Wong, 2018; Evgenidou et al., 2015; Gracia-Lor et al., 2014; Kovalova et al., 2013).

The mechanisms involved in drugs metabolism in humans mainly correspond to oxidations, reductions and hydrolysis (phase I metabolism), or to the transference of a polar group to the pharmaceuticals basic structure forming conjugates such as glucuronides or sulfates (phase II metabolism) (Frédéric and Yves, 2014). In some cases, the drugs only become bioactive after the transformation of the parent compound into metabolites (Mompelat et al., 2009). Several factors such as age, gender, ethnicity or the time of administration may influence the pharmacokinetics of these compounds (Chikhani and Hardman, 2016). Commonly, with greater polarity (mobility) and stability, the metabolites may still contain structural similarities to the parent compounds and present pharmacophore groups attaining similar (or unknown) activity, being hazardous to the environment from an (eco)toxicological point of view (Trontelj, 2012).

According to Santos et al. (2013), concentrations higher than tens ng L^{-1}, for some substances, might result in relevant ecotoxicological impact, presenting a risk to aquatic organisms. For these reasons, the implementation of tests for identifying the potential toxicity of different drugs, as well as of their metabolites and transformation products, in different trophic levels has gained more and more attention in scientific community (Cruz-morató et al., 2014; Li et al., 2014). According to Al Aukidy et al. (2014) the degree of risk of a compound present in wastewater is site-specific, and depends on a combination of several factors: (i) compound concen-tration and toxicity, (ii) compound removal efficiency in the wastewater treatment plant (WWTP), and (iii) the dilution factor. Considering the expected high concentrations of pharmaceuticals in raw HWW released from hospitals and health care centers, a comprehensive insight in monitoring and, therefore, a more realis-tic knowledge on the occurrence of these compounds in this type of samples, is essential to contribute to an adequate selection of treatment processes for the total or partial elimination of the recal-citrant or more toxic compounds.

The presence of metabolites in the aquatic environment is a fact that needs to be studied in more depth. Assessing only the parent compounds in the aquatic environment may not be sufficient for a proactive risk assessment. The first step for evaluating the presence of metabolites in environmental samples is their reliable identification. This is an analytical challenge due to the huge number of compounds that may be present, many of them still unknown or nor sufficiently documented. They are commonly present at low concentrations in highly complex matrices, and reference standards are not available for many of them (Camacho-Muñoz and Kasprzyk-Hordern, 2015). Thus, the screening of these compoundsin hospital wastewater demands powerful techniques for their detection and reliable identification. The use of liquid chromatogra- phy coupled to mass spectrometry (LC-MS) allows the detection, identification and quantification of a wide range of organic pollu- tants at the low concentration levels required (Mosekiemang et al., 2019; Wang et al., 2017). When dealing with emerging contami- nants, there is a clear trend towards the use of high resolution MS (HRMS), which allows wide-scope screening of a large number of compounds thanks to the valuable information contained in accurate-mass full-spectrum data (Hernández et al., 2019a, 2019b; Singer et al., 2016). With the same instrument, target, suspect and non-target analyses can be performed, as well as retrospective data treatment at any time, without the need of additional analysis or ref- erence standards (Partridge et al., 2018). Thus, the identification ofpharmaceuticals metabolites and TPs has been efficiently made by retrospective analysis of QTOF MS data in wastewater samples (Hernández et al., 2014; Ibáñez et al., 2017).

In addition to the concentration data reported, the risk assessment of pharmaceuticals and metabolites in the aquatic environment should be further investigated. For this purpose, predicted environmental concentrations and *in silico* (quantitative) structure-activity relationships ((Q)SAR) predictions are useful tools (Mansour et al., 2016; Roos et al., 2012; Thomas et al., 2019).

Only few studies have reported the contribution of wastewater from a large health care institution to the environment contamination in developing countries (Botero-Coy et al., 2018; Diwan et al., 2009; Mansour et al., 2016; Martins et al., 2008; Wilde et al., 2012). This is an issue of concern because raw wastewater is sometimes directly discharged to the aquatic environment, without any treatment. Therefore, negative environmental impact can be expected due to the presence of pharmaceuticals, among other compounds. In the present work, an analytical strategy for wide-scope screening of a large number of pharmaceuticals and metabolites in raw HWW has been applied based on the use of LC-QTOF MS. A target screening for compounds which reference standards were available, together with a suspect screening using a purpose-built database containing around 1000 pharmaceuticals and 250 metabolites, has been applied for both detection and identification of the compounds present in raw HWW. An investigation of non-expected/non reported metabolites has been also performed by searching for common fragments shared with the parent compound. Finally, a preliminary risk assessment of the pharmaceutical human metabolites was carried out by means of *in silico* quantitative structure-activity relationship (QSAR).

2. Experimental

2.1. Chemicals

HPLC grade methanol (MeOH) was purchased from Scharlab (Barcelona, Spain) and formic acid (purity 98%) was provided by Merck (Darmstadt, Germany). All analytical pharmaceutical stan-

dards used were purchased from different providers with purity >98.99% and used as received.

For QTOF MS analysis, HPLC-grade water was obtained from a Milli-Q water purification system (Millipore Ltd., Bedford, MA, USA). HPLC-grade MeOH and sodium hydroxide (>99%) were obtained from Scharlab (Barcelona, Spain). Leucine-enkephalin and formic acid were purchased from Sigma-Aldrich.

2.2. Hospital wastewater and study site characterization

The studied hospital is considered as a large health center in Brazil. It has 128,000 m^2 of built area with 699 inpatient units, 87 units of intensive care treatment, 47 emergency rooms and 188 outpatient rooms. Besides the metropolitan population (~4.3 million), the investigated hospital is responsible for treating patients from all over the state of Rio Grande do Sul, and therefore predicting its entire coverage population is quite difficult. According to the data provided by the hospital, along 2018, 31,288 patients were hospitalized and >569,000 people had medical appointments. Additionally, >3 million clinical diagnostic tests, 47,546 surgical procedures, 3515 births and 425 transplants were carried out in this institution.

Six raw HWW samples were collected monthly from February to July 2017, in a hospital located in the city of Porto Alegre (in south Brazil). The raw HWW was collected periodically in the earlyhours of the morning (8:00–9:00 am).This raw HWW is directly discarded into the public sewage system to be subsequently trea- ted in municipal wastewater treatment plants. The main physicochemical parameters monitored in raw HWW samples collected were: pH, conductivity, biochemical oxygen demand (BOD), dissolved organic carbon (DOC), chemical oxygen demand (COD), BOD/COD ratio, phosphate and chloride concentrations, total suspended solids (TSS) and total solids (TS) (see Table S1 in Supplementary Material). All methods employed to characterise this wastewater matrix were taken from Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1998).

2.3. Sample preparation and instrumentation

Raw HWW samples were collected in suitable glass bottles and immediately stored in refrigerated thermal boxes during transport from the study site to the laboratory. Upon arrival in the laboratory, the samples were filtered through cellulose acetate membranes of two different porosities (1 μm and 0.45 μm) and stored at —20 °C. Solid phase extraction (SPE) was performed according to Hernández et al. (2015). Instrumentation used in sample analysis was based on LC-QTOF MS. More details about sample preparation and instrumentation are shown on Section S2 (Supplementary Material).

2.4. General strategy

The identification of metabolites and pharmaceuticals parent compounds was performed by using the customised database associated to commercial software, which extracted from the raw data the potential compounds (pharmaceuticals and metabolites) and their fragments, when known. This approach has been successfully employed by Ibáñez et al. (2017) for the screening of a large number of compounds. The initial database was supplemented with 118 pharmaceuticals routinely used in the studied hospital and which had not been included in the original database. For each pharmaceutical, the main metabolites, when information was available, were also included. Large free-search databases, such as METLIN (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage), or scientific literature were checked. When characteristic fragments of pharmaceuticals and/or of metabolites were found, they were also added to the database to facilitate faster and more reliable identification of the compounds.

The extended database of pharmaceuticals (1111 compounds) and metabolites (272) employed in this study contained: i) 202 compounds for which the analytical reference standard was available at the laboratory, *i.e.* the retention time was known and there was information on empirical fragment ions (target screening); ii) 107 compounds for which the reference standard was not available at the laboratory, but whose fragmentation profiles were known (suspect screening); iii) 1074 compounds for which only the elemental composition was known (*i.e.* the theoretical exact mass). In this case, for potential positives in samples it was necessary to consider the information about molecular ion (generally in the low energy function (LE)) as well about the fragments (typically observed in high energy function (HE)), and to evaluate whether the potential fragments were consistent with the chemical struc- ture of the compound (suspect screening). It should be noted that for all compounds, the presence of a characteristic isotopic pattern (mainly due to the presence of a chlorine or bromine atom in the molecule), was also taken into account.

In a final step, the presence of additional chromatographic peaks (usually in the HE acquisition) in the narrow-window extracted ion chromatograms (nw-XICs) at m/z fragments corresponding to the parent compound was also evaluated in order to investigate the presence of potential metabolites not included in the database. This strategy, which assumes that many metabolites/TPs share the fragmentation pathway with the parent compound (Ibáñez et al., 2016, 2017), was applied for all pharmaceuticals detected in the raw HWW samples analysed.

In the present work, metabolites and parent compounds found in the samples were divided into two groups: group 'c', which stands for 'confirmed', *i.e.* compounds which were confirmed by retention time, and at least one fragment ion in addition to the (de)protonated molecule (*i.e.* compounds for which the analytical reference standard was available at laboratory); group 's', which stands for 'suspect', *i.e.* compounds tentatively identified by the presence of two or more plausible fragments, but for which no ref- erence standard was available.

2.5. Prediction of the environmental concentrations in the raw hospital wastewater

A worst-case scenario was used for the PEC calculations in the influent wastewater of the studied hospital. It is important to note that the influent samples studied did not undergo any treatment prior to analysis, fact that can justified high PEC values (μg L^{-1}). The following premises were considered: (i) the amount of pharmaceuticals used in the hospital considering the maximum influxes; (ii) the maximum fraction of the pharmaceuticals excreted unchanged were released through the sewer system; (iii) a negligible elimination in the sewer system, and (iv) the pattern of use was evenly temporally and spatially distributed (Wilde et al., 2012). The water consumption, according data provided by the hospital management, reaches 25,000 m^3 per month. The PEC of the studied pharmaceuticals was calculated by the Eq. (1) (Escher et al., 2011).

$$PEC_{RHWW} = \frac{A \times E \times 10^6}{365 \times V_{HWW}} \quad (1)$$

where: PEC_{HWW} is the predicted concentration in the raw HWW (μg L^{-1}). A is the amount of pharmaceutical used per year (g yr^{-1}). E is the excretion factor of the unchanged pharmaceutical (%). V is the volume of wastewater generated per day (L day^{-1}). 365 is the number of days in a year, *i.e.* a daily release is considered, and 10^6

is the conversion factor from g L^{-1} to lg L^{-1}. The same approach was used to calculate the PEC for the metabolites.

The estimation of the Predicted No Effect Concentration (PNEC) was based on toxicity screening by means of Quantitative Structure Activity Relationship (QSAR) obtained by the software US EPA ECOSAR V2.0 (ECOSAR, 2012). It was considered the lowest predicted value of LC/LD/EC_{50} for each pharmaceutical and metabolite calculated according to Eq. (2) (Mansour et al., 2016). For the PNEC calculation, three different ecotoxicological endpoints were considered, such as Green algae, Daphnid and Fish.

$$PNEC = \frac{ECOSAREcotoxicity_{Greenalgae=daphnid=fish}}{1000} \quad (2)$$

The theoretical risk quotient (RQ) of each pharmaceutical released in the health institution influent wastewater was calculated according to Eq. (3), whereas the RQ of the mixture of pharmaceuticals was accounted by the model of addition toxicity according to Eq. (4) (Escher et al., 2011).

$$RQ = \frac{PEC}{PNEC} \quad (3)$$

$$RQ_{mixt.} = \sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} \quad (4)$$

2.6. In silico quantitative structure-activity relationship (QSAR) models used for the predictions and risk assessment

The structure of the pharmaceuticals and metabolites were transformed into SMILES strings by means of ChemBioDraw Ultra (v.12) and subjected to *in silico* predictions by different software and models. The log K_{OW} and log BCF values were predicted by EPI Suite™ KOWWIN v1.68 and BCFBAF v3.01 programs, respec- tively. The persistence and biodegradability was predicted by EPI Suite™ BIOWIN v4.10. The predicted ready biodegradability of the combined BIOWIN 1–7 models (Boethling and Costanza, 2010; Pavan and Worth, 2008)is pointed out as "yes" or "no" based on the following criteria: "yes" if the BIOWIN 3 result is "weeks","days" or "days to weeks" and in the case of BIOWIN 5 the probability is ≥0.5. If these criteria were not met the prediction is "no" (US EPA, 2012). The Prometheus software (Pizzo et al., 2016) was used for predicting and ranking the pharmaceuticals/metabolites depending on its persistence (P), bioaccumulation (B), and toxicity (T), *i.e.* PBT compounds, for the hazard assessment according to Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (REACH Commission regulation, 2011). Moreover, the mutagenicity activity was predicted by means of VEGA QSAR v.1.1.4 software (Benfenati et al., 2013).A consensus approach was used by applying the Mutagenicity (Ames test) CONSENSUS model (v1.0.2). It performs an analysis among the CAESAR model (v.2.1.13), ISS model (v.1.0.2), KNN/Read-Across model (v.1.0.0), and SarPy/IRFMN model (v.1.0.7) to predict the mutagenicity bya consensus score from 0 to 1 (as the consensus score approach to 1 more reliable is the prediction). Concerning carcinogenicity activity, VEGA QSAR software (Benfenati et al., 2013) was applied for the predictions. It provides the results in four different models for carcinogenicity: (i) Carcinogenicity model (IRFMN/Antares) (version 1.0.0), (ii) Carcinogenicity model (CAESAR) (version 2.1.9), (iii) Carcinogenicity model (ISS) (version 1.0.2), and (iii) Carcinogenicity model (IRFMN/ISSCAN-CGX) (version 1.0.0). In general, VEGA QSAR provides an applicability domain index (ADI). If the ADI is lower than 0.75, it might indicate that there are differences in the target compound compared to the similar one found in the database.

3. Results and discussion

3.1. Pharmaceuticals parent compounds screening

Up to 43 pharmaceutical parent compounds were identified in the raw HWW (Table S2 in Supplementary Material), corresponding to different therapeutic groups. Caffeine, a natural alkaloid present in coffee and other beverages, products containing cocoa or chocolate, dietary supplements, and in some medications, such as some analgesic formulations (Gracia-Lor et al., 2017), was also found. In addition to caffeine, acetaminophen, atenolol, codeine, diazepam, metoclopramide and trimethoprim were observed in all the 6 samplings. Metronidazole was identified in five out of six samples, and clindamycin, metformin, fluconazole, lidocaine, metoprolol and tramadol were found in four of the collected samples.

It is worth noticing that the group of pharmaceuticals with the highest occurrence were antibiotics. The presence of these compounds in wastewater is of concern, since they can promote bacterial resistance. Besides, they are not completely removed by conventionally wastewater treatment systems (Kümmerer, 2009a, 2009b). Ben et al. (2017) demonstrated the prevalence of antibiotic resistance in WWTP effluents from China and indicated the risk of dissemination of antibiotic resistance genes into the environment with the discharge or reuse of those effluents. The authors also pointed out those disinfection systems play an important role in eliminating antibiotic resistance in effluents, but additional studies related to this process are needed. Similar situations of antibiotic resistance associated to wastewaters/effluents have been reported in different countries, such as Romania, Canada and, Italy, among others (Neudorf et al., 2017; Szekeres et al., 2017; Turolla et al., 2018), and even in pristine areas such as the Antarctic (Hernández et al., 2019a, 2019b).

3.2. Metabolites screening

As stated in the experimental section, the large group of compounds included into the database was searched using two different strategies. Fig. 1 shows the identification of a metabolite when information about retention time and fragment ions was available. A chromatographic peak was observed at 10.01 min, which might correspond to losartan carboxylic acid (shift <0.1 min in relation to the reference standard). The LE spectrum showed the *m/z* corresponding to the protonated molecule ($[M + H]^+$, $C_{22}H_{22}N_6O_2Cl^+$ of *m/z* 437.1493) with a measured mass error of 1.4 ppm in relation to the theoretical exact mass. The isotopic pattern characteristic of a chlorine atom was also observed. Similarly, the XICs of the main fragment ions were extracted from the HE function at the same retention time. The two main fragment ions were found at *m/z* 235.0978 Da (corresponding to $C_{14}H_{11}N_4^+$) and *m/z* 190.0657 ($C_{14}H_8N^+$), with mass errors lower than 3 ppm.

However, the above described ideal situation did not occur for many metabolites, as ion fragments were not available in the database. Fig. 2 illustrates the tentative identification of 4- methylamino antipyrine (4-MAA). The LE spectrum in ESI positive ion mode of the chromatographic peak at 2.44 min, showed an abundant signal at *m/z* 218.1288 (Fig. 2a, bottom) which might correspond to the protonated molecule of 4-MAA ($C_{12}H_{16}N_3O^+$, expressed as $[M + H]^+$, with a mass error of 0.5 ppm). The HE spectrum showed two fragment ions at *m/z* 159.0917 ($C_{10}H_{11}N_2^+$, corresponding to the loss of C_2H_5NO) and *m/z* 97.076 ($C_5H_9N^+$), both with mass errors below 2 ppm (Fig. 2a, top). The structure of these fragment ions was justified on the basis of their measured accurate masses, and all were compatible with the structure of the candidate. Moreover, the fragment ions were in accordance to the infor-

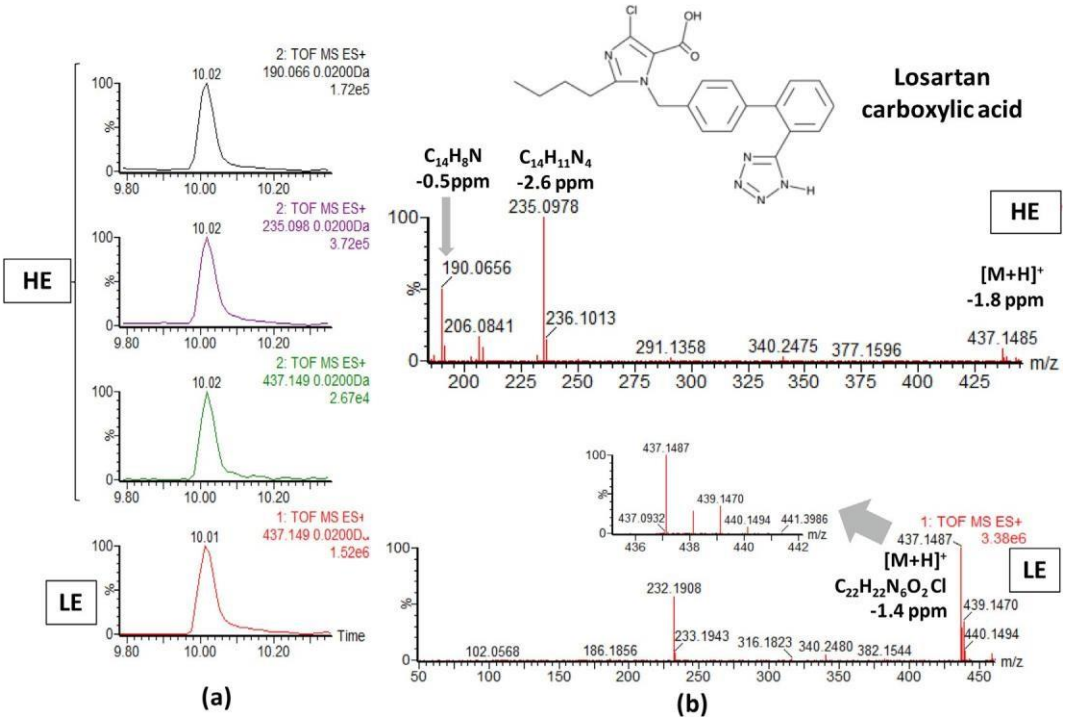

Fig. 1. Identification of the metabolite losartan carboxylic acid in RHWW: (a) nw-XICs (0.02 Da mass window) for m/z corresponding to the protonated molecule in LE function and for its two main fragment ions in HE function. (b) Combined LE and HE spectra for chromatographic peak at 10.0 min. Elemental composition and mass error for the observed ions are shown.

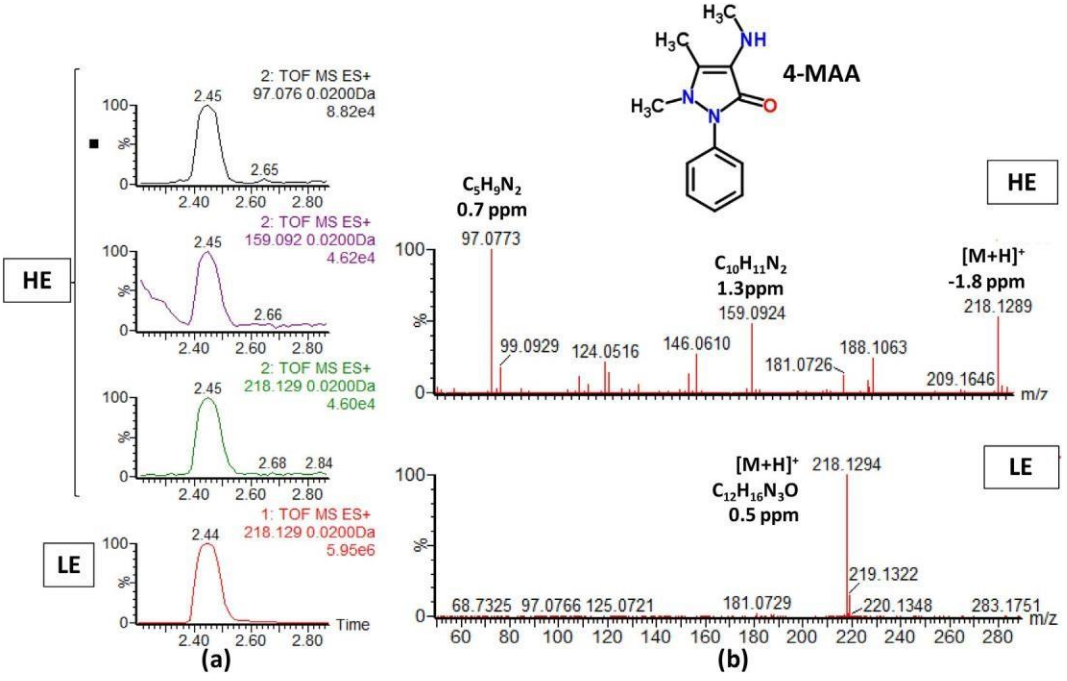

Fig. 2. Tentative identification of 4-methylamino antipyrine (4-MAA): (a) nw-XICs at 0.02 Da for m/z corresponding to the protonated molecule in LE function and main fragment ions in HE: (b) Combined LE and HE spectra of the potential metabolite.

mation reported by Gómez et al. (2010). Accordingly, all these data strongly supported the tentative identification of the compound as 4-MAA.

In addition, the common fragmentation pattern approach was applied in order to investigate other metabolites not included in the list of suspects. This strategy allowed the identification of 4 additional metabolites. For all pharmaceuticals and metabolites found in the samples, the presence of additional chromatographic peaks at m/z of the parent compound in the LE function and/or of the different fragments, commonly in the HE function, was evaluated. These signals could be related to the presence of other possible new metabolites (Ibáñez et al., 2016, 2017).

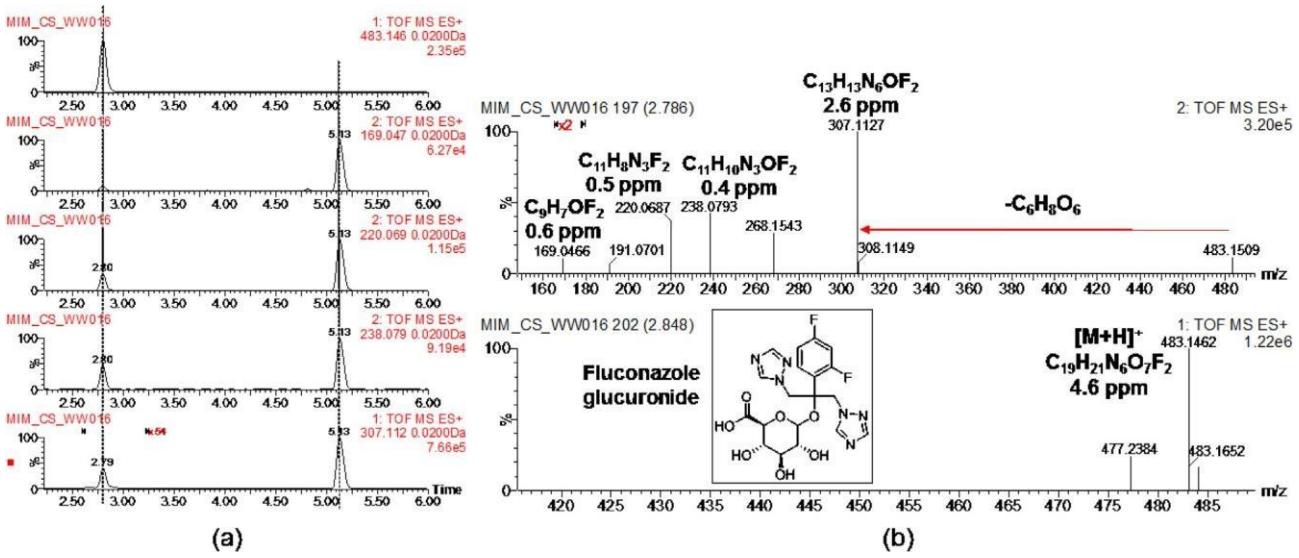

Fig. 3. Detection and tentative identification of fluconazole glucuronide: (a) from bottom to top, nw-XICs at 0.02 Da mass window for *m/z* 307.112 in LE function (corresponding to the protonated molecule of fluconazole, 5.11 min), *m/z* 238.0792 and 220.069 (corresponding to its main fragments in HE), *m/z* 483.1463 in LE function (corresponding to fluconazole glucuronide, 2.8 min). (b) LE and HE spectra of chromatographic peak at 2.8 min.

An illustrative example of positive findings using the common fragmentation strategy is shown in Fig. 3. The XIC at the *m/z* of fluconazole ($C_{13}H_{13}N_6OF^+$ *m/z* 307.1119, as $[M + H]^+$) in the LE function, showed a chromatographic peak at the expected retention time of the parent pharmaceutical (5.11 min), but also a minor peakat 2.80 min (Fig. 3a, bottom). Moreover, XICs at the positive fragment ions of fluconazole (*m/z* 238.079 and 220.069) indicated that both compounds shared these fragment ions too (Fig. 3a, middle). Therefore, the peak at 2.80 min was treated as a potential metabolite/TP. After investigating the LE function (Fig. 3a, top), the accurate mass was assigned to *m/z* 483.1463 (Fig. 3b, bottom), which corresponded to the elemental composition $C_{19}H_{21}N_6O_7F_2^+$ (error 4.8 ppm). This implied an increase of $C_6H_8O_6$ from fluconazole, which could be associated to a glucuronide moiety. Therefore, fluconazole-glucuronide was proposed as a reasonable structure for this potential metabolite. The HE spectrum confirmed the presence of the glucuronide moiety by the typical loss of 176 Da in positive ionization mode (Fabregat et al., 2013). Similar situations were observed for codeine glucuronide, hydrocodone glucuronide and azithromycin metabolite 591.

Glucuronide metabolites are important for both toxicology and pharmacokinetics of many drugs (Trontelj, 2012). Although a drug bound to a glucuronide losses its pharmacological activity, there may be situations where deconjugation occurs (*e.g.* in the wastewater treatment plant), releasing the original compound and recovering its pharmacological activity. For this reason, conjugated compounds could also represent an environmental risk due to its back transformation.

Considering this approach 31 metabolites were identified by the use of the expanded home-made database (Table 1). Five metabolites (4-AAA, 4-FAA and 4-MAA, from dipyrone; atenolol acid; and D,L,*O*-desmethyl venlafaxine) were identified in all raw HWW samples analysed. Also, the dipyrone metabolite 4-AA was found in 5 out of the 6 analysed samples. It is interesting to note that although dipyrone (a non-steroidal anti-inflammatory drug) was not detected in the samples (Section 3.3), its metabolites (4-AAA, 4-FAA, 4-MAA, 4-AA (Gómez et al., 2008) were identified and four of them were among the most frequently seen, indicating the importance of this complementary analysis of drugs and metabolites. In half of the samples analysed, metabolites originated from trimethoprim hydroxylation and also *N*,*O*-didesmethyl venlafaxine b-D-glucuronide were identified. In addition, for nine of the metabolites identified in this study (4-AAA, 4-AA, 4-FAA and 4- MAA from dipyrone; albendazole sulfoxide from albendazole; 4- hydroxy omeprazole sulfide, 5-hydroxy omeprazole and omeprazole sulfone N-oxide from omeprazole, and desalkyl vera- pamil D617 from verapamil) their parent compounds (active prin- ciple) were not identified in the raw HWW samples. These results illustrate the importance of performing metabolites screening when investigating the occurrence of pharmaceuticals in the aquatic environment, because metabolites can be present in the samples even if the parent compounds are not.

Han and Lee (2017) evaluated the significance of the metabolites in the environmental risk assessment of pharmaceuticals consumed by humans. These authors concluded that some metabolites may potentially present similar or even greater risk than their parent active pharmaceutical ingredient in the aquatic environment. Consequently, the inclusion of metabolites in qualitative (screening) and quantitative monitoring of environmental and wastewater samples is essential, including effluent samples, because they are frequently discharged directly into the aquatic environment.

In addition to human metabolites, some compounds found in the samples may be the result of biotic and abiotic processes inthe aqueous media (Michael et al., 2014). The raw HWW sample analysed in this work do not undergo any treatment before it is discarded into the public sewage system. It is possible that some aerobic or anaerobic organism are present in the sewer pipeline, but considering the high flow rate of sewage discharged and the lack of sunlight, the biotic process is predominant and it could be inferred that just few transformation products (TPs). Evgenidou et al. (2015) provided an extensive literature survey about the occurrence of biotic and abiotic TPs in influents and effluents of WWTPs. Some metabolites found in this study, such as the ones of dipyrone (4-AAA, 4-AA, 4-FAA, and 4-MAA), atorvastatin (o-/p-hydroxyatovastatin), metoprolol (a-hydroxy metoprolol), metronidazole (hydroxy metronidazole), and venlafaxine (D.L.O.-desmethyl venlafaxine) were also found in influents and effluents of WWTPs.

In the present work, seven phase II metabolites were identified in the influent raw HWW, such as the glucuronide derivatives of codeine, hydrocodone, fluconazole, diphenhydramine, and

Table 1
Metabolites identified in the RHWW samples evaluated.

Therapeutic class	Pharmaceutical	Metabolite	February	March	April	May	June	July	Status[a]
Analgesics/anti-inflammatory	Dipyrone (CAS: 68-89-3)	4-AAA (CAS:83-15-8)	x	x	x	x	x	x	c
		4-AA (CAS:83-07-8)	x	x	x	x	x		c
		4-FAA (CAS: 1672-58-8)	x	x	x	x	x	x	c
		4-MAA (CAS: 856307-27-2)	x	x	x	x	x	x	s
	Acetaminophen (CAS: 103-90-2)	3-cysteinyl acetaminophen (CAS: 53446-10-9)				x		x	s
		3-methoxy acetaminophen (CAS: 3251-55-6)	x			x			s
	Codeine (CAS: 76-57-3)	*Codeine-glucuronide*[c] (CAS: 20736-11-2)					x		s
	Hydrocodone (CAS: 125-29-1)	*Hydrocodone-glucuronide*[c]	x						s
Anthelmintic drug	Albendazole (CAS: 54965-21-8)	Albendazole sulfoxide (CAS: 54029-12-8)				x			s
Cardiovascular disease	Atenolol (CAS: 29122-68-7)	Atenolol acid (CAS: 56392-14-4)	x	x	x	x	x	x	s
	Metoprolol (CAS: 37350-58-6)	a-Hydroxy metoprolol (CAS: 56392-16-6)			x		x		s
	Losartan (CAS: 114798-26-4)	Losartan carboxylic acid	x						c
		Losartan Metabolite 1 (CAS: 141675-57-2)	x				x		s
		Losartan Metabolite 2 (CAS:141675-59-4)	x				x		s
	Atorvastatin (CAS: 134523-00-5)	o/p-hydroxyatorvastatin[b]					x		s
Antibiotics	*Azithromycin* (CAS: 83905-01-5)	*Azithromycin met 591*[c] (*Descladinose*)					x		s
	Clindamycin (CAS: 18323-44-9)	*N*-desmethyl clindamycin (CAS: 22431-45-4)					x		s
		Clindamycin sulfoxide (CAS: 22431-46-5)					x		s
	Fluconazole (CAS: 86386-73-4)	*Fluconazole-glucuronide*[c] (CAS: 136134-23-1)				x			s
	Metronidazole (CAS: 443-48-1)	Hydroxy metronidazole (CAS: 4812-40-2)		x					c
	Trimethoprim (CAS: 738-70-5)	a-Hydroxy trimethoprim 29606-06-2			x	x			s
		Trimethoprim 1-N-oxide (CAS: 27653-68-5)			x				s
		Trimethoprim 3-N-oxide (CAS: 27653-67-4)			x				s
Antihistaminic	Diphenhydramine (CAS: 58-73-1)	Diphenhydramine N-oxide 3922-74-5				x			s
		Diphenhydramine N-b-D-glucuronide (CAS: 137908-78-2)				x	x	x	s
Psychiatric drugs	Venlafaxine (CAS: 93413-69-5)	D,L,*O*-Desmethylvenlafaxine	x	x	x	x	x	x	c
		N,*O*-Didesmethylvenlafaxineb-D-glucuronide		x		x		x	s
Calcium antagonist	Verapamil (CAS: 52-53-9)	Desalkyl verapamil D617 (CAS: 77326-93-3)					x		s
Proton pump inhibitors	Omeprazole (CAS: 73590-58-6)	4-Hydroxy omeprazole sulfide (CAS: 103876-98-8)						x	s
		5-Hydroxy omeprazole (CAS: 103876-99-9)			x				c
		Omeprazolesulfone N-oxide (CAS: 158812-85-2)			x				s

4-AAA: *N*-acetyl-4-aminoantipyrine; 4-AA: 4-aminoantipyrine; 4-FAA: N-formyl-4-aminoantipyrine; 4-MAA: 4-methylaminoantipyrine.
[a] Status: 'c' for 'confirmed' and 's' for 'suspect'.
[b] Identified in positive and negative ionization modes.
[c] Compound not included in the database applied in this study. Their identification was made possible after applying the common fragmentation pathway strategy.

venlafaxine. A phase II sulfide derivative metabolite of omeprazole was also identified. Brown and Wong (2018) have evaluated the occurrence and distribution of pharmaceuticals and their phase II metabolite conjugates in a WWTPs reflecting the importance of analyzing the contribution of such metabolites in the discharge of emerging contaminants from wastewaters in the environment. Thus, raw HWW might be one of the main contributor of phase II metabolites to WWTPs.

3.3. Preliminary risk assessment of pharmaceuticals and metabolites

3.3.1. Exposure assessment

The PECs (lg L^{-1}) of pharmaceuticals and metabolites in the influent wastewater are shown in Fig. S1 (Supplementary Material). The excretion rates and predicted eco-toxicity used for the PEC calculations can be seen in Tables S3–S6 (Supplementary Material). For some metabolites, values of PEC in the influent

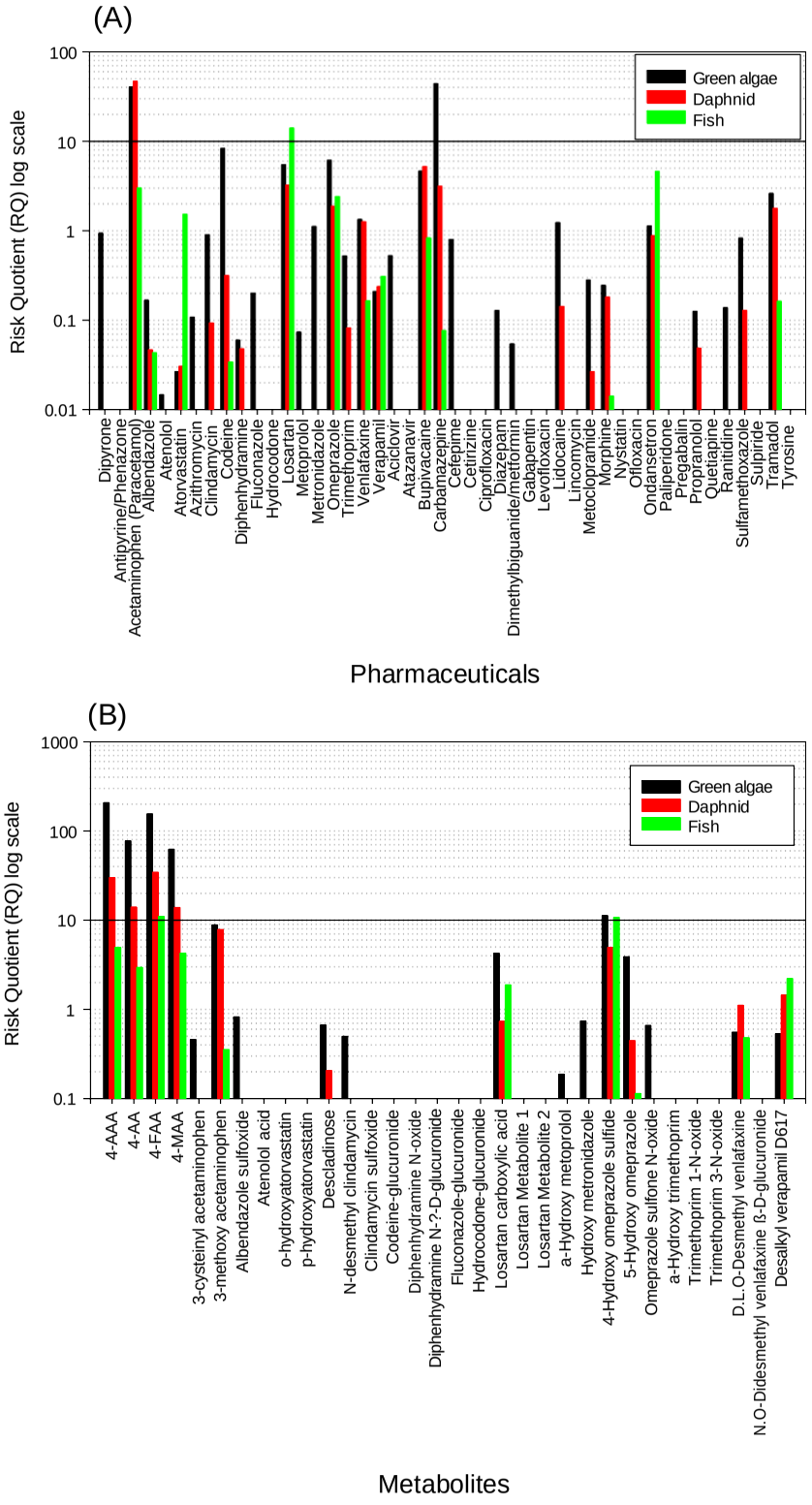

Fig. 4. Estimated risk quotient (RQ) of (A) pharmaceuticals and (B) metabolites released into hospital wastewater considering the different ecotoxicological endpoints '*Greenalgae*', '*Daphnid*' and '*Fish*'.* Not found in the list of pharmaceuticals provided by the hospital institution;** Excretion rate not found.

wastewater higher than those corresponding to the parent compounds were predicted because of their higher excretion rates after metabolism. Most of pharmaceuticals were predicted with PEC between 1 and 50 μg L^{-1}, with some exceptions such as dipyrone, acetaminophen and cefepime, which were predicted with values higher than 50 μg L^{-1}. Concerning the PECs for metabolites, the highest values were for the metabolites of dipyrone 4-AAA, 4-AA, 4-FAA, and 4-MAA (predicted at concentrations>100 μg L^{-1}). Due to the lack of information about the excretion rate of some metabolites, such as o/p-hydroxyatorvastatin, hydrocodone-glucuronide, and losartan metabolites 1 and 2, their PEC could not be estimated. Most of metabolites were predicted with PEC within the range of

1–50 μg L^{-1}. The higher values for dipyrone and its metabolites in raw HWW in this study could be compared with those stated by Feldmann et al. (2008), who reported the occurrence of dipyrone and metabolites in different locations of the municipal sewage system of Berlin (Germany) in concentrations above 50 μg L^{-1}.

Usually, a PEC/PNEC ratio with a log of ~10 is seen as a trigger value (European Commission, 1996). A more accurate criterion for risk assessment was proposed using log RQ < 1 as a minimum risk for aquatic organisms, 1 < log RQ < 10 as medium risk, and log RQ > 10 as high risk (Franquet-Griell et al., 2015; Hernando et al., 2006). As shown in Fig. 4, acetaminophen, losartan, and carbamazepine exceed the trigger value for high risk. Besides, acetaminophen exceeded the RQ for the two-ecotoxicological endpoints *Green algae* and *Daphnid*. Due to the environmental con-cern, such compounds should be checked in further biotests. Some pharmaceuticals could be considered as medium risk such as dipy-

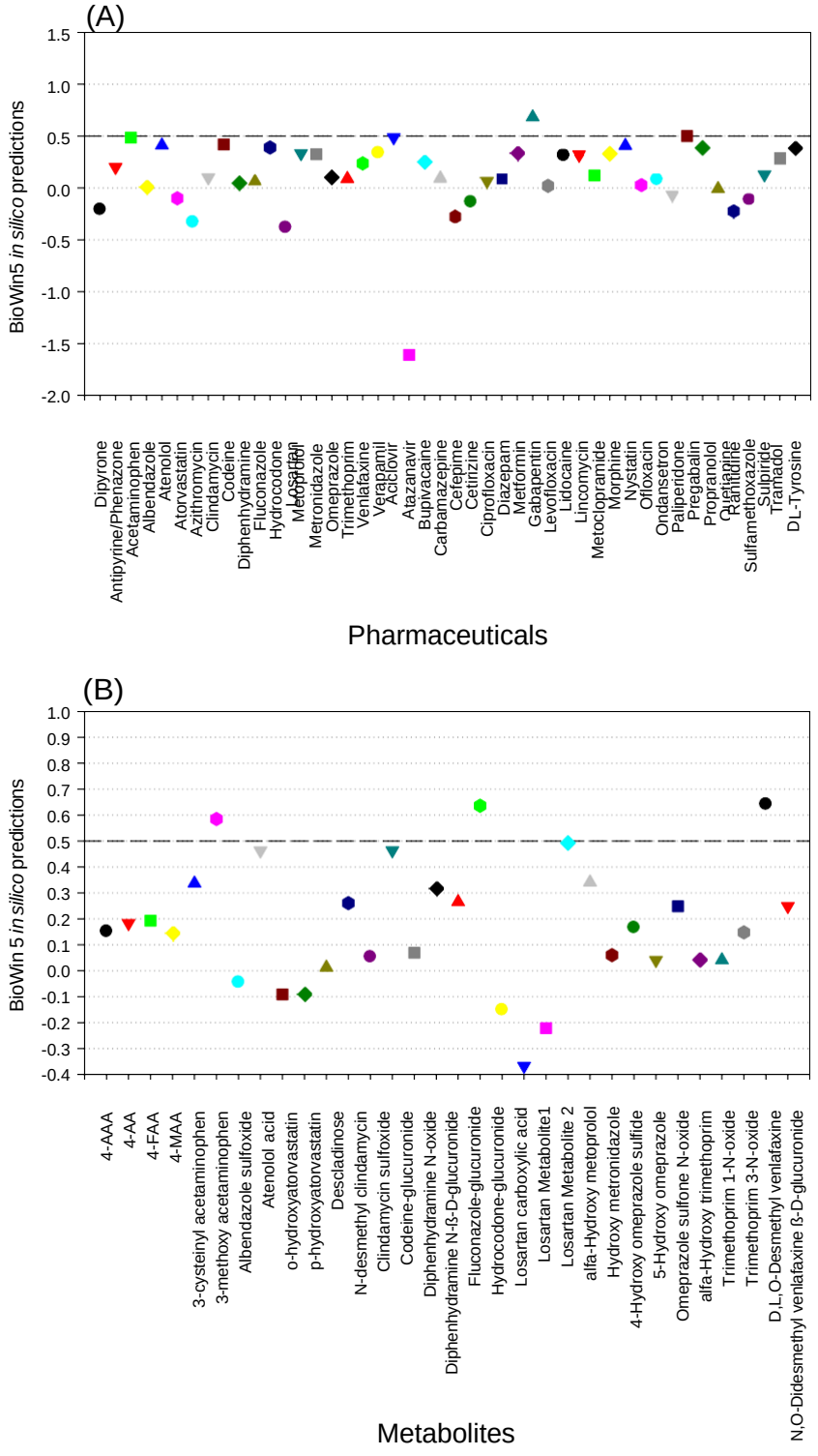

Fig. 5. BIOWIN 5 (linear MITI model) *in silico* QSAR predictions for ready biodegradability (0 means not biodegradable and 1 biodegradable) of the (A) pharmaceuticals parent compounds and (B) human metabolites found in the RHWW.(—) is the trigger value of 0.5.

rone, atorvastatin, codeine, metronidazole, omeprazole, venlafaxine, bupivacaine, and lidocaine as their log RQ exceeded the trigger value of 1 for at least one endpoint. The RQ_{mixt} for the pharmaceuticals achieved 117.47, 62.325, and 22.45 for the end points *Green algae*, *Daphnid* and *Fish*, respectively. Considering the *Green algae* and *Daphnid*, the RQ_{mixt} achieved levels found by Escher et al. (2011) in their study for a general hospital, the RQ_{mixt} found in the present work was lower. To understand this difference, it mustbe taken into account the distinct number and type of pharmaceu- ticals investigated in both studies.

Concerning the metabolites, 4-AAA, 4-AA, 4-FAA, 4-MAA, and 4-hydroxy omeprazole sulfide exceeded the trigger value of log RQ 10. The metabolites 3-methoxy acetaminophen, losartan carboxylic acid, D.L.O.-desmethyl venlafaxine, and desalkyl verapamil D617 were classified as medium risk (1 < log RQ > 10).The RQ_{mixt} for the metabolites achieved 532.58, 106.11, and 36.17 for the endpoints *Green algae*, *Daphnid* and *Fish*, respectively.

Investigating the occurrence of pharmaceuticals in influent raw HWW is of relevance, especially in some developing countries where raw HWW may be directly discharged into the aquatic environment. It has been reported that several compounds in WWTPs were found at the same level as in HWW, indicating a similarity in usage pattern in hospitals and households (Azuma et al., 2019). The PEC of pharmaceuticals in the influent raw HWW were at levels from few ng L^{-1} (atorvastatin) up to >400 lg L^{-1} (cefepime). Measured concentrations of pharmaceuticals hospital effluent at lg L^{-1} levels were also reported by Azuma et al. (2019). The PEC of psychiatric compounds screened in this study such as quetiapine, venlafaxine and the metabolite D.L.*O*-desmethyl venlafaxine were predicted in concentrations lower than 1 lg L^{-1}, which is at the same level as the measured concentrations reported in hospital WWTP influent in Greece (Kosma et al., 2019). Oliveira et al. (2015) analysed 185 pharmaceuticals and personal care products (PPCPs) belonging to >20 therapeutic categories in raw HWW of mid-sized hospitals, and the concentrations found for some pharmaceuticals were in the same level as the PEC provided by our study.

3.4. In silico risk assessment by QSAR

The persistence of the parent compounds and human metabolites found in the raw HWW was assessed by *in silico* predictions provided by the BIOWIN models of the U.S. EPA EPI Suite software. The predicted values can be seen in Table S7 (Supplementary Material). Fig. 5 shows the BIOWIN 5 MITI (Japanese Ministry of International Trade and Industry biodegradation database) predictions for the parent compounds and human metabolites. Among the parent compounds, only gabapentin was predicted with >0.5 and by combining the BIOWIN 1–7 predictions, it was expected as probably ready biodegradable. However, Herrmann et al. (2015), who studied the biodegradability of gabapentin by means of OECD 301D reported that this compound is not readily biodegradable.

Concerning metabolites, the wide majority were predicted as not readily biodegradable. In general, by comparing the BIOWIN 5 values of the parent compounds and of human metabolites, an increase was observed for metabolites. Such behavior could be explained according to the generalized ‘‘rules of thumb” for biodegradation, such as hydroxylation and insertion of groups susceptible to enzymatic hydrolysis. Values of BIOWIN 5 > 0.5 were predicted for the metabolites 3-methoxi acetaminophen, hydrocodone-glucuronide and *N*,*O*-didesmethyl venlafaxine b-D-glucuronide. The predicted increase in biodegradability might be due the phase II metabolism of the glucuronidation process. The metabolite a-hydroxy metoprolol was predicted with a value of 0.4926, which is around the trigger values of BIOWIN 5. However, by combining the BIOWIN 1–7 predictions, a-hydroxy metoprolol was not predicted as readily biodegradable. Studies concerning the biodegradation of beta-blockers TPs have identified some of them as ready biodegradable compounds (Rastogi et al., 2014, 2015a, 2015b). These authors tested 4-hydroxypropranolol glucuronide by means of OECD 301D test showing that the glucuronide derivative of 4-hydroxypropranolol is not readily biodegradable, but it undergoes a biotic transformation. Such behavior could also take place for the glucuronide metabolite derivatives.

The hazard concerning pharmaceuticals and human metabolites found in this work was assessed by evaluating their PBT, mutagenicity and carcinogenicity predictions. The software Prometheus was used to rank the parent compounds and metabolites as PBT compounds. The *in silico* values predicted can be seen in Table S8 (Supplementary Material). A threshold of 0.5 of the total score was used to discriminate non-PBT (<0.5) and the potentially PBT or vPvB ($\geq$0.5) compounds (Pizzo et al., 2016). Fig. S2 (Supplementary Material) shows the predicted PBT values for the parent compounds and metabolites. Although most of the structures were ranked below the threshold value of 0.5, some compounds such as the pharmaceuticals albendazole, fluconazole, cetirizine, diazepam and ondanestron were around this value (>0.45). The metabolite fluconazole-glucuronide was predicted with 0.494 and therefore might be classified as PBT taking into account the uncertainty of the predictions (Pizzo et al., 2016).

The predicted values and applied VEGA QSAR models for Mutagenicity (Ames test) CONSENSUS model are summarized in Tables S9–S10 (Supplementary Material) and Table 2. It is worth to mention that the end-point does not address any dose-dependence on the results. The predictions provide a consensus score from 0 to 1. The score 1 usually corresponds to structures inside the ADI and due to presence of experimental value in at least one of the four models comprised into the CONSENSUS model. Among the parent compounds, mutagenicity alerts with *Exp. values* were predicted for dipyrone, metronidazole, antipyrine/phenazone and ciprofloxa-cin. Conversely, non-mutagenicity alerts by consensus score of 1 were pointed out for 8 compounds. The metabolite hydroxy metronidazole was pointed out with a consensus score 1, being classified as mutagenic compound. Most of the parent compounds were predicted with a consensus score below 0.75, indicating structural differences among the predicted compounds and the structures present in the predictive models. Accordingly, such predictions could be classified as of moderate or low reliability. Taking those structures of parent compounds (see Table S10, Supplementary Material) and metabolites (see Table 2) with a consensus score of 1 aside, and analyzing the compounds which have presented a consensus score >0.75 (*i.e.* good reliability), most of compounds were predicted as non-mutagenic among the parent compounds calculated with high RQ, acetaminophen, losartan, and carbamazepine were predicted as non-mutagenic compounds.

Concerning the carcinogenicity assessment, different VEGA QSAR models were applied as can be seen in Table S11 (Supplementary Material). In regard to the analgesics, dipyrone presented contrasted predictions in ANTARES and ISS models and were reported as Exp. values for non-carcinogenic (NC) and carcinogenic (C). In relation to the dipyrone metabolites, 4-AAA, 4-AA, 4-FAA, 4-MAA most of alerts predicted the metabolites as carcinogenic compounds. Acetaminophen was predicted with contradicting alerts as Exp. values towards carcinogenicity in two different models. The same behavior was also predicted for its human metabolites 3-cisteinyl acetaminophen and 3-methoxy acetaminophen, probably because their predictions are based on the structure of the parent compound. Codeine and hydrocodone and its metabolites were predicted as non-carcinogenic with good and moderate reliability according to some models. The antihelmintic drug albendazole and the metabolite albendazole sulfoxide presented contradicting predictions, which could be pointed out as inconclusive predictions.

Table 2
In silico QSAR predictions for Mutagenicity (Ames test) CONSENSUS model of the pharmaceuticals and metabolites investigated in the present study according to VEGA QSAR v.1.1.4 software.

Pharmaceutical	Mutagenicity	Consensus score	Metabolite	Mutagenicity	Consensus score
Dipyrone	Mut.	1[a]	4-AAA	Mut.	0.35
			4-AA	Non-Mut.	0.3
			4-FAA	Mut.	0.35
			4-MAA	Non-Mut.	0.3
Acetaminophen	Non-Mut.	1[a]	3-Cysteinyl acetaminophen	Mut.	0.35
			3-Methoxy acetaminophen	Non-Mut.	0.45
Albendazole	Mut.	0.25	Albendazole sulfoxide	Non-Mut.	0.2
Atenolol	Non-Mut.	1[a]	Atenolol acid	Non-Mut.	0.82
Atorvastatin	Non-Mut.	1[a]	o/p-Hydroxyatorvastatin	Non-Mut.	0.4
Azithromycin	Non-Mut.	1[a]	Descladinose	Non-Mut.	0.9
Clindamycin	Mut.	0.25	*N*-desmethyl clindamycin	Mut.	0.25
			Clindamycin sulfoxide	Mut.	0.25
Codeine	Non-Mut.	1[a]	Codeine-glucuronide	Non-Mut.	0.6
Diphenhydramine	Non-Mut.	1[a]	Diphenhydramine N-oxide	Non-Mut.	0.47
			Diphenhydramine N-b-D-glucuronide	Non-Mut.	0.35
Fluconazole	Non-Mut.	0.35	Fluconazole-glucuronide	Non-Mut.	0.3
Hydrocodone	Non-Mut.	0.82	Hydrocodone-glucuronide	Non-Mut.	0.25
Losartan	Non-Mut.	0.35	Losartan carboxylic acid	Non-Mut.	0.4
			Losartan Metabolite 1	Non-Mut.	0.4
			Losartan Metabolite 2	Non-Mut.	0.4
Metoprolol	Non-Mut.	1[a]	a-Hydroxy metoprolol	Non-Mut.	0.72
Metronidazole	Mut.	1[a]	Hydroxy metronidazole	Mut.	1[a]
Omeprazole	Mut.	0.2	4-Hydroxy omeprazole sulfide	Mut.	0.3
			5-Hydroxy omeprazole	Non-Mut.	0.35
			Omeprazole sulfone N-oxide	Mut.	0.2
Trimethoprim	Mut.	0.43	a-Hydroxy trimethoprim	Mut.	0.35
			Trimethoprim 1-N-oxide	Mut.	0.43
			Trimethoprim 3-N-oxide	Mut.	0.43
Venlafaxine	Non-Mut.	0.75	D,L,*O*-Desmethylvenlafaxine	Non-Mut.	0.75
			N,*O*-Didesmethylvenlafaxineb-D-glucuronide	Non-Mut.	0.57
Verapamil	Non-Mut.	0.82	Desalkyl verapamil D617	Non-Mut.	0.82

Mut.: positive alert for mutagenicity. Non-Mut.: negative alert for mutagenicity.
Consensus score: 0–1.
[a] Means that the alert and consensus are based in an Exp. value.

Among the cardiovascular drugs assessed in this study, atenolol was predicted as carcinogenic by the ANTARES models with experimental value, whereas the metabolite atenolol acid was predicted as non-carcinogenic by 3 models and as carcinogenic with moderate reliability by the ISSCAN-CGX model. Metoprolol and its metabolite a-hydroxy metoprolol were predicted as non-carcinogenic by most of the models. Atorvastatin and its metabolites o/p-hydroxy atorvastatin were predicted as carcinogenic compounds by the ANTARES model with good reliability. Losartan and its metabolites were predicted as non-carcinogenic with moderate reliability by the ANTARES model.

The different models applied predicted the most of antibiotics and its metabolites as carcinogenic compounds. The calcium antagonist verapamil was predicted as non-carcinogenic with experimental value by the ANTARES model, whereas its metabolite desalkyl verapamil D617 was predicted with contradicting alerts according to the model assessed.

Several pharmaceuticals found in the raw HWW were predicted with carcinogenicity alert with experimental value in at least one model such as carbamazepine, gabapentin, quetiapine, sulfamethoxazole. The other pharmaceuticals were predicted with different alerts in different models, which could in some extent indicate that the prediction for these compounds by applying a consensus model, are inconclusive.

In summary, the combination of exposure and *in silico* QSAR models as complimentary tools can improve the risk assessment of pharmaceuticals and metabolites. For instance, the three human metabolites of trimethoprim (a-hydroxy trimethoprim, trimethoprim 1-N-oxide, and trimethoprim 3-N-oxide) were predicted with lower PEC values (0.89, 0.38 and 0.38 1g L^{-1}) and, consequently, lower RQ values. If the risk assessment would only be based on the exposure assessment (*i.e.* PEC and EC_{50}/LC_{50} values), the potential hazard might be underestimated, because of the *in silico* (Q)SAR predictions provided by different VEGA QSAR models have pointed out these three metabolites with positive alerts regarding mutagenicity and carcinogenicity. Nevertheless, the alerts do not provide any dose response relationship. Besides, the metabolites of trimethoprim were predicted as not biodegradable (*i.e.* persistent in the environment), which is problematic due to the continuous release in the environment.

By combining the analytical methodology applied for monitoring the occurrence in HWW with a risk assessment based on exposure (*i.e.* PEC and Risk Quotient), hazardous and fate in the environment predictions by means of *in silico* (Q)SAR models, a list of priority pharmaceuticals and metabolites for further monitoring studies could be pointed out: albendazole, fluconazole, cetirizine, diazepam, ondanestron, metronidazole, dipyrone, carbamazepine, ciprofloxacin, levofloxacin, ofloxacin, 4-AAA, 4-AA, 4-FAA, 4-MAA, fluconazole-glucuronide, 4-hydroxy omeprazole sulfide. However, according to uncertainties in the risk assessment, some pharmaceuticals and metabolites could be included in a list of pharmaceuticals and metabolites under attention and also considered for further studies such as clindamycin, omeprazole, trimethropim, metoclopramide, ondansetron, 3-cysteinyl acetaminophen, *N*-desmethyl clindamycin, clindamycin sulfoxide, a-hydroxy trimethoprim, trimethoprim 1-N-oxide, trimethoprim 3-N-oxide.

4. Conclusions

In this study, a purpose-built database has been used to investigate the presence of metabolites and pharmaceuticals in raw

HWW samples by LC-HRMS. With the strategy applied, consisting on a combination of target and suspect screening, it was possible to identify 31 metabolites and 43 pharmaceuticals in the samples. The group of drugs most detected were antibiotics. In addition to many known/reported metabolites included in the database, four additional metabolites (fluconazole glucuronide, codeine glucuronide, hydrocodone glucuronide and azithromycin metabolite 591) were found after applying the common fragmentation pathway approach with their parents. This study highlights the importance of using wide-scope screening, including both pharmaceuticals and metabolites, for comprehensive evaluation of pharmaceuticals occurrence in wastewaters. This is of great relevance to propose additional treatment systems in conventional WWTPs for an efficient removal of all these compounds.

The risk assessment of pharmaceuticals and its metabolites based only on PEC and RQ (PEC/PNEC) might underestimate their risks to the environment and humans. Although some pharmaceuticals and metabolites were pointed out as lower or medium RQ, several of them were predicted as mutagen and carcinogen compounds. The inclusion of *in silico* QSAR predictions allowed assessing the environmental fate and effect of such compounds in terms of biodegradability, as possible PBT compounds and their potential hazard to the aqueous environment. By adding such kind of approach in the environmental risk assessment of pharmaceuticals and metabolites it is possible to perform a more proactive prioritization of such complex mixture of compounds. In addition, reliable and freely available QSAR models should be further improved and implemented for the risk assessment of metabolites and biotic and abiotic transformation products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134218.

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SUPPLEMENTARY MATERIAL I

SUPPLEMENTARY MATERIAL

Investigation of pharmaceuticals and their metabolites in Brazilian hospital wastewater by LC-QTOF MS screening combined with a preliminary exposure and *in silico* risk assessment

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S1. Physical-chemical wastewater characterisation

Several physical-chemical parameters were monitored for each sample (Table S1). In general, all parameters showed variable results over the sampling period. Particular attention has to be paid to parameters related to organic matter (COD, BOD, DOC) and conductivity, which are of relevance in the processes applied in wastewater treatment plants (WWTPs). In addition, the presence of a representative amount of solids (SST and, especially ST) was determined in some samples.

According to Carraro et al. (2016), the variability in some physical-chemical parameters is recurrent in raw HWW samples. As highlighted in that review, some factors, such as size of hospital, bed density, the number of inpatients and outpatients, the number and types of wards, the number and types of services, the country and the seasonality contribute to the high variability of this matrix. That work also noticed the high COD values commonly found in most hospital wastewaters. The values obtained in our study can be considered similar to those compiled by Carraro et al. (2016).

An additional remark could be derived from the observed BOD/COD ratio. According to Kumar et al. (2010), an ideal biodegradability index is a BOD/COD ratio close to 1.0. However, Esplugas et al. (2004), considered that values close to 0.5 represent a considerable biodegradability index. In the present work, four samples (March, May, June and July) had BOD/COD ratios below 0.5. This fact would indicate a reduced biodegradability and, therefore, these wastewater samples might present some recalcitrant fractions that could limit the application of biological treatment systems. In turn, Kajitvichyanukul and Suntronvipart (2006) found BOD/COD ratios close to 0.3 for hospital wastewater sample. These authors concluded that the application of a chemical pre-treatment favours the reduction of toxicity of pollutants and the increase in biodegradability, enhancing the possibility of success of subsequent biological treatment (Kajitvichyanukul and Suntronvipart, 2006).

Table S1. Physical-chemical parameters monitored for raw HWW samples studied.

Parameter	Months sampled						Method	LOD	LOQ
	Feb.	Mar.	Apr.	May	Jun.	Jul.			
pH	8.02	7.52	7.80	8.06	7.98	8.21	*SMEWW 4500-H^+ B		
Conductivity ($\mu S\ cm^{-1}$)	944.0	326.0	956.0	539.3	471.0	781.0	SMEWW 2510 B	1	0.2
COD ($mg\ L^{-1}\ O_2$)	203	211	473	277	273	706	SMEWW 5220 B	5	0.8
BOD ($mg\ L^{-1}\ O_2$)	132	45	236	108	83	319	SMEWW 5210 B	2	0.6
BOD/COD	0.65	0.21	0.50	0.39	0.30	0.45	≥ 0.5 biodegradable(Lopez et al., 2004)		
DOC ($mg\ L^{-1}$)	65.0	63.1	70.2	93.2	41.34	82.74	SMEWW 5310	1.68	3.99
Total Chloride ($mg\ L^{-1}$)	72.9	27.9	4.3	37.7	41.6	9.0	SMEWW 4110 B	0.5	0.02
Total Phosphate ($mg\ L^{-1}\ PO_4^{3-}$)	21.36	3.93	22.07	8.56	2.99	0.45	SMEWW 4500 P E	0.03	0.006
TSS ($mg\ L^{-1}$)	128	52	98	63	27	165	SMEWW 2540 D	10	5
TS ($mg\ L^{-1}$)	525	222	521	244	225	611	SMEWW 2540 B	10	5

*SMEWW – Standard Methods for the examination of water and wastewater (American Public Health Association et al., 1998).

S2. Sample preparation, instrumentation and general strategy

Sample preparation

Solid phase extraction (SPE) was performed according to Hernández et al. (2015). Briefly, 100 mL of the effluent samples were passed by gravity through 200 mg OASIS HLB cartridges, conditioned with 10 mL methanol and 10 mLwater. After that, SPE material was dried by passing airfor 15 minutes. This part was performed at Universidade Federal do Rio Grande do Sul (Brazil). Cartridges were packed separately and frozen for transport to the University Jaume I (UJI, Castellón-Spain). The analytes were eluted with 10 mL of methanol. The extracts were then evaporated to dryness under a constant, gentle stream of nitrogen at 40◦C and reconstituted with 0.5 mL of water:methanol (v/v, 90:10) (the pre-concentration factor along SPE was 200). Finally, the extracts were filtered through PTFE membranes (0.22 µm), collected in vials and further analysed by means of LC-QTOF MS.

LC-QTOFMS instrumentation

For analysis, a Waters Acquity UHPLC system (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), with an orthogonal Z-spray-ESI interface operating in both positive and negative ionisation modes, was used. Two acquisition functions were selected for MS^E experiments: a low energy function (LE) with a fixed collision energy at 4 eV and a high energy function (HE), with a collision energy ramp from 15 to 40 eV. A scan time of 0.4 s was employed. Mass data was acquired with MassLynx v 4.1 (Waters) and processed by ChromaLynx application manager software (within MassLynx v 4.1).

The chromatographic separation was performed using aCortecsC18 analytical column (2.1 × 100 mm, 2.7 µm) from Waters. The mobile phase was composed by water (A) and methanol (B), both acidified at 0.01% with formic acid, at a flow rate of 300 µL/min. The initial percentage of B was 10%, which was linearly increased to 90% in 14 min, followed by a 2 min isocratic period, and then returned to initial conditions over 2 min. The QTOF mass spectrometer was

operated under the following conditions: capillary voltages of 700 and 2000 V were used in positive and negative ionisation modes, respectively; a cone voltage of 20 V was employed; the desolvation temperature was set to 600 °C, the source temperature to 130 °C, and the column temperature to 40 °C. In all analyses, the injection volume was 25 µL.

S3 Pharmaceuticals parent compounds screening

Table S2. Pharmaceuticals identified for raw HWW samples evaluated.

Therapeutic class	Pharmaceutical	February	March	April	May	June	July	Status*
Amino acid	Tyrosine	x						s
Analgesic	Acetaminophen	x	x	x	x	X	x	c
	Antipyrine/Phenazone				x	X		s
	Codeine	x	x	x	x	X	x	s
	Hydrocodone				x	X	x	s
	Morphine		x	x				s
	Tramadol		x	x	x		x	s
Anesthetic	Bupivacaine	x		x				s
	Lidocaine	x	x	x		X		c
Angiotensin-receptor blocker	Losartan	x				X		c
Antibiotic	Azithromycin				x	X		c
	Cefepime					x	x	s
	Ciprofloxacin					x		c
	Clindamycin	x	x	x		x		c
	Levofloxacin					x	x	s
	Lincomycin					x		c
	Metronidazole		x	x	x	x	x	c
	Ofloxacin					x	x	s
	Sulfamethoxazole			x	x		x	c
	Trimethoprim	x	x	x	x	x	x	c
Anticonvulsant	Carbamazepine		x					c
	Diazepam	x	x	x	x	x	x	s
	Gabapentin	x					x	s
	Pregabalin				x			s
Antidepressant	Sulpiride			x	x		x	s
	Venlafaxine	x						c
Antiemetic	Ondansetron			x			x	s
Antifungal	Fluconazole		x		x	x	x	c
	Nystatin						x	s
Antihistamine	Cetrizine			x		x		s
	Diphenhydramine	x						s
Antihyperglycemic	Dimethylbiguanide/ Metformin	x			x	x	x	c
Antilipemic	Atorvastatin					x		c
Antipsychotic	Paliperidone						x	s
	Quetiapine	x						c
Antiretroviral	Atazanavir	x						c
Antiulcerative	Ranitidine		x		x	x		s
Antiviral	Aciclovir				x	x		s
Beta-blocker	Atenolol	x	x	x	x	x	x	c
	Metoprolol	x		x	x		x	s
	Propranolol	x		x				c
CNS stimulant	Caffeine	x	x	x	x	x	x	s
Prokinetic agents	Metoclopramide	x	x	x	x	x	x	s

*Status: ‘c’for ‘confirmed’ and ‘s’ for ‘suspect’

S4. Data and *in silico* QSAR predictions used for the risk assessment of pharmaceuticals and metabolites

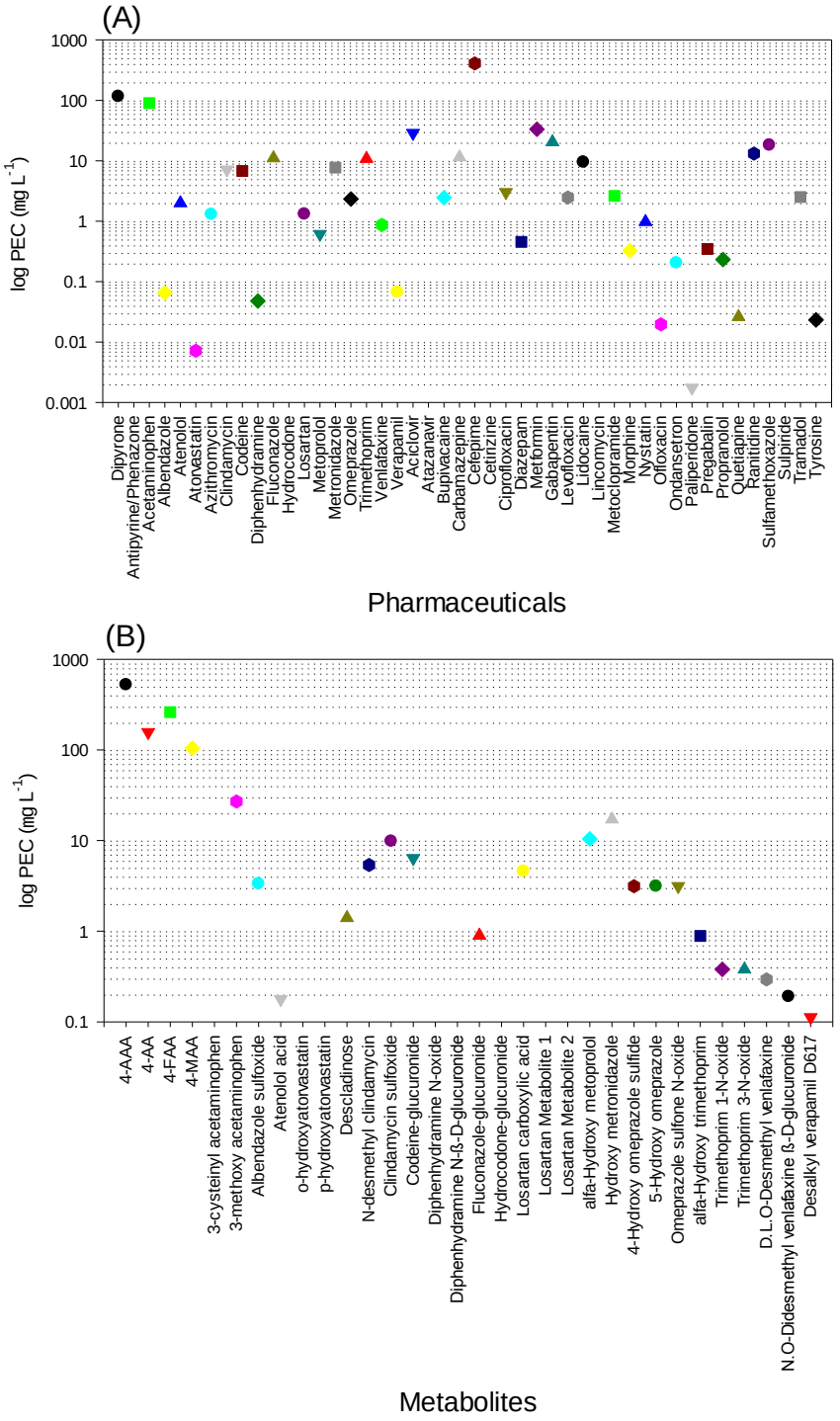

Figure S1. Predicted environmental concentration (PEC) (µg L^{-1}) of the studied (A) pharmaceuticals and (B) metabolites. X-axis is in log scale.* Not found in thelist of pharmaceuticals provided by the hospital institution; ** Excretion rate not found.

Table S3. Emission related data of the selected pharmaceuticals investigated.

Pharmaceutical	Anual Amount (kg yr^{-1})*	Excretion rate as active compound (%)	Excretion rate reference
Dipyrone	353.04	10	(Nikolova et al., 2012)
Antipyrine/Phenazone	0	nf	nf
Acetaminophen (Paracetamol)	136.821	20	(Steventon et al., 2011)
Albendazole	1.98672	1	https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020666s005s006lbl.pdf
Atenolol	0.6744	90	(Wilde et al., 2012)
Atorvastatin	0.10908	2	(Williams and Feely, 2002)
Azithromycin	3.2832	12	(Bakheit et al., 2017)
Clindamycin	10.836	20	(Mitrano et al., 2009)
Codeine	2.39904	86.1	(Chen et al., 1991)
Diphenhydramine	0.3636	4	(Albert et al., 1975)
Fluconazole	4.2048	80	(Brammer et al., 1991)
Hydrocodone	0	50	(Gómez-Canela et al., 2019)
Losartan	9.96	4	(Al-Majed et al., 2015)
Metoprolol	3.726444	5	(Wilde et al., 2012)
Metronidazole	19.55016	12	(Lamp et al., 1999)
Omeprazole	3.67128	19.3	(Regårdh et al., 1990)
Trimethoprim	5.49552	60	(Lamp et al., 1999)
Venlafaxine	0.30375	87	https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020699s081lbl.pdf
Verapamil	0.5088	4	https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/018817s021lbl.pdf
Aciclovir	9.7092	90	https://www.medicines.org.uk/EMC/medicine/23721/SPC/Aciclovir+Tablets+BP+400mg
Atazanavir	0	50	(Gómez-Canela et al., 2019)
Bupivacaine	0.7485	100	https://en.wikipedia.org/wiki/Bupivacaine
Carbamazepine	4.836	72	https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=7a1e523a-b377-43dc-b231-7591c4c888ea
Cefepime	125.52	99	https://en.wikipedia.org/wiki/Cefepime
Cetirizine	0	50	(Gómez-Canela et al., 2019)
Ciprofloxacin	1.83144	50	https://www.drugbank.ca/drugs/DB00537
Diazepam	0.27774	50	(Gómez-Canela et al., 2019)
Dimethylbiguanide/metformin	11.2128	90	(Dunn and Peters, 1995)
Gabapentin	6.2064	100	(Herrmann et al., 2015)
Levofloxacin	0.906	83	(Zhanel et al., 2006)
Lidocaine	28.87056	10	https://www.accessdata.fda.gov/drugsatfda_docs/label/2006/020612s008lbl.pdf
Lincomycin	0	24.8	https://www.drugbank.ca/drugs/DB01627
Metoclopramide	0.938904	85	https://pubchem.ncbi.nlm.nih.gov/compound/metoclopramide#section=Absorption-Distribution-and-Excretion
Morphine	0.9896088	10	(Yeh, 1975)
Nystatin	0.2966436	100	https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+3138
Ofloxacin	0.00702	85	https://www.drugbank.ca/drugs/DB01165
Ondansetron	1.238064	5	(Stoltz et al., 2004)
Paliperidone	0.0009	59	(Vermeir et al., 2008)
Pregabalin	0.108	98	(Ben-Menachem, 2004)
Propranolol	0.70692	10	(Wilde et al., 2012)

Pharmaceutical	Anual Amount (kg yr^{-1})*	Excretion rate as active compound (%)	Excretion rate reference
Quetiapine	0.7941	1	https://www.drugbank.ca/drugs/DB01224
Ranitidine	5.0694	79	(van Hecken et al., 1982)
Sulfamethoxazole	27.4776	20	https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~ujlZRD:3
Sulpiride	0	93.1	(Blanchin and Gomeni, 1984)
Tramadol	2.5452	30	(Guthrie and Teter, 2016)
Tyrosine	1.6833	0.42	(Agharanya et al., 1981)

* calculated from data provided by the hospital

Table S4. Predicted eco-toxicity data of the pharmaceuticals investigated based on the ECOSAR predictions for EC50 and LC50 towards *Green algae*, *Daphnid* and *Fish*.

Pharmaceutical	$EC50_{Green\ algae}$ (mg L^{-1})	$LC50_{Daphnid}$(mg L^{-1})	$LC50_{Fish}$(mg L^{-1})	$PNEC_{Green\ algae}$ (mg L^{-1})	$PNEC_{Daphnid}$(mg L^{-1})	$PNEC_{Fish}$(mg L^{-1})
Dipyrone	123.65	376.94	823.97	0.12365	0.37694	0.82397
Antipyrine/Phenazone	1.31	3.47	2.32	0.00131	0.00347	0.00232
Acetaminophen (Paracetamol)	2.22	0.87	15.47	0.00222	0.00087	0.01547
Albendazole	0.39	3.59	1.08	0.00039	0.00359	0.00108
Atenolol	135.78	103.26	1096.37	0.13578	0.10326	1.09637
Atorvastatin	0.27	0.87	0.02	0.00027	0.00087	0.00002
Azithromycin	11.97	34.25	18.82	0.01197	0.03425	0.01882
Clindamycin	7.94	9.63	80.75	0.00794	0.00963	0.08075
Codeine	0.82	26.3	9.24	0.00082	0.0263	0.00924
Diphenhydramine	0.8	1.25	9.2	0.0008	0.00125	0.0092
Fluconazole	55.45	530.28	1631.3	0.05545	0.53028	1.6313
Hydrocodone	4.33	5.45	44.84	0.00433	0.00545	0.04484
Losartan	0.24	1.69	0.23	0.00024	0.00169	0.00023
Metoprolol	8.31	9.38	81.56	0.00831	0.00938	0.08156
Metronidazole	6.92	179.75	878.33	0.00692	0.17975	0.87833
Omeprazole	0.38	3.26	0.78	0.00038	0.00326	0.00078
Trimethoprim	20.74	6.38	211.62	0.02074	0.00638	0.21162
Venlafaxine	0.65	1.06	7.68	0.00065	0.00106	0.00768
Verapamil	0.32	0.88	0.77	0.00032	0.00088	0.00077

Table S5. Emission related data of the selected metabolites investigated.

Metabolites	Anual Amount of parent compound(kg yr^{-1})*	Excretion rate as active compound(%)	Excretion rate reference
4-AAA	353.04	45	(Nikolova et al., 2012)
4-AA	353.04	13.5	(Nikolova et al., 2012)
4-FAA	353.04	22.5	(Nikolova et al., 2012)
4-MAA	353.04	9	(Nikolova et al., 2012)
3-cysteinyl acetaminophen	136.821	55	(McGill and Jaeschke, 2013)
z3-methoxy acetaminophen	136.821	6	(Hamilton and Kissinger, 1982)
Albendazole sulfoxide	1.98672	51	(Pharmacology and Marrinefl, 1986)
Atenolol acid	0.6744	8	(Reeves et al., 1978)
o-hydroxyatorvastatin	0.10908	nf	nf
p-hydroxyatorvastatin	0.10908	nf	nf
Descladinose	3.2832	13	(Luke and Foulds, 1997)
N-desmethyl clindamycin	10.836	15.1	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/050801s000_Evoclin_PharmR.pdf
Clindamycin sulfoxide	10.836	27.57	https://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/050801s000_Evoclin_PharmR.pdf
Codeine-glucuronide	2.39904	81	(Vree and Verwey-Van Wissen, 1992)
Diphenhydramine N-oxide	0.3636	2	(Sharma and Hamelin, 2005)
Diphenhydramine N-β-D-glucuronide	0.3636	7	(Sharma and Hamelin, 2005)
Fluconazole-glucuronide	4.2048	6.5	(Brammer et al., 1991)
Hydrocodone-glucuronide	0	4	(Bluth, 2016)
Losartan carboxylic acid	9.96	14	(Lo et al., 1995)
Losartan Metabolite 1	9.96	nf	nf
Losartan Metabolite 2	9.96	nf	nf
α-Hydroxy metoprolol	3.726444	85	(Barclay et al., 2012)
Hydroxy metronidazole	19.55016	26.7	(Han and Lee, 2017)
4-Hydroxy omeprazole sulfide	3.67128	26	(Boix et al., 2014)
5-Hydroxy omeprazole	3.67128	26	(Boix et al., 2014)
Omeprazole sulfone N-oxide	3.67128	26	(Boix et al., 2014)
α-Hydroxy trimethoprim	5.49552	4.9	(Sigel et al., 1973)
Trimethoprim 1-N-oxide	5.49552	2.1	(Sigel et al., 1973)
Trimethoprim 3-N-oxide	5.49552	2.1	(Sigel et al., 1973)
D.L.O-Desmethyl venlafaxine	0.30375	29.4	(Gurke et al., 2015)
N.O-Didesmethyl venlafaxine β-D-glucuronide	0.30375	19	http://primarypsychiatry.com/desvenlafaxine-frequently-asked-questions/
Desalkyl verapamil D617	0.5088	6.7	(Han and Lee, 2017)

* calculated from data provided by the hospital for the parent compound;

nf: not found

Table S6. Predicted eco-toxicity data of the metabolites investigated based on the ECOSAR predictions for EC50 and LC50 towards *Green algae*, *Daphnid* and *Fish*.

Pharmaceutical	$EC50_{Green\ algae}$ (mg L^{-1})	$LC50_{Daphnid}$(mg L^{-1})	$LC50_{Fish}$(mg L^{-1})	$PNEC_{Green\ algae}$ (mg L^{-1})	$PNEC_{Daphnid}$(mg L^{-1})	$PNEC_{Fish}$(mg L^{-1})
4-AAA	2.53	6.91	6.05	0.00253	0.00691	0.00605
4-AA	2.03	5.53	4.74	0.00203	0.00553	0.00474
4-FAA	1.69	4.51	3.13	0.00169	0.00451	0.00313
4-MAA	1.68	4.5	3.25	0.00168	0.0045	0.00325
3-cysteinyl acetaminophen	539.54	67.29	6999.18	0.53954	0.06729	6.99918
3-methoxy acetaminophen	3.06	1.13	22.1	0.00306	0.00113	0.0221
Albendazole sulfoxide	4.05	76.5	157.35	0.00405	0.0765	0.15735
Atenolol acid	59071.87	26363.02	366460.03	59.07187	26.36302	366.46003
o-hydroxyatorvastatin	0.34	1.15	0.02	0.00034	0.00115	0.00002
p-hydroxyatorvastatin	0.67	2.83	0.1	0.00067	0.00283	0.0001
Descladinose	2.11	3.24	24.09	0.00211	0.00324	0.02409
N-desmethyl clindamycin	10.82	12.51	107.48	0.01082	0.01251	0.10748
Clindamycin sulfoxide	268.12	198.89	2138.43	0.26812	0.19889	2.13843
Codeine-glucuronide	182282.17	75252.78	1088074.25	182.28217	75.25278	1088.07425
Diphenhydramine N-oxide	4.78	5.85	48.81	0.00478	0.00585	0.04881
Diphenhydramine N-β-D-glucuronide	13017.81	29311.01	58499.24	13.01781	29.31101	58.49924
Fluconazole-glucuronide	2080.16	23855.29	99865.09	2.08016	23.85529	99.86509
Hydrocodone-glucuronide	610468.62	211527.97	3341637.75	610.46862	211.52797	3341.63775
Losartan carboxylic acid	1.08	5.79	0.39	0.00108	0.00579	0.00039
Losartan Metabolite 1	11.58	18.88	22.19	0.01158	0.01888	0.02219
Losartan Metabolite 2	11.58	18.88	22.19	0.01158	0.01888	0.02219
α-Hydroxy metoprolol	55.49	48.31	479.03	0.05549	0.04831	0.47903
Hydroxy metronidazole	23.19	852.41	10657.11	0.02319	0.85241	10.65711
4-Hydroxy omeprazole sulfide	0.28	2.27	0.46	0.00028	0.00227	0.00046
5-Hydroxy omeprazole	0.81	8.71	3.91	0.00081	0.00871	0.00391
Omeprazole sulfone N-oxide	4.76	86.19	158.24	0.00476	0.08619	0.15824
α-Hydroxy trimethoprim	70.87	16.71	2254.53	0.07087	0.01671	2.25453
Trimethoprim 1-N-oxide	31.69	8.97	461.06	0.03169	0.00897	0.46106
Trimethoprim 3-N-oxide	31.69	8.97	461.06	0.03169	0.00897	0.46106
D.L.O-Desmethyl venlafaxine	0.53	0.5	2.31	0.00053	0.0005	0.00231
N.O-Didesmethyl venlafaxine β-D-glucuronide	129153.97	55108.9	783629.88	129.15397	55.1089	783.62988
Desalkyl verapamil D617	0.21	0.37	0.65	0.00021	0.00037	0.00065

Table S7. *In silico* QSAR predictions for ready biodegradability (0 means not biodegradable and 1 biodegradable) of the pharmaceuticals parent compounds and human metabolites found in the raw HWW.

Compound	SMILES	CAS No	Log Kow Theor.	Log Kow Exp.	BioWin 1	BioWin 2	BioWin 3	BioWin 4	BioWin 5	BioWin 6	BioWin 7	BioWin RB Prediction
Dipyrone	O=S(CN(C1=C(C)N(C)N(c2ccccc2)C1(=O))C)(O)=O	68-89-3	-2.61		0.6305	0.9933	2.4709	3.2926	-0.2072	0.002	-0.7105	NO
4-AAA	CC(=O)(NC1=C(C)N(C)N(c2ccccc2)C1(=O))	83-15-8	-0.13		0.969	0.9823	2.6249	3.7042	0.1514	0.047	-0.2295	NO
4-AA	O=C1N(c2ccccc2)N(C)C(C)=C1N	83-07-8	-0.07		0.9327	0.954	2.7965	3.6027	0.1827	0.048	0.5953	NO
4-FAA	CC(N(N(C1(=O))c2ccccc2)C)=C1NC=O	-	0.5		0.9757	0.9854	2.6559	3.7244	0.1927	0.0675	-0.1499	NO
4-MAA	O=C1N(c2ccccc2)N(C)C(C)=C1NC	-	0.39		0.926	0.9444	2.7655	3.5824	0.1414	0.0332	0.5157	NO
Antipyrine/Phenazone	O=C1N(c2ccccc2)N(C)C(C)=C1	60-80-0	0.59	0.38	0.786	0.8943	2.8052	3.5811	0.2003	0.0962	0.3444	NO
Acetaminophen	O=C(C)Nc1ccc(O)cc1	103-90-2	0.27	0.46	1.0015	0.9886	2.8673	3.8748	0.4866	0.509	-0.1124	NO
3-cysteinyl acetaminophen	O=C(O)C(N)CSc1cc(NC(=O)(C))ccc1O	53446-10-9	-2.95		1.1713	0.9893	2.993	4.1317	0.3371	0.0876	0.2073	NO
3-methoxy acetaminophen	CC(=O)(Nc1ccc(O)c(OC)c1)	3251-55-6	0.09		1.1192	0.9981	2.7428	3.9086	**0.5847**	0.5965	0.0815	NO
Albendazole	O=C(OC)Nc1nc2ccc(SCCC)cc2n1	54965-21-8	3.14	3.07	0.7008	0.5623	2.5661	3.6586	0.0036	0.0306	0.4426	NO
Albendazole sulfoxide	O=C(OC)Nc1nc2ccc(S(CCC)=O)cc2n1	54029-12-8	0.97	1.27	0.6932	0.5058	2.5308	3.6355	-0.044	0.0195	0.4426	NO
Atenolol	O=C(Cc1ccc(OCC(CNC(C)C)O)cc1)N	29122-68-7	-0.03	0.16	1.33	0.9991	2.6078	3.8502	0.4115	0.2349	-0.1861	NO
Atenolol acid	CC(C)NCC(O)COc1ccc(CC(=O)(O))cc1	56392-14-4	-2.34		1.1921	0.9927	3.0245	4.0289	0.4631	0.2768	0.5686	NO
Atorvastatin	O=C(O)CC(O)CC(O)CCn1c(c2ccc(F)cc2)c(c3ccccc3)c(C(=O)(Nc4ccccc4))c1C(C)C	134523-00-5	6.36		0.5827	0.0033	2.1572	3.8463	-0.1	0	-0.6474	NO
o-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn1c(c2ccc(F)cc2)c(c3ccccc3)c(C(=O)(Nc4ccccc4O))c1C(C)C	-	6.18		0.5628	0.0011	2.1562	3.8581	-0.0916	0	-0.6894	NO
p-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn1c(c2ccc(F)cc2)c(c3ccccc3)c(C(=O)(Nc4ccc(O)cc4))c1C(C)C	-	5.53		0.5628	0.0011	2.1562	3.8581	-0.0916	0	-0.6894	NO
Azithromycin	O=C1C(C)C(OC2OC(C)C(O)C(C)(OC)C2)C(C)C(OC3OC(C)CC(N(C)C)C3O)C(C)(O)CC(C)CN(C)C(C)C(O)C(C)(O)C(CC)O1	83905-01-5	3.24	4.02	-1.6578	0	0.9748	2.2994	-0.3277	0	-4.0087	NO
Azithromycin metabolite 591 (Descladinose)	CCC1C(C)(O)C(O)C(C)N(C)CC(C)CC(C)(O)C(OC2C(O)C(N(C)C)CC(C)O2)C(C)C(O)C(C)C(=O)(O1)	-	3.02		-0.3565	0	1.5625	2.7103	0.0126	0	-2.6624	NO
Clindamycin	CLC(C(C1OC(C(C(C1O)O)O)SC)NC(=O)(C2N(CC(C2)CCC)C))C	18323-44-9	2.01	2.16	0.5676	0.0111	2.2491	3.43	0.1013	0.0008	-1.2421	NO
N-desmethyl clindamycin	O=C(C1NCC(CCC)C1)NC(C2C(O)C(O)C(O)C(SC)O2)C(CL)C	22431-45-4	1.8		0.9333	0.278	2.5593	3.7815	0.2607	0.0021	0.0896	NO
Clindamycin sulfoxide	O=C(C1N(C)CC(CCC)C1)NC(C2OC(S(C)=O)C(O)C(O)C2O)C(CL)C	22431-46-5	-0.13		0.5599	0.0089	2.2137	3.4069	0.0537	0.0005	-1.2421	NO
Codeine	COc1c2OC3C45c2c(cc1)CC(N(CC5)C)C4C=CC3O	76-57-3	1.28	1.19	0.6931	0.7316	2.0396	3.1895	0.4191	0.0606	-0.7853	NO
Codeine-glucuronide	CN1CCC23C4C1Cc5c2c(OC3C(OC6C(O)C(O)C(O)C(C(=O)(O))O6)C=C4)c(OC)cc5	20736-11-2	-2.68		0.3046	0.0042	2.3175	3.5604	0.4637	0.0039	-0.6502	NO
Diphenhydramine	CN(C)CCOC(C1=CC=CC=C1)C2=CC=CC=C2	58-73-1	3.11	3.27	0.3295	0.0647	2.4154	3.1914	0.0412	0.0313	-1.088	NO
Diphenhydramine N-oxide	C[N+](CCOC(C1=CC=CC=C1)C2=CC=CC=C2)([O-])C	3922-74-5	2.34		0.5272	0.3372	2.6348	3.4563	0.0691	0.0574	-0.4509	NO
Diphenhydramine N-β-D-glucuronide	C[N+](C)(CCOC(C1=CC=CC=C1)C2=CC=CC=C2)C3C(C(C(C(O3)C(=O)[O-])O)O)O	137908-78-2	0.65		0.6515	0.0823	3.1124	3.9865	0.315	0.0165	0.0742	NO
Fluconazole	Fc1cc(F)c(C(O)(Cn2ncnc2)Cn3ncnc3)cc1	86386-73-4	0.25	0.5	-1.2022	0	1.4963	3.2793	0.0597	0	-0.1142	NO

Compound	SMILES	CAS No	Log Kow Theor.	Log Kow Exp.	BioWin 1	BioWin 2	BioWin 3	BioWin 4	BioWin 5	BioWin 6	BioWin 7	BioWin RB Prediction
Fluconazole-glucuronide	Fc1cc(F)c(C(Cn2cncn2)(OC3C(O)C(O)C(O)C(C(=O)(O))O3)Cn4cncn4)cc1	136134-23-1	-0.5		-1.4319	0	1.9343	3.7797	0.2655	0	0.1536	NO
Hydrocodone	CN1CCC23C4C1Cc5c2c(c(cc5)OC)OC3C(=O)CC4	125-29-1	2.16		0.5412	0.3617	1.8571	3.0379	0.3903	0.0858	-1.4424	NO
Hydrocodone-glucuronide	CN1CCC23C4C1Cc5c2c(OC3C(=O)(CC4))c(OC6C(C(C(C(=O)(O))O6)O)O)O)cc5	-	-3.44		0.6655	0.0909	2.3347	3.5682	**0.6359**	0.0248	-0.8377	NO
Losartan	OCc1c(nc(n1Cc2ccc(c3c(c4nnnn4)cccc3)cc2)CCCC)CL	114798-26-4	4.01		0.6856	0.1864	2.4414	3.4022	-0.3808	0.0006	-0.6786	NO
Losartan carboxylic acid	CCCCc1nc(c(n1Cc2ccc(cc2)c3ccccc3c4nnnn4)C(=O)O)CL	124750-92-1	4.81		0.6971	0.4091	2.3384	3.2603	-0.1509	0.0019	-0.5385	NO
Losartan Metabolite 1	OCc1c(nc(n1Cc2ccc(c3c(c4nnnn4)cccc3)cc2)CCC(O)C)CL	141675-57-2	2.88		0.7283	0.0812	2.2677	3.2395	-0.3674	0.0006	-0.42	NO
Losartan Metabolite 2	OCc1c(nc(n1Cc2ccc(c3c(c4nnnn4)cccc3)cc2)C(O)CCC)CL	141675-59-4	2.88		0.6736	0.0473	2.3426	3.308	-0.2214	0.0015	-0.0733	NO
Metoprolol	COCCc1ccc(cc1)OCC(CNC(C)C)O	37350-58-6	1.69	1.88	0.772	0.6976	2.6511	3.6336	0.3331	0.1475	0.0709	NO
α-Hydroxy metoprolol	CC(C)NCC(COc1ccc(cc1)C(COC)O)O	56392-16-6	0.56		0.8685	0.7594	2.8505	3.8085	0.4926	0.3046	0.3586	NO
Metronidazole	Cc1ncc(n1CCO)N(=O)(=O)	443-48-1	0	-0.02	0.5744	0.4414	2.7365	3.5533	0.325	0.0739	0.3396	NO
Hydroxy metronidazole	c1c(n(c(n1)CO)CCO)N(=O)(=O)	4812-40-2	-1.06		0.6709	0.5195	2.9359	3.7282	0.3414	0.0818	0.8368	NO
Omeprazole	Cc1cnc(c(c1OC)C)CS(=O)c2nc3c(n2)cc(cc3)OC	73590-58-6	3.4	2.23	0.8017	0.8925	1.9557	3.3478	0.1024	0.014	0.5414	NO
4-Hydroxy omeprazole sulfide	Oc1c(C)c(CSc2nc3ccc(OC)cc3n2)ncc1C	**103876-98-8**	3.59		0.7999	0.7691	2.1366	3.3537	0.0603	0.0142	0.5237	NO
5-Hydroxy omeprazole	COc1ccc2c(nc(SCc3ncc(CO)c(OC)c3C)n2)c1	103876-99-9	2.69		0.9058	0.9345	2.1905	3.5458	0.1664	0.0246	1.0386	NO
Omeprazole sulfone N-oxide	O=S(c1nc2ccc(OC)cc2n1)(Cc3c(C)c(OC)c(C)cn3=O)=O	158812-85-2	-0.56		0.941	0.9644	2.0992	3.3204	0.0407	0.0089	-0.0996	NO
Trimethoprim	COc1cc(cc(c1OC)OC)Cc2cnc(nc2N)N	738-70-5	0.73	0.91	0.5922	0.9164	2.0385	3.3749	0.0889	0.0172	0.1677	NO
α-Hydroxy trimethoprim	OC(c1cc(OC)c(OC)c(OC)c1)c2cnc(N)nc2N	29606-06-2	-0.81		0.6887	0.9375	2.2379	3.5498	0.2484	0.0424	0.4553	NO
Trimethoprim 1-N-oxide	COc1c(OC)c(OC)cc(Cc2cn(=O)c(N)nc2N)c1	27653-68-5	0.58		0.5846	0.8972	2.0031	3.3519	0.0413	0.0109	0.1677	NO
Trimethoprim 3-N-oxide	COc1c(OC)c(OC)cc(Cc2cnc(N)n(=O)c2N)c1	27653-67-4	1.58		0.5846	0.8972	2.0031	3.3519	0.0413	0.0109	0.1677	NO
Venlafaxine	CN(C)CC(c1ccc(cc1)OC)C2(CCCCC2)O	93413-69-5	3.28		0.4129	0.1139	1.9862	3.0147	0.2373	0.1009	-1.6709	NO
D,L,O-Desmethyl venlafaxine	CN(CC(C1(O)CCCCC1)c2ccc(O)cc2)C	-	2.72		0.4034	0.0395	2.1317	2.9975	0.1476	0.0668	-1.6886	NO
N,O-Didesmethyl venlafaxine β-D-glucuronide	CNCC(c1ccc(cc1)OC2C(C(C(C(O2)C(=O)O)O)O)O)C3(CCCCC3)O	-	-2.54		0.903	0.4368	2.7741	3.8965	**0.6423**	0.0722	0.2655	YES
Verapamil	CC(C)C(CCCN(C)CCc1cc(c(cc1)OC)OC)(C(#N))c2cc(c(cc2)OC)OC	52-53-9	4.8	3.79	1.0312	0.9989	1.3379	2.9251	0.3385	0.0405	-1.1742	NO
Desalkyl verapamil D617	CC(C)C(CCCN(C)CCc1cc(c(cc1)O)OC)(C(#N))c2cc(c(cc2)OC)OC	77326-93-3	4.5		1.0218	0.9966	1.4834	2.908	0.2488	0.0263	-1.1919	NO
Aciclovir	c1nc2c(n1COCCO)N=C(NC2(=O))N	59277-89-3	-1.7	-1.56	0.6618	0.5475	2.7986	3.848	0.4878	0.3134	0.1263	NO
Atazanavir	CC(C)(C)C(C(=O)NC(Cc1ccccc1)C(CN(Cc2ccc(cc2)c3ccccn3)NC(=O)C(C(C)(C)C)NC(=O)OC)O)NC(=O)OC	198904-31-3	2.88		0.6003	0.0202	0.9626	3.1637	-1.6098	0	-2.0519	NO
Bupivacaine	CCCCN1CCCCC1C(=O)Nc2c(cccc2C)C	38396-39-3	3.44	3.41	0.8329	0.9152	2.4014	3.481	0.2434	0.0656	-2.5964	NO
Carbamazepine	c1ccc2c(c1)C=Cc3ccccc3N2C(=O)N	298-46-4	2.25	2.45	0.6351	0.4143	2.677	3.5068	0.0873	0.0364	-0.0744	NO
Cefepime	CN1(H)(CCCC1)CC2=C(N3C(C(C3(=O))NC(=O)C(=NOC)c4csc(n4)N)SC2)C(=O)O	88040-23-7	0.2		0.777	0.5683	2.254	3.8395	-0.2741	0.0003	-1.5782	NO

Compound	SMILES	CAS No	Log Kow Theor.	Log Kow Exp.	BioWin 1	BioWin 2	BioWin 3	BioWin 4	BioWin 5	BioWin 6	BioWin 7	BioWin RB Prediction
Cetirizine	C1CN(CCN1CCOCC(=O)O)C(c2ccccc2)c3ccc(cc3)CL	83881-51-0	-0.61	1.7	-0.1771	0	2.0015	2.926	-0.1343	0.0025	-2.7961	NO
Ciprofloxacin	C1CC1N2C=C(C(=O)c3cc(c(cc32)N4CCNCC4)F)C(=O)O	85721-33-1	0	0.28	-0.3974	0	1.917	3.2138	0.0597	0.0001	-2.2865	NO
Diazepam	CN1C(=O)CN=C(c2c1ccc(c2)CL)c3ccccc3	439-14-5	2.7	2.82	0.7678	0.8085	2.3311	3.4819	0.0837	0.0217	-0.8789	NO
Metformin	CN(C)C(=N)N=C(N)N	1115-70-4	-1.4		0.6861	0.764	2.9137	3.6614	0.3287	0.2379	0.6769	NO
Gabapentin	C1CCC(CC1)(CC(=O)O)CN	60142-96-3	-1.37	-1.1	0.7086	0.6472	2.9977	3.8761	**0.6822**	0.7053	0.3178	YES
Levofloxacin	CC1COc2c3N1C=C(C(=O)c3cc(c2N4CCN(CC4)C)F)C(=O)O	100986-85-4	-0.2	-0.39	-0.6388	0	1.5132	2.9163	0.0204	0.0001	-3.3043	NO
Lidocaine	CCN(CC)CC(=O)Nc1c(cccc1C)C	137-58-6	1.66	2.44	0.7502	0.7864	2.2226	3.29	0.3134	0.1311	-1.9177	NO
Lincomycin	CCCC1CC(N(C1)C)C(=O)NC(C2C(C(C(C(O2)SC)O)O)O)C(C)O	154-21-2	0.29	0.2	0.8465	0.222	2.623	3.6867	0.3162	0.007	-1.0947	NO
Metoclopramide	CCN(CC)CCNC(=O)C1=CC(=C(C=C1OC)N)Cl	364-62-5	1.69	2.62	0.3254	0.0791	1.828	3.136	0.1211	0.0149	-1.6343	NO
Morphine	CN1CCC23C4C1CC5=C2C(=C(C=C5)O)OC3C(C=C4)O	57-27-2	0.72	0.89	0.6836	0.4657	2.1851	3.1723	0.3294	0.0395	-0.803	NO
Nystatin	CC1C=CC=CCCC=CC=CC=CC=CC(CC2C(C(CC(O2)(CC(C(CCC(CC(CC(CC(=O)OC(C(C1O)C)C)O)O)O)O)O)O)O)C(=O)O)OC3C(C(C(C(O3)C)O)N)O	1400-61-9	-3.33		0.9099	0.0019	2.8834	4.1518	0.4083	0	-0.6403	NO
Ofloxacin	CC1COC2=C3N1C=C(C(=O)C3=CC(=C2N4CCN(CC4)C)F)C(=O)O	82419-36-1	-0.2	-0.39	-0.6388	0	1.5132	2.9163	0.0204	0.0001	-3.3043	NO
Ondansetron	CC1=NC=CN1CC2CCC3=C(C2=O)C4=CC=CC=C4N3C	99614-02-5	3.95		0.724	0.3879	2.3787	3.2652	0.0742	0.0253	-0.7561	NO
Paliperidone	CC1=C(C(=O)N2CCCC(C2=N1)O)CCN3CCC(CC3)C4=NOC5=C4C=CC(=C5)F	144598-75-4	1.95		-0.0472	0	1.6258	3.2242	-0.0717	0.0001	-1.8698	NO
Pregabalin	CC(C)CC(CC(=O)O)CN	148553-50-8	-1.78		0.8983	0.9242	3.2363	4.0469	0.5006	0.4767	0.7873	YES
Propranolol	CC(C)NCC(COC1=CC=CC2=CC=CC=C21)O	235-867-6	2.6	3.48	1.0685	0.9782	2.7523	3.7234	0.3861	0.201	0.2173	NO
Quetiapine	C1CN(CCN1CCOCCO)C2=NC3=CC=CC=C3SC4=CC=CC=C42	111974-69-7	1.94		0.1711	0.0009	2.2482	3.1261	-0.0087	0.0077	-1.5028	NO
Ranitidine	CNC(=C[N+](=O)[O-])NCCSCC1=CC=C(O1)CN(C)C	66357-35-5	0.29	0.27	0.7003	0.1886	2.2985	3.1927	-0.2302	0.0009	-0.35	NO
Sulfamethoxazole	CC1=CC(=NO1)NS(=O)(=O)C2=CC=C(C=C2)N	723-46-6	0.48	0.89	0.4479	0.1281	2.4297	3.3054	-0.1165	0.006	-0.2907	NO
Sulpiride	CCN1CCCC1CNC(=O)C2=C(C=CC(=C2)S(=O)(=O)N)OC	15676-16-1	0.65	0.57	0.7218	0.7062	2.0775	3.3497	0.1294	0.0159	-1.3427	NO
Tramadol	CN(C)CC1CCCCC1(C2=CC(=CC=C2)OC)O	27203-92-5	3.01	2.51/2.63	0.3649	0.081	2.0921	3.1034	0.2815	0.0823	-1.43	NO
DL-Tyrosine	C1=CC(=CC=C1CC(C(=O)O)N)O	556-03-6	-1.76	0.950757576	1.0583	0.9753	3.1693	3.9863	0.3783	0.2478	0.6115	NO

Table S8. *In silico* QSAR prediction of PBT values by the Prometheus software for the pharmaceuticals and human metabolites investigated in this study.

Compounds	SMILES	LogP	LogP rel.	P	P rel.	B	B rel.	T	T rel.	Score P	Score B	Score T	PBT	PB
Dipyrone	O=C1C(=C(N(N1c2ccccc2)C)C)N(C)CS(=O)(=O)[O-]	1.28	0.2	P/vP	0.5	1.17	0.8	0.101	0.3	0.712	0.166	0.499	**0.371**	0.344
4-AAA	O=C(NC=2C(=O)N(c1ccccc1)N(C=2C)C)C	1.72	0.2	P/vP	0.5	1.24	0.8	1.89	0.3	0.712	0.175	0.346	**0.351**	0.353
4-AA	O=C1C(N)=C(N(N1c2ccccc2)C)C	1.03	0.2	P/vP	0.5	0.56	0.4	11	0.3	0.712	0.235	0.288	**0.381**	0.409
4-FAA	O=CNC=2C(=O)N(c1ccccc1)N(C=2C)C	3.84	0.2	P/vP	0.5	1.2	0.4	10	0.3	0.712	0.273	0.291	**0.406**	0.441
4-MAA	O=C2C(NC)=C(N(N2(c1ccccc1))C)C	2.24	0.2	P/vP	0.5	0.88	0.4	1.93	0.3	0.712	0.252	0.345	**0.406**	0.423
Antipyrine/Phenazone	O=C1C=C(N(N1c2ccccc2)C)C	0.38	1	nP	0.5	0.75	0.4	3.51	0.3	0.359	0.244	0.322	**0.301**	0.296
Acetaminophen	O=C(Nc1ccc(O)cc1)C	0.46	1	nP/P	0.5	0.39	0.8	54	0.7	0.571	0.102	0.133	**0.214**	0.242
3-cysteinyl acetaminophen	O=C(O)C(N)CSc1cc(ccc1(O))NC(=O)C	0.35	0.4	P/vP	0.5	0.47	0.4	2.39	0.3	0.712	0.23	0.336	**0.39**	0.405
3-methoxy acetaminophen	O=C(Nc1ccc(O)c(OC)c1)C	0.75	0.4	vP	0.5	0.15	0.8	8.01	0.4	0.854	0.09	0.266	**0.274**	0.277
Albendazole	O=C(OC)Nc1nc2ccc(cc2([nH]1))SCCC	2.52	0.4	P/vP	0.5	1.35	0.4	0.135	0.4	0.712	0.286	0.479	**0.457**	0.451
Albendazole sulfoxide	O=C(OC)Nc1nc2ccc(cc2([nH]1))S(=O)CCC	2.01	0.4	P/vP	0.5	0.62	0.4	0.057	0.3	0.712	0.237	0.533	**0.433**	0.411
Atenolol	O=C(N)Cc1ccc(OCC(O)CNC(C)C)cc1	0.16	1	P/vP	0.5	0.86	0.8	1.43	0.4	0.712	0.136	0.335	**0.316**	0.311
Atenolol acid	O=C(O)Cc1ccc(OCC(O)CNC(C)C)cc1	1.15	0.8	nP/P	0.7	0.87	0.8	1.44	0.4	0.584	0.137	0.335	**0.292**	0.282
Atorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccccc3)c4C(C)C	6.66	0.2	P/vP	0.5	0.97	0.4	-	0.5	0.712	0.257	0.5	**0.442**	0.428
o-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccccc3(O))c4C(C)C	6.18	0.2	nP	0.5	0.99	0.8	-	0.5	0.359	0.148	0.5	**0.269**	0.23
p-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccc(O)cc3)c4C(C)C	5.47	0.2	nP	0.5	1.07	0.8	-	0.5	0.359	0.156	0.5	**0.275**	0.237
Azithromycin	O=C3OC(CC)C(O)(C)C(O)C(N(C)CC(C)CC(O)(C)C(OC1OC(C)CC(N(C)C)C1(O))C(C)C(OC2OC(C)C(O)C(OC)(C)C2)C3C)C	4.02	1	nP	0.5	0.86	0.4	0.351	0.3	0.359	0.25	0.427	**0.322**	0.3
Azithromycin metabolite 591	O=C2OC(CC)C(O)(C)C(O)C(N(C)CC(C)CC(O)(C)C(OC1OC(C)CC(N(C)C)C1(O))C(C)C(O)C2C)C	1.02	0.2	nP	0.5	1.22	0.8	0.953	0.3	0.359	0.172	0.376	**0.27**	0.249
Clindamycin	O=C(NC(C1OC(C(O)C(O)C1(O))SC)C(C)Cl)C2N(C)CC(CCC)C2	2.16	1	nP	0.5	0.62	0.4	0.37	0.3	0.359	0.237	0.424	**0.314**	0.292
N-desmethyl clindamycin	O=C(NC(C1OC(C(O)C(O)C1(O))SC)C(C)Cl)C2NCC(CCC)C2	-0.6	0.2	nP	0.5	0.38	0.4	0.366	0.3	0.359	0.227	0.425	**0.309**	0.285
Clindamycin sulfoxide	O=C(NC(C1OC(C(O)C(O)C1(O))S(=O)C)C(C)Cl)C2N(C)CC(CCC)C2	-2.53	0.2	nP	0.5	0.39	0.4	0.573	0.3	0.359	0.227	0.401	**0.305**	0.285
Codeine	OC1C=CC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4)	1.19	1	nP	0.7	1.18	0.4	0.131	0.6	0.333	0.272	0.477	**0.33**	0.301
Codeine-glucuronide	O=C(O)C6OC(OC1C=CC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4))C(O)C(O)C6(O)	0.54	0.2	nP	0.5	1.68	0.8	-	0.5	0.359	0.234	0.5	**0.323**	0.29
Diphenhydramine	O(CCN(C)C)C(c1ccccc1)c2ccccc2	3.27	1	nP	0.5	2.02	0.4	0.241	0.3	0.359	0.356	0.448	**0.374**	0.357
Diphenhydramine N-oxide	[O-][N+](C)(C)CCOC(c1ccccc1)c2ccccc2	-1.66	0.2	nP/P	0.5	1.13	0.4	0.163	0.3	0.571	0.268	0.471	**0.406**	0.391
Diphenhydramine N-β-D-glucuronide	O=C([O-])C1OC(C(O)C(O)C1(O))[N+](C)(C)CCOC(c2ccccc2)c3ccccc3	-1.94	0.2	nP/P	0.5	0.7	0.4	-	0.5	0.571	0.241	0.5	**0.394**	0.371
Fluconazole	Fc1ccc(c(F)c1)C(O)(Cn2ncnc2)Cn3ncnc3	0.25	0.2	vP	0.5	1.72	0.8	-	0.5	0.854	0.24	0.5	**0.462**	0.453
Fluconazole-glucuronide	O=C(O)C4OC(OC(c1ccc(F)cc1(F))(Cn2ncnc2)Cn3ncnc3)C(O)C(O)C4(O)	-1.5	0.2	P/vP	0.5	2.28	0.8	-	0.5	0.712	0.341	0.5	**0.494**	0.493
Hydrocodone	O=C1CCC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4)	1.9	0.4	nP	0.7	1.36	0.4	0.094	0.6	0.333	0.287	0.505	**0.341**	0.309
Hydrocodone-glucuronide	O=C(O)C6OC(Oc5ccc4c1c5(OC2C(=O)CCC3C(N(C)CCC123)C4))C(O)C(O)C6(O)	-1.4	0.2	nP	0.5	2.11	0.8	-	0.5	0.359	0.307	0.5	**0.36**	0.332
Losartan	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))CCCC)Cl	3.25	0.4	nP	0.5	1.54	0.4	0.00007	0.3	0.359	0.303	0.752	**0.389**	0.33
Losartan carboxylic acid	O=C(O)c1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nn[nH]n3))CCCC)Cl	3.91	0.2	nP	0.5	1.16	0.4	0.00007	0.3	0.359	0.271	0.752	**0.372**	0.312
Losartan Metabolite 1	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))CCC(O)C)Cl	1.98	0.2	nP	0.5	1.14	0.4	0.00007	0.3	0.359	0.269	0.752	**0.37**	0.31

Losartan Metabolite 2	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))C(O)CCC)Cl	2.31	0.4	nP	0.5	1.18	0.4	0.00007	0.3	0.359	0.272	0.752	**0.372**	0.312
Metoprolol	OC(COc1ccc(cc1)CCOC)CNC(C)C	1.88	1	nP	0.7	0.62	0.8	0.922	0.3	0.333	0.117	0.377	**0.225**	0.197
α-Hydroxy metoprolol	OC(c1ccc(OCC(O)CNC(C)C)cc1)COC	0.78	0.8	nP/P	0.7	0.3	0.4	2.39	0.3	0.584	0.224	0.336	**0.356**	0.361
Metronidazole	O=[N+]([O-])c1cnc(n1CCO)C	-0.12	0.8	nP	0.9	0.5	0.8	16	0.3	0.31	0.109	0.28	**0.2**	0.184
Hydroxy metronidazole	O=[N+]([O-])c1cnc(n1CCO)CO	-0.89	0.8	nP	0.5	0.54	0.8	1.83	0.3	0.359	0.112	0.347	**0.223**	0.2
Omeprazole	O=S(c1nc2ccc(OC)cc2([nH]1))Cc3ncc(c(OC)c3C)C	2.23	1	P/vP	0.5	0.88	0.4	0.181	0.3	0.712	0.252	0.465	**0.431**	0.423
4-Hydroxy omeprazole sulfide	Oc1c(cnc(c1C)CSc2nc3ccc(OC)cc3([nH]2))C	3	0.2	nP	0.9	2.4	0.8	0.201	0.3	0.31	0.365	0.459	**0.358**	0.336
5-Hydroxy omeprazole	OCc1cnc(c(c1(OC))C)CSc2nc3cc(OC)ccc3([nH]2)	3.14	0.4	P/vP	0.5	1.98	0.8	0.361	0.3	0.712	0.283	0.426	**0.444**	0.449
Omeprazole sulfone N-oxide	O=S(=O)(c1nc2ccc(OC)cc2([nH]1))Cc3c(c(OC)c(c[n+]3([O-]))C)C	2.42	0.4	P/vP	0.5	0.65	0.4	0.003	0.3	0.712	0.239	0.682	**0.456**	0.412
Trimethoprim	n1cc(c(nc1N)N)Cc2cc(OC)c(OC)c(OC)c2	0.91	1	nP	0.5	1.96	0.8	0.196	0.4	0.359	0.279	0.454	**0.34**	0.316
α-Hydroxy trimethoprim	OC(c1cc(OC)c(OC)c(OC)c1)c2cnc(nc2(N))N	0.58	0.4	nP	0.5	0.79	0.4	0.622	0.4	0.359	0.247	0.381	**0.312**	0.297
Trimethoprim 1-N-oxide	[O-][n+]1cc(c(nc1(N))N)Cc2cc(OC)c(OC)c(OC)c2	0.83	0.4	nP	0.9	0.99	0.4	0.625	0.4	0.31	0.259	0.38	**0.301**	0.283
Trimethoprim 3-N-oxide	[O-][n+]1c(ncc(c1(N))Cc2cc(OC)c(OC)c(OC)c2)N	0.83	0.4	nP	0.9	0.99	0.4	0.625	0.4	0.31	0.259	0.38	**0.3**	0.283
Venlafaxine	OC1(CCCCC1)C(c2ccc(OC)cc2)CN(C)C	3.15	0.4	nP/P	0.7	1.53	0.4	0.17	0.4	0.584	0.302	0.464	**0.428**	0.42
D,L,O-Desmethyl venlafaxine	Oc1ccc(cc1)C(CN(C)C)C2(O)(CCCCC2)	2.6	0.8	nP/P	0.7	2.16	0.8	0.143	0.3	0.584	0.316	0.479	**0.439**	0.43
N,O-Didesmethyl venlafaxine β-D-glucuronide	O=C(O)C3OC(Oc1ccc(cc1)C(CNC)C2(O)(CCCCC2))C(O)C(O)C3(O)	-0.34	0.2	nP/P	0.5	1.12	0.4	-	0.5	0.571	0.267	0.5	**0.41**	0.391
Verapamil	N#CC(c1ccc(OC)c(OC)c1)(CCCN(C)CCc2ccc(OC)c(OC)c2)C(C)C	3.79	1	nP	0.7	0.64	0.8	-	0.5	0.333	0.119	0.5	**0.239**	0.199
Desalkyl verapamil D617	N#CC(c1ccc(OC)c(OC)c1)(CCCN(C)CCc2ccc(O)c(OC)c2)C(C)C	3.03	0.4	nP	0.9	0.68	0.8	-	0.5	0.31	0.121	0.5	**0.235**	0.194
Aciclovir	O=C2NC(=Nc1c2(ncn1COCCO))N	-0.63	0.4	P/vP	0.5	0.27	0.4	10	0.3	0.712	0.222	0.291	**0.374**	0.398
Atazanavir	O=C(OC)NC(C(=O)NN(Cc1ccc(cc1)c2ncccc2)CC(O)C(NC(=O)C(NC(=O)OC)C(C)(C)C)Cc3ccccc3)C(C)(C)C	4.81	0.4	nP	0.5	0.57	0.8	-	0.5	0.359	0.113	0.5	**0.242**	0.202
Bupivacaine	O=C(Nc1c(cccc1C)C)C2N(CCCC)CCCC2	3.11	0.4	P/vP	0.5	1.07	0.8	0.038	0.4	0.712	0.156	0.565	**0.37**	0.333
Carbamazepine	O=C(N)N2c3ccccc3(C=Cc1ccccc12)	2.45	1	P/vP	0.5	1.26	0.4	0.817	0.3	0.712	0.278	0.383	**0.432**	0.445
Cefepime	O=C([O-])C4=C(C[N+]1(C)(CCCC1))CSC3N4(C(=O)C3(NC(=O)C(=NOC)c2nc(N)sc2))	-5	0.2	nP	0.5	0.16	0.4	-	0.5	0.359	0.219	0.5	**0.314**	0.28
Cetirizine	O=C(O)COCCN1CCN(CC1)C(c2ccccc2)c3ccc(cc3)Cl	2.59	0.2	P/vP	0.5	1.15	0.4	-	0.5	0.712	0.27	0.5	**0.45**	0.438
Ciprofloxacin	O=C(O)C1=CN(c2cc(c(F)cc2(C1(=O)))N3CCNCC3)C4CC4	-1.03	0.4	nP/P	0.7	1	0.4	0.069	0.3	0.584	0.259	0.522	**0.413**	0.389
Diazepam	O=C1N(c3ccc(cc3(C(=NC1)c2ccccc2))Cl)C	2.82	1	P/vP	0.5	1.57	0.4	0.087	0.3	0.712	0.306	0.509	**0.475**	0.467
Metformin	N=C(N=C(N)N)N(C)C	-0.6	0.4	-	0.5	0.14	0.8	425	0.3	0.5	0.089	0.24	**0.217**	0.211
Gabapentin	O=C(O)CC1(CN)(CCCCC1)	-1.1	1	nP	0.7	0.3	0.8	1.99	0.4	0.333	0.097	0.319	**0.202**	0.18
Levofloxacin	O=C(O)C2=CN1c3c(OCC1C)c(c(F)cc3(C2(=O)))N4CCN(C)CC4	-0.39	1	nP	0.9	0.56	0.4	-	0.5	0.31	0.235	0.5	**0.305**	0.27
Lidocaine	O=C(Nc1c(cccc1C)C)CN(CC)CC	2.44	1	P/vP	0.5	1.01	0.8	0.365	0.4	0.712	0.15	0.413	**0.342**	0.327
Lincomycin	O=C(NC(C(O)C)C1OC(C(O)C(O)C1(O))SC)C2N(C)CC(CCC)C2	0.56	1	nP	0.5	0.45	0.4	0.376	0.3	0.359	0.229	0.423	0.31	0.287
Metoclopramide	O=C(NCCN(CC)CC)c1cc(c(N)cc1(OC))Cl	2.62	1	vP	0.5	1.57	0.8	1.19	0.4	0.854	0.217	0.345	0.412	0.431
Morphine	Oc5ccc4c1c5(OC2C(O)C=CC3C(N(C)CCC123)C4)	0.89	1	nP	0.7	1.15	0.4	0.132	0.6	0.333	0.27	0.477	0.329	0.3
Nystatin	O=C(O)C3C(O)CC2(O)(OC3(CC(OC1OC(C)C(O)C(N)C1(O))C=CC=CC=CCCC=CC=CC(C)C(O)C(C)C(OC(=O)CC(O)CC(O)CCC(O)C(O)C2)C))	-0.7	0.2	nP	0.5	0.54	0.4	-	0.5	0.359	0.234	0.5	0.323	0.289
Ofloxacin	O=C(O)C2=CN1c3c(OCC1C)c(c(F)cc3(C2(=O)))N4CCN(C)CC4	-0.39	1	nP	0.9	0.56	0.4	-	0.5	0.31	0.235	0.5	0.305	0.27
Ondansetron	O=C3c2c1ccccc1n(c2CCC3Cn4ccnc4C)C	2.68	0.4	vP	0.5	2.03	0.8	0.799	0.3	0.854	0.293	0.384	0.474	0.5

Paliperidone	O=C1C(=C(N=C2N1CCCC2(O))C)CCN5CCC(c4noc3cc(F)ccc34)CC5	3.75	0.2	nP	0.5	1.5	0.4	0.045	0.3	0.359	0.299	0.546	0.363	0.327
Pregabalin	O=C(O)CC(CN)CC(C)C	-1.78	0.4	nP	0.7	0.5	0.8	8.01	0.4	0.333	0.109	0.266	0.204	0.19
Propranolol	OC(COc1cccc2ccccc12)CNC(C)C	3.48	1	nP	0.5	1.89	0.8	1.22	0.6	0.359	0.267	0.308	0.309	0.309
Quetiapine	OCCOCCN4CCN(C2=Nc1ccccc1Sc3ccccc23)CC4	-0.09	0.2	P/vP	0.5	0.99	0.4	0.114	0.3	0.712	0.258	0.492	0.441	0.429
Ranitidine	O=[N+]([O-])C=C(NC)NCCSCc1oc(cc1)CN(C)C	0.27	1	nP/P	0.5	0.39	0.4	-	0.5	0.571	0.227	0.5	0.384	0.36
Sulfamethoxazole	O=S(=O)(Nc1noc(c1)C)c2ccc(N)cc2	0.89	1	P/vP	0.5	0.38	0.4	4	0.4	0.712	0.227	0.29	0.376	0.402
Sulpiride	O=C(NCC1N(CC)CCC1)c2cc(ccc2(OC))S(=O)(=O)N	0.57	1	nP	0.7	0.59	0.4	0.172	0.4	0.333	0.236	0.463	0.31	0.28
Tramadol	OC2(c1cccc(OC)c1)(CCCCC2(CN(C)C))	2.57	1	nP/P	0.7	1.31	0.4	0.236	0.3	0.584	0.282	0.45	0.414	0.406
DL-Tyrosine	O=C(O)C(N)Cc1ccc(O)cc1	-2.15	1	nP	0.7	0.31	0.8	3.02	0.4	0.333	0.097	0.301	0.2	0.18

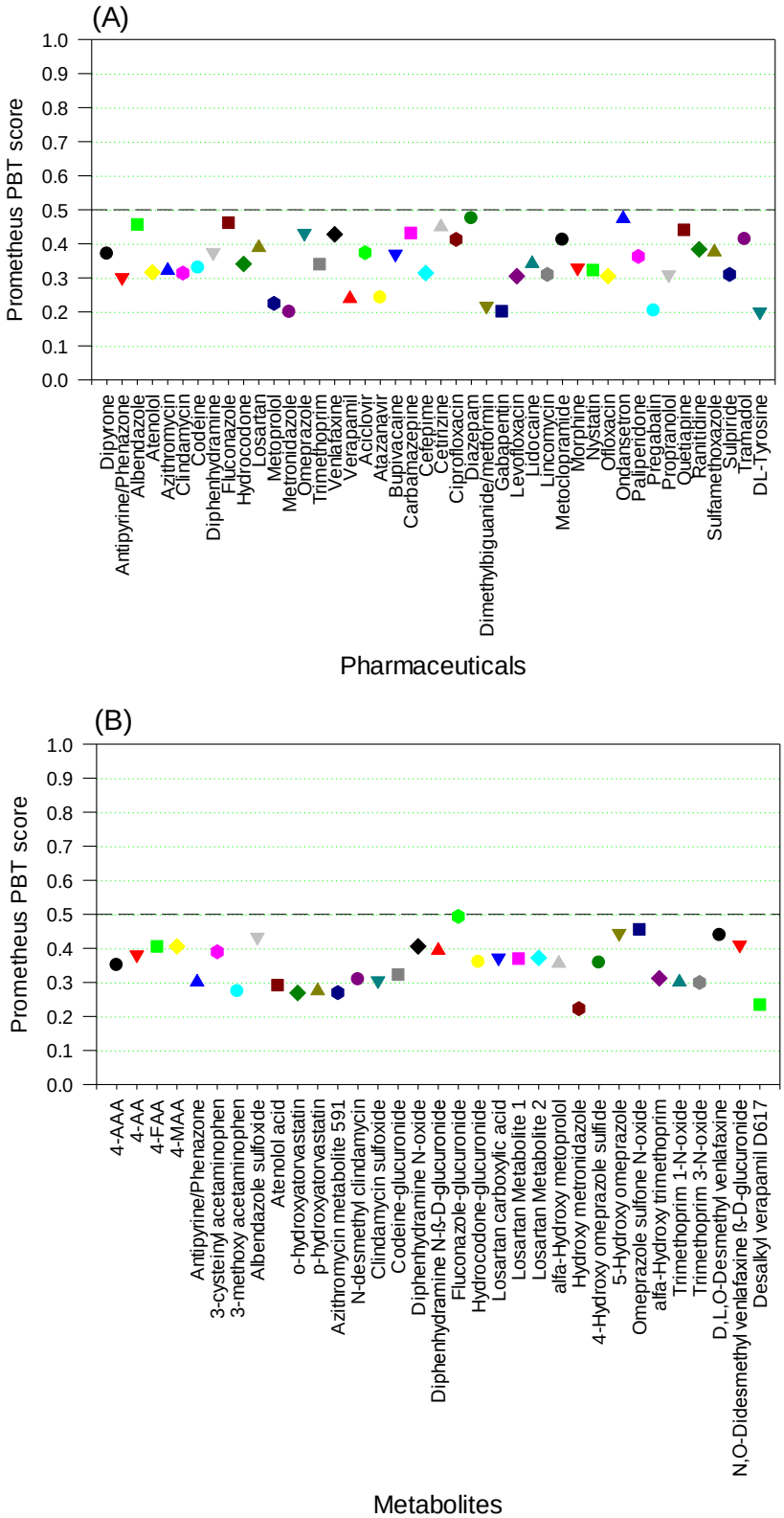

Figure S2. *In silico* QSAR prediction of PBT values by the Prometheus software for the (A) pharmaceuticals and (B) human metabolites investigated in this study.

Table S9. *In silico* QSAR predictions for Mutagenicity (Ames test) CONSENSUS model of the pharmaceuticals and metabolites investigated in the present study according to VEGA QSAR v.1.1.4 software.

Compound	SMILES	Assessment	Used models	Predicted Consensus Mutagen activity	Mutagenic Score	Non-Mutagenic Score	Model Caesar assessment	Model ISS assessment	Model SarPy assessment	Model KNN assessment
Dipyrone	O=C1C(=C(N(N1c2ccccc2)C)C)N(C)CS(=O)(=O)[O-]	Mutagenic	1	Mutagenic	1	0	Mutagenic (moderate reliability)	Mutagenic (experimental value)	Mutagenic (moderate reliability)	Mutagen (moderate reliability)
4-AAA	O=C(NC=2C(=O)N(c1ccccc1)N(C=2C)C)C	Mutagenic	4	Mutagenic	0.35	0.15	NON-Mutagenic (moderate reliability)	Mutagenic (low reliability)	Mutagenic (moderate reliability)	Mutagen (moderate reliability)
4-AA	O=C1C(N)=C(N(N1c2ccccc2)C)C	NON-Mutagenic	4	NON-Mutagenic	0.1	0.3	NON-Mutagenic (moderate reliability)	Mutagenic (low reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
4-FAA	O=CNC=2C(=O)N(c1ccccc1)N(C=2C)C	Mutagenic	4	Mutagenic	0.35	0.15	NON-Mutagenic (moderate reliability)	Mutagenic (low reliability)	Mutagenic (moderate reliability)	Mutagen (moderate reliability)
4-MAA	O=C2C(NC)=C(N(N2(c1ccccc1))C)C	NON-Mutagenic	4	NON-Mutagenic	0.1	0.3	NON-Mutagenic (moderate reliability)	Mutagenic (low reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Antipyrine/ Phenazone	O=C1C=C(N(N1c2ccccc2)C)C	NON-Mutagenic	4	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (Experimental value)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)
Acetaminophen	O=C(Nc1ccc(O)cc1)C	NON-Mutagenic	2	NON-Mutagenic	0	1	NON-Mutagenic (moderate reliability)	NON-Mutagenic (experimental value)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (experimental value)
3-cysteinyl acetaminophen	O=C(O)C(N)CSc1cc(ccc1(O))NC(=O)C	Mutagenic	4	Mutagenic	0.35	0.15	Mutagenic (low reliability)	Mutagenic (moderate reliability)	Possible NON-Mutagenic (moderate reliability)	Mutagen (moderate reliability)
3-methoxy acetaminophen	O=C(Nc1ccc(O)c(OC)c1)C	NON-Mutagenic	4	NON-Mutagenic	0.15	0.45	NON-Mutagenic (moderate reliability)	Mutagenic (moderate reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)
Albendazole	O=C(OC)Nc1nc2ccc(cc2([nH]1))SCCC	Mutagenic	4	Mutagenic	0.25	0.15	Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	Mutagenic (low reliability)	Mutagen (moderate reliability)
Albendazole sulfoxide	O=C(OC)Nc1nc2ccc(cc2([nH]1))S(=O)CCC	NON-Mutagenic	4	NON-Mutagenic	0.1	0.2	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Atenolol	O=C(N)Cc1ccc(OCC(O)CNC(C)C)cc1	NON-Mutagenic	1	NON-Mutagenic	0	1	NON-Mutagenic (moderate reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (experimental value)
Atenolol acid	O=C(O)Cc1ccc(OCC(O)CNC(C)C)cc1	NON-Mutagenic	4	NON-Mutagenic	0	0.82	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (good reliability)
Atorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccccc3)c4C(C)C	NON-Mutagenic (Consensus score: 1)	1	NON-Mutagenic	0	1	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (experimental value)
o-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccccc3(O))c4C(C)C	NON-Mutagenic (Consensus score: 0.4)	4	NON-Mutagenic	0	0.4	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
p-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccc(O)cc3)c4C(C)C	NON-Mutagenic (Consensus score: 0.4)	4	NON-Mutagenic	0	0.4	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (moderate reliability)

Compound	SMILES	Assessment	Used models	Predicted Consensus Mutagen activity	Mutagenic Score	Non-Mutagenic Score	Model Caesar assessment	Model ISS assessment	Model SarPy assessment	Model KNN assessment
Azithromycin	O=C3OC(CC)C(O)(C)C(O)C(N(C)CC(C)CC(O)(C)C(OC1OC(C)CC(N(C)C)C1(O))C(C)C(OC2OC(C)C(O)C(OC)(C)C2)C3C)C	NON-Mutagenic (Consensus score: 1)	3	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (good reliability)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)
Descladinose (Azithromycin metabolite 591)	O=C2OC(CC)C(O)(C)C(O)C(N(C)CC(C)CC(O)(C)C(OC1OC(C)CC(N(C)C)C1(O))C(C)C(O)C2C)C	NON-Mutagenic (Consensus score: 0.9)	4	NON-Mutagenic	0	0.9	NON-Mutagenic (good reliability)	NON-Mutagenic (good reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (good reliability)
Clindamycin	O=C(NC(C1OC(C(O)C(O)C1(O))SC)C(C)Cl)C2N(C)CC(CCC)C2	Mutagenic (Consensus score: 0.25)	4	Mutagenic	0.25	0.15	Suspect Mutagenic (low reliability)	Mutagenic (moderate reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
N-desmethyl clindamycin	O=C(NC(C1OC(C(O)C(O)C1(O))SC)C(C)Cl)C2NCC(CCC)C2	Mutagenic (Consensus score: 0.25)	4	Mutagenic	0.25	0.15	Suspect Mutagenic (low reliability)	Mutagenic (moderate reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Clindamycin sulfoxide	O=C(NC(C1OC(C(O)C(O)C1(O))S(=O)C)C(C)Cl)C2N(C)CC(CCC)C2	Mutagenic (Consensus score: 0.25)	4	Mutagenic	0.25	0.15	Suspect Mutagenic (low reliability)	Mutagenic (moderate reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Codeine	OC1C=CC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4)	NON-Mutagenic (Consensus score: 1)	4	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (experimental value)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)
Codeine-glucuronide	O=C(O)C6OC(OC1C=CC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4))C(O)C(O)C6(O)	NON-Mutagenic (Consensus score: 0.6)	4	NON-Mutagenic	0	0.6	NON-Mutagenic (moderate reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)
Diphenhydramine	O(CCN(C)C)C(c1ccccc1)c2ccccc2	NON-Mutagenic (Consensus score: 1)	2	NON-Mutagenic	0	1	NON-Mutagenic (low reliability)	NON-Mutagenic (experimental value)	NON-Mutagenic (low reliability)	NON-Mutagen (experimental value)
Diphenhydramine N-oxide	[O-][N+](C)(C)CCOC(c1ccccc1)c2ccccc2	NON-Mutagenic (Consensus score: 0.47)	4	NON-Mutagenic	0	0.47	NON-Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (good reliability)
Diphenhydramine N-ß-D-glucuronide	O=C([O-])C1OC(C(O)C(O)C1(O))[N+](C)(C)CCOC(c2ccccc2)c3ccccc3	NON-Mutagenic (Consensus score: 0.35)	4	NON-Mutagenic	0.05	0.35	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Fluconazole	Fc1ccc(c(F)c1)C(O)(Cn2ncnc2)Cn3ncnc3	NON-Mutagenic (Consensus score: 0.35)	4	NON-Mutagenic	0.15	0.35	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	Mutagen (moderate reliability)
Fluconazole-glucuronide	O=C(O)C4OC(OC(c1ccc(F)cc1(F))(Cn2ncnc2)Cn3ncnc3)C(O)C(O)C4(O)	NON-Mutagenic (Consensus score: 0.3)	4	NON-Mutagenic	0	0.3	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Hydrocodone	O=C1CCC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4)	NON-Mutagenic (Consensus score: 0.82)	4	NON-Mutagenic	0	0.82	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (good reliability)
Hydrocodone-glucuronide	O=C(O)C6OC(Oc5ccc4c1c5(OC2C(=O)CCC3C(N(C)CCC123)C4))C(O)C(O)C6(O)	NON-Mutagenic (Consensus score: 0.25)	4	NON-Mutagenic	0.15	0.25	NON-Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Mutagen (moderate reliability)

Compound	SMILES	Assessment	Used models	Predicted Consensus Mutagen activity	Mutagenic Score	Non-Mutagenic Score	Model Caesar assessment	Model ISS assessment	Model SarPy assessment	Model KNN assessment
Losartan	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))CCCC)Cl	NON-Mutagenic (Consensus score: 0.35)	4	NON-Mutagenic	0.15	0.35	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (moderate reliability)	Mutagen (moderate reliability)
Losartan carboxylic acid	O=C(O)c1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nn[nH]n3))CCCC)Cl	NON-Mutagenic (Consensus score: 0.4)	4	NON-Mutagenic	0	0.4	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (low reliability)
Losartan Metabolite 1	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))CCC(O)C)Cl	NON-Mutagenic (Consensus score: 0.4)	4	NON-Mutagenic	0	0.4	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (low reliability)
Losartan Metabolite 2	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))C(O)CCC)Cl	NON-Mutagenic (Consensus score: 0.4)	4	NON-Mutagenic	0	0.4	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (low reliability)
Metoprolol	OC(COc1ccc(cc1)CCOC)CNC(C)C	NON-Mutagenic (Consensus score: 1)	1	NON-Mutagenic	0	1	NON-Mutagenic (good reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (experimental value)
a-Hydroxy metoprolol	OC(c1ccc(OCC(O)CNC(C)C)cc1)COC	NON-Mutagenic (Consensus score: 0.72)	4	NON-Mutagenic	0	0.72	NON-Mutagenic (good reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (good reliability)
Metronidazole	O=[N+]([O-])c1cnc(n1CCO)C	Mutagenic (Consensus score: 1)	4	Mutagenic	1	0	Mutagenic (experimental value)	Mutagenic (experimental value)	Mutagenic (experimental value)	Mutagen (experimental value)
Hydroxy metronidazole	O=[N+]([O-])c1cnc(n1CCO)CO	Mutagenic (Consensus score: 1)	3	Mutagenic	1	0	Mutagenic (experimental value)	Mutagenic (moderate reliability)	Mutagenic (experimental value)	Mutagen (experimental value)
Omeprazole	O=S(c1nc2ccc(OC)cc2([nH]1))Cc3ncc(c(OC)c3C)C	Mutagenic (Consensus score: 0.2)	4	Mutagenic	0.2	0.1	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (low reliability)	Mutagen (moderate reliability)
4-Hydroxy omeprazole sulfide	Oc1c(cnc(c1C)CSc2nc3ccc(OC)cc3([nH]2))C	Mutagenic (Consensus score: 0.3)	4	Mutagenic	0.3	0.1	Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (low reliability)	Mutagen (moderate reliability)
5-Hydroxy omeprazole	OCc1cnc(c(c1(OC))C)CSc2nc3cc(OC)ccc3([nH]2)	NON-Mutagenic (Consensus score: 0.35)	4	NON-Mutagenic	0.05	0.35	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)
Omeprazole sulfone N-oxide	O=S(=O)(c1nc2ccc(OC)cc2([nH]1))Cc3c(c(OC)c(c[n+]3([O-]))C)C	Mutagenic (Consensus score: 0.2)	4	Mutagenic	0.2	0.1	NON-Mutagenic (low reliability)	Mutagenic (low reliability)	Possible NON-Mutagenic (low reliability)	Mutagen (moderate reliability)
Trimethoprim	n1cc(c(nc1N)N)Cc2cc(OC)c(OC)c(OC)c2	Mutagenic (Consensus score: 0.43)	4	Mutagenic	0.43	0.05	Mutagenic (good reliability)	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagen (moderate reliability)
a-Hydroxy trimethoprim	OC(c1cc(OC)c(OC)c(OC)c1)c2cnc(nc2(N))N	Mutagenic (Consensus score: 0.35)	4	Mutagenic	0.35	0.05	Mutagenic (moderate reliability)	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagen (moderate reliability)
Trimethoprim 1-N-oxide	[O-][n+]1cc(c(nc1(N))N)Cc2cc(OC)c(OC)c(OC)c2	Mutagenic (Consensus score: 0.43)	4	Mutagenic	0.43	0.05	Mutagenic (good reliability)	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagen (moderate reliability)

Compound	SMILES	Assessment	Used models	Predicted Consensus Mutagen activity	Mutagenic Score	Non-Mutagenic Score	Model Caesar assessment	Model ISS assessment	Model SarPy assessment	Model KNN assessment
Trimethoprim 3-N-oxide	[O-][n+]1c(ncc(c1(N))Cc2cc(OC)c(OC)c(OC)c2)N	Mutagenic (Consensus score: 0.43)	4	Mutagenic	0.43	0.05	Mutagenic (good reliability)	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagen (moderate reliability)
Venlafaxine	OC1(CCCCC1)C(c2ccc(OC)cc2)CN(C)C	NON-Mutagenic (Consensus score: 0.75)	4	NON-Mutagenic	0	0.75	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
D,L,O-Desmethyl venlafaxine	Oc1ccc(cc1)C(CN(C)C)C2(O)(CCCCC2)	NON-Mutagenic (Consensus score: 0.75)	4	NON-Mutagenic	0	0.75	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
N,O-Didesmethyl venlafaxine ß-D-glucuronide	O=C(O)C3OC(Oc1ccc(cc1)C(CNC)C2(O)(CCCCC2))C(O)C(O)C3(O)	NON-Mutagenic (Consensus score: 0.57)	4	NON-Mutagenic	0	0.57	NON-Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagen (good reliability)
Verapamil	N#CC(c1ccc(OC)c(OC)c1)(CCCN(C)CCc2ccc(OC)c(OC)c2)C(C)C	NON-Mutagenic (Consensus score: 0.82)	4	NON-Mutagenic	0	0.82	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (good reliability)
Desalkyl verapamil D617	N#CC(c1ccc(OC)c(OC)c1)(CCCN(C)CCc2ccc(O)c(OC)c2)C(C)C	NON-Mutagenic (Consensus score: 0.82)	4	NON-Mutagenic	0	0.82	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (good reliability)
Aciclovir	O=C2NC(=Nc1c2(ncn1COCCO))N	Mutagenic (Consensus score: 0.15)	4	Mutagenic	0.15	0.05	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagenic (low reliability)	Mutagen (low reliability)
Atazanavir	O=C(OC)NC(C(=O)NN(Cc1ccc(cc1)c2ncccc2)CC(O)C(NC(=O)C(NC(=O)OC)C(C)(C)C)Cc3ccccc3)C(C)(C)C	Mutagenic (Consensus score: 0.15)	4	Mutagenic	0.15	0.15	Mutagenic (low reliability)	Mutagenic (low reliability)	Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Bupivacaine	O=C(Nc1c(cccc1C)C)C2N(CCCC)CCCC2	NON-Mutagenic (Consensus score: 0.65)	4	NON-Mutagenic	0	0.65	NON-Mutagenic (good reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagen (good reliability)
Carbamazepine	O=C(N)N2c3ccccc3(C=Cc1ccccc12)	NON-Mutagenic (Consensus score: 0.35)	4	NON-Mutagenic	0.15	0.35	Mutagenic (moderate reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)
Cefepime	O=C([O-])C4=C(C[N+]1(C)(CCCC1))CSC3N4(C(=O)C3(NC(=O)C(=NOC)c2nc(N)sc2))	NON-Mutagenic (Consensus score: 0.38)	4	NON-Mutagenic	0.1	0.38	Mutagenic (low reliability)	Mutagenic (low reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
Cetirizine	O=C(O)COCCN1CCN(CC1)C(c2ccccc2)c3ccc(cc3)Cl	NON-Mutagenic (Consensus score: 0.43)	4	NON-Mutagenic	0.05	0.43	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
Ciprofloxacin	O=C(O)C1=CN(c2cc(c(F)cc2(C1(=O)))N3CCNCC3)C4CC4	Mutagenic (Consensus score: 1)	2	Mutagenic	1	0	Mutagenic (experimental value)	Mutagenic (low reliability)	Mutagenic (experimental value)	Mutagen (moderate reliability)
Diazepam	O=C1N(c3ccc(cc3(C(=NC1)c2ccccc2))Cl)C	NON-Mutagenic (Consensus score: 1)	4	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (experimental value)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)
Metformin	N=C(N=C(N)N)N(C)C	NON-Mutagenic (Consensus score: 0.35)	4	NON-Mutagenic	0.05	0.35	Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)

Compound	SMILES	Assessment	Used models	Predicted Consensus Mutagen activity	Mutagenic Score	Non-Mutagenic Score	Model Caesar assessment	Model ISS assessment	Model SarPy assessment	Model KNN assessment
Gabapentin	O=C(O)CC1(CN)(CCCCC1)	NON-Mutagenic (Consensus score: 0.83)	4	NON-Mutagenic	0	0.83	NON-Mutagenic (good reliability)	NON-Mutagenic (good reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
Levofloxacin	O=C(O)C2=CN1c3c(OCC1C)c(c(F)cc3(C2(=O)))N4CCN(C)CC4	Mutagenic (Consensus score: 0.72)	4	Mutagenic	0.72	0	Mutagenic (good reliability)	Mutagenic (low reliability)	Mutagenic (good reliability)	Mutagen (good reliability)
Lidocaine	O=C(Nc1c(cccc1C)C)CN(CC)CC	NON-Mutagenic (Consensus score: 0.5)	4	NON-Mutagenic	0	0.5	NON-Mutagenic (moderate reliability)	NON-Mutagenic (moderate reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (low reliability)
Lincomycin	O=C(NC(C(O)C)C1OC(C(O)C(O)C1(O))SC)C2N(C)CC(CCC)C2	NON-Mutagenic (Consensus score: 0.3)	4	NON-Mutagenic	0	0.3	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (moderate reliability)
Metoclopramide	O=C(NCCN(CC)CC)c1cc(c(N)cc1(OC))Cl	Mutagenic (Consensus score: 0.35)	4	Mutagenic	0.35	0.15	Mutagenic (moderate reliability)	Mutagenic (low reliability)	Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)
Morphine	Oc5ccc4c1c5(OC2C(O)C=CC3C(N(C)CCC123)C4)	NON-Mutagenic (Consensus score: 1)	3	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)
Nystatin	O=C(O)C3C(O)CC2(O)(OC3(CC(OC1OC(C)C(O)C(N)C1(O))C=CC=CC=CCCC=CC=CC(C)C(O)C(C)C(OC(=O)CC(O)CC(O)CC(O)CCC(O)C(O)C2)C))	NON-Mutagenic (Consensus score: 0.67)	4	NON-Mutagenic	0	0.67	NON-Mutagenic (moderate reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagen (good reliability)
Ofloxacin	O=C(O)C2=CN1c3c(OCC1C)c(c(F)cc3(C2(=O)))N4CCN(C)CC4	Mutagenic (Consensus score: 0.72)	4	Mutagenic	0.72	0	Mutagenic (good reliability)	Mutagenic (low reliability)	Mutagenic (good reliability)	Mutagen (good reliability)
Ondansetron	O=C3c2c1ccccc1n(c2CCC3Cn4ccnc4C)C	Mutagenic (Consensus score: 0.3)	4	Mutagenic	0.3	0.1	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	Mutagenic (moderate reliability)	Mutagen (moderate reliability)
Paliperidone	O=C1C(=C(N=C2N1CCCC2(O))C)CCN5CCC(c4noc3cc(F)ccc34)CC5	NON-Mutagenic (Consensus score: 0.45)	4	NON-Mutagenic	0.05	0.45	NON-Mutagenic (moderate reliability)	Mutagenic (low reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagen (moderate reliability)
Pregabalin	O=C(O)CC(CN)CC(C)C	NON-Mutagenic (Consensus score: 0.83)	4	NON-Mutagenic	0	0.83	NON-Mutagenic (good reliability)	NON-Mutagenic (good reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
Propranolol	OC(COc1cccc2ccccc12)CNC(C)C	NON-Mutagenic (Consensus score: 1)	3	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)
Quetiapine	OCCOCCN4CCN(C2=Nc1ccccc1Sc3ccccc23)CC4	NON-Mutagenic (Consensus score: 0.2)	4	NON-Mutagenic	0	0.2	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagenic (low reliability)	NON-Mutagen (low reliability)
Ranitidine	O=[N+]([O-])C=C(NC)NCCSCc1oc(cc1)CN(C)C	NON-Mutagenic (Consensus score: 1)	3	NON-Mutagenic	0	1	NON-Mutagenic (experimental value)	NON-Mutagenic (low reliability)	NON-Mutagenic (experimental value)	NON-Mutagen (experimental value)

Compound	SMILES	Assessment	Used models	Predicted Consensus Mutagen activity	Mutagenic Score	Non-Mutagenic Score	Model Caesar assessment	Model ISS assessment	Model SarPy assessment	Model KNN assessment
Sulfamethoxazole	O=S(=O)(Nc1noc(c1)C)c2ccc(N)cc2	NON-Mutagenic (Consensus score: 1)	1	NON-Mutagenic	0	1	NON-Mutagenic (good reliability)	Mutagenic (low reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (experimental value)
Sulpiride	O=C(NCC1N(CC)CCC1)c2cc(ccc2(OC))S(=O)(=O)N	NON-Mutagenic (Consensus score: 0.75)	4	NON-Mutagenic	0	0.75	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
Tramadol	OC2(c1cccc(OC)c1)(CCCCC2(CN(C)C))	NON-Mutagenic (Consensus score: 0.75)	4	NON-Mutagenic	0	0.75	NON-Mutagenic (good reliability)	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	NON-Mutagen (moderate reliability)
D,L-Tyrosine	O=C(O)C(N)Cc1ccc(O)cc1	NON-Mutagenic (Consensus score: 1)	1	NON-Mutagenic	0	1	NON-Mutagenic (moderate reliability)	NON-Mutagenic (good reliability)	Possible NON-Mutagenic (moderate reliability)	NON-Mutagen (experimental value)

Table S10. *In silico* QSAR predictions for Mutagenicity (Ames test) CONSENSUS model of the pharmaceuticals investigated in the present study according to VEGA QSAR v.1.1.4 software.

Pharmaceutical	Mutagenicity	Consensus score
Antipyrine/Phenazone	Non-Mut.	1*
Aciclovir	Mut.	0.15
Atazanavir	Mut.	0.15
Bupivacaine	Non-Mut.	0.65
Carbamazepine	Non-Mut.	0.35
Cefepime	Non-Mut.	0.38
Cetirizine	Non-Mut.	0.43
Ciprofloxacin	Mut.	1*
Diazepam	Non-Mut.	1*
Metformin	Non-Mut.	0.35
Gabapentin	Non-Mut.	0.83
Levofloxacin	Mut.	0.72
Lidocaine	Non-Mut.	0.5
Lincomycin	Non-Mut.	0.3
Metoclopramide	Mut.	0.35
Morphine	Non-Mut.	1*
Nystatin	Non-Mut.	0.67
Ofloxacin	Mut.	0.72
Ondansetron	Mut.	0.3
Paliperidone	Non-Mut.	0.45
Pregabalin	Non-Mut.	0.83
Propranolol	Non-Mut.	1*
Quetiapine	Non-Mut.	0.2
Ranitidine	Non-Mut.	1*
Sulfamethoxazole	Non-Mut.	1*
Sulpiride	Non-Mut.	0.75
Tramadol	Non-Mut.	0.75
DL-Tyrosine	Non-Mut.	1*

Mut.: positive alert for mutagenicity
Non-Mut.: negative alert for mutagenicity.
Consensus score: 0-1.
* means that the alert and consensus are based in an Exp. value.

Table S11. *In silico* QSAR predictions of the pharmaceuticals and human metabolites found in raw HWW concerning the Carcinogenicity as endpoint by different models provided by the VEGA QSAR v.1.1.4 software.

Compound	SMILES	Assessment CARC_ANTARES	Assessment CARC_CAESAR	Assessment CARC_ISS	Assessment CARC_ISSCAN-CGX
Dipyrone	O=C1C(=C(N(N1c2ccccc2)C)C)N(C)CS(=O)(=O)[O-]	NON-Carcinogen (experimental value)	NON-Carcinogen (low reliability)	Carcinogen (experimental value)	Carcinogen (moderate reliability)
4-AAA	O=C(NC=2C(=O)N(c1ccccc1)N(C=2C)C)C	Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
4-AA	O=C1C(N)=C(N(N1c2ccccc2)C)C	Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
4-FAA	O=CNC=2C(=O)N(c1ccccc1)N(C=2C)C	Carcinogen (moderate reliability)	Carcinogen (good reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
4-MAA	O=C2C(NC)=C(N(N2(c1ccccc1))C)C	Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
Antipyrine/Phenazone	O=C1C=C(N(N1c2ccccc2)C)C	Carcinogen (experimental value)	Carcinogen (experimental value)	Carcinogen (experimental value)	Carcinogen (experimental value)
Acetaminophen	O=C(Nc1ccc(O)cc1)C	NON-Carcinogen (experimental value)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (experimental value)
3-cysteinyl acetaminophen	O=C(O)C(N)CSc1cc(ccc1(O))NC(=O)C	Possible NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (good reliability)
3-methoxy acetaminophen	O=C(Nc1ccc(O)c(OC)c1)C	Possible NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (good reliability)	Carcinogen (good reliability)
Albendazole	O=C(OC)Nc1nc2ccc(cc2([nH]1))SCCC	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)	Possible NON-Carcinogen (low reliability)
Albendazolesulfoxide	O=C(OC)Nc1nc2ccc(cc2([nH]1))S(=O)CCC	Carcinogen (good reliability)	Carcinogen (low reliability)	Carcinogen (low reliability)	Possible NON-Carcinogen (low reliability)
Atenolol	O=C(N)Cc1ccc(OCC(O)CNC(C)C)cc1	Carcinogen (experimental value)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Atenolol acid	O=C(O)Cc1ccc(OCC(O)CNC(C)C)cc1	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Atorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccccc3)c4C(C)C	Carcinogen (good reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (low reliability)
o-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccccc3(O))c4C(C)C	Carcinogen (good reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (low reliability)
p-hydroxyatorvastatin	O=C(O)CC(O)CC(O)CCn4c(c1ccc(F)cc1)c(c2ccccc2)c(C(=O)Nc3ccc(O)cc3)c4C(C)C	Carcinogen (good reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (low reliability)
Azithromycin	O=C3OC(CC)C(O)(C)C(O)C(N(C)CC(C)CC(O)(C)C(OC1OC(C)CC(N(C)C)C1(O))C(C)C(OC2OC(C)C(O)C(OC)(C)C2)C3C)C	Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)
Descladinose (Azithromycin met 591)	O=C2OC(CC)C(O)(C)C(O)C(N(C)CC(C)CC(O)(C)C(OC1OC(C)CC(N(C)C)C1(O))C(C)C(O)C2C)C	Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)
Clindamycin	O=C(NC(C1OC(C(O)C(O)C1(O))SC)C(C)Cl)C2N(C)CC(CCC)C2	Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)	Possible NON-Carcinogen (low reliability)
N-desmethylclindamycin	O=C(NC(C1OC(C(O)C(O)C1(O))SC)C(C)Cl)C2NCC(CCC)C2	Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (moderate reliability)	Possible NON-Carcinogen (low reliability)
Clindamycinsulfoxide	O=C(NC(C1OC(C(O)C(O)C1(O))S(=O)C)C(C)Cl)C2N(C)CC(CCC)C2	Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)	Possible NON-Carcinogen (low reliability)
Codeine	OC1C=CC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4)	NON-Carcinogen (experimental value)	NON-Carcinogen (experimental value)	NON-Carcinogen (experimental value)	NON-Carcinogen (experimental value)

Compound	SMILES	Assessment CARC_ANTARES	Assessment CARC_CAESAR	Assessment CARC_ISS	Assessment CARC_ISSCAN-CGX
Codeine-glucuronide	O=C(O)C6OC(OC1C=CC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4))C(O)C(O)C6(O)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)
Diphenhydramine	O(CCN(C)C)C(c1ccccc1)c2ccccc2	Possible NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (experimental value)
Diphenhydramine N-oxide	[O-][N+](C)(C)CCOC(c1ccccc1)c2ccccc2	Carcinogen (moderate reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Possible NON-Carcinogen (moderate reliability)
Diphenhydramine N-ß-D-glucuronide	O=C([O-])C1OC(C(O)C(O)C1(O))[N+](C)(C)CCOC(c2ccccc2)c3ccccc3	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Fluconazole	Fc1ccc(c(F)c1)C(O)(Cn2ncnc2)Cn3ncnc3	Carcinogen (experimental value)	Carcinogen (experimental value)	NON-Carcinogen (low reliability)	Carcinogen (experimental value)
Fluconazole-glucuronide	O=C(O)C4OC(OC(c1ccc(F)cc1(F))(Cn2ncnc2)Cn3ncnc3)C(O)C(O)C4(O)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Possible NON-Carcinogen (low reliability)
Hydrocodone	O=C1CCC5C4N(C)CCC25(c3c(OC12)c(OC)ccc3C4)	Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	NON-Carcinogen (good reliability)	Carcinogen (low reliability)
Hydrocodone-glucuronide	O=C(O)C6OC(Oc5ccc4c1c5(OC2C(=O)CCC3C(N(C)CCC123)C4))C(O)C(O)C6(O)	Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
Losartan	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))CCCC)Cl	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Losartan carboxylic acid	O=C(O)c1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nn[nH]n3))CCCC)Cl	Possible NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Losartan Metabolite 1	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))CCC(O)C)Cl	Possible NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Losartan Metabolite 2	OCc1c(nc(n1Cc2ccc(cc2)c4ccccc4(c3nnn[nH]3))C(O)CCC)Cl	Possible NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Metoprolol	OC(COc1ccc(cc1)CCOC)CNC(C)C	NON-Carcinogen (experimental value)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
a-Hydroxy metoprolol	OC(c1ccc(OCC(O)CNC(C)C)cc1)COC	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Metronidazole	O=[N+]([O-])c1cnc(n1CCO)C	Carcinogen (experimental value)	Carcinogen (experimental value)	Carcinogen (experimental value)	Carcinogen (experimental value)
Hydroxy metronidazole	O=[N+]([O-])c1cnc(n1CCO)CO	Carcinogen (good reliability)	NON-Carcinogen (low reliability)	Carcinogen (good reliability)	Carcinogen (good reliability)
Omeprazole	O=S(c1nc2ccc(OC)cc2([nH]1))Cc3ncc(c(OC)c3C)C	Carcinogen (experimental value)	Carcinogen (experimental value)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
4-Hydroxy omeprazole sulfide	Oc1c(cnc(c1C)CSc2nc3ccc(OC)cc3([nH]2))C	Carcinogen (good reliability)	Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
5-Hydroxy omeprazole	OCc1cnc(c(c1(OC))C)CSc2nc3cc(OC)ccc3([nH]2)	Carcinogen (good reliability)	Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
Omeprazole sulfone N-oxide	O=S(=O)(c1nc2ccc(OC)cc2([nH]1))Cc3c(c(OC)c(c[n+]3([O-]))C)C	Carcinogen (good reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
Trimethoprim	n1cc(c(nc1N)N)Cc2cc(OC)c(OC)c(OC)c2	Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
a-Hydroxy trimethoprim	OC(c1cc(OC)c(OC)c(OC)c1)c2cnc(nc2(N))N	Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
Trimethoprim 1-N-oxide	[O-][n+]1cc(c(nc1(N))N)Cc2cc(OC)c(OC)c(OC)c2	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
Trimethoprim 3-N-oxide	[O-][n+]1c(ncc(c1(N))Cc2cc(OC)c(OC)c(OC)c2)N	Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)

Compound	SMILES	Assessment CARC_ANTARES	Assessment CARC_CAESAR	Assessment CARC_ISS	Assessment CARC_ISSCAN-CGX
Venlafaxine	OC1(CCCCC1)C(c2ccc(OC)cc2)CN(C)C	Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
D,L,O-Desmethyl venlafaxine	Oc1ccc(cc1)C(CN(C)C)C2(O)(CCCCC2)	Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
N,O-Didesmethyl venlafaxine ß-D-glucuronide	O=C(O)C3OC(Oc1ccc(cc1)C(CNC)C2(O)(CCCCC2))C(O)C(O)C3(O)	Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Verapamil	N#CC(c1ccc(OC)c(OC)c1)(CCCN(C)CCc2ccc(OC)c(OC)c2)C(C)C	NON-Carcinogen (experimental value)	NON-Carcinogen (good reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Desalkyl verapamil D617	N#CC(c1ccc(OC)c(OC)c1)(CCCN(C)CCc2ccc(O)c(OC)c2)C(C)C	Carcinogen (low reliability)	NON-Carcinogen (good reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Aciclovir	O=C2NC(=Nc1c2(ncn1COCCO))N	NON-Carcinogen (EXPERIMENTAL value)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Atazanavir	O=C(OC)NC(C(=O)NN(Cc1ccc(cc1)c2ncccc2)CC(O)C(NC(=O)C(NC(=O)OC)C(C)(C)C)Cc3ccccc3)C(C)(C)C	Carcinogen (moderate reliability)	NON-Carcinogen (good reliability)	Carcinogen (low reliability)	Carcinogen (good reliability)
Bupivacaine	O=C(Nc1c(cccc1C)C)C2N(CCCC)CCCC2	Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)
Carbamazepine	O=C(N)N2c3ccccc3(C=Cc1ccccc12)	Carcinogen (EXPERIMENTAL value)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (good reliability)
Cefepime	O=C([O-])C4=C(C[N+]1(C)(CCCC1))CSC3N4(C(=O)C3(NC(=O)C(=NOC)c2nc(N)sc2))	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)	Carcinogen (moderate reliability)
Cetirizine	O=C(O)COCCN1CCN(CC1)C(c2ccccc2)c3ccc(cc3)Cl	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Ciprofloxacin	O=C(O)C1=CN(c2cc(c(F)cc2(C1(=O)))N3CCNCC3)C4CC4	NON-Carcinogen (EXPERIMENTAL value)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (good reliability)
Diazepam	O=C1N(c3ccc(cc3(C(=NC1)c2ccccc2))Cl)C	NON-Carcinogen (EXPERIMENTAL value)	NON-Carcinogen (EXPERIMENTAL value)	NON-Carcinogen (EXPERIMENTAL value)	NON-Carcinogen (EXPERIMENTAL value)
Metformin	N=C(N=C(N)N)N(C)C	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	NON-Carcinogen (low reliability)	Possible NON-Carcinogen (low reliability)
Gabapentin	O=C(O)CC1(CN)(CCCCC1)	Carcinogen (EXPERIMENTAL value)	Carcinogen (EXPERIMENTAL value)	Carcinogen (EXPERIMENTAL value)	Carcinogen (EXPERIMENTAL value)
Levofloxacin	O=C(O)C2=CN1c3c(OCC1C)c(c(F)cc3(C2(=O)))N4CCN(C)CC4	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
Lidocaine	O=C(Nc1c(cccc1C)C)CN(CC)CC	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)
Lincomycin	O=C(NC(C(O)C)C1OC(C(O)C(O)C1(O))SC)C2N(C)CC(CCC)C2	Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Possible NON-Carcinogen (low reliability)
Metoclopramide	O=C(NCCN(CC)CC)c1cc(c(N)cc1(OC))Cl	Carcinogen (good reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)	Carcinogen (good reliability)
Morphine	Oc5ccc4c1c5(OC2C(O)C=CC3C(N(C)CCC123)C4)	Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)
Nystatin	O=C(O)C3C(O)CC2(O)(OC3(CC(OC1OC(C)C(O)C(N)C1(O))C=CC=CC=CC=CCCC=CC=CC(C)C(O)C(C)C(OC(=O)CC(O)CC(O)CC(O)CCC(O)C(O)C2)C))	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
Ofloxacin	O=C(O)C2=CN1c3c(OCC1C)c(c(F)cc3(C2(=O)))N4CCN(C)CC4	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)	Carcinogen (moderate reliability)
Ondansetron	O=C3c2c1ccccc1n(c2CCC3Cn4ccnc4C)C	NON-Carcinogen (EXPERIMENTAL value)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (moderate reliability)

Compound	SMILES	Assessment CARC_ANTARES	Assessment CARC_CAESAR	Assessment CARC_ISS	Assessment CARC_ISSCAN-CGX
Paliperidone	O=C1C(=C(N=C2N1CCCC2(O))C)CCN5CCC(c4noc3cc(F)ccc34)CC5	Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (low reliability)
Pregabalin	O=C(O)CC(CN)CC(C)C	Possible NON-Carcinogen (low reliability)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Possible NON-Carcinogen (low reliability)
Propranolol	OC(COc1cccc2ccccc12)CNC(C)C	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (good reliability)
Quetiapine	OCCOCCN4CCN(C2=Nc1ccccc1Sc3ccccc23)CC4	Carcinogen (EXPERIMENTAL value)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Ranitidine	O=[N+]([O-])C=C(NC)NCCSCc1oc(cc1)CN(C)C	NON-Carcinogen (EXPERIMENTAL value)	NON-Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Carcinogen (low reliability)
Sulfamethoxazole	O=S(=O)(Nc1noc(c1)C)c2ccc(N)cc2	Possible NON-Carcinogen (moderate reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	Carcinogen (EXPERIMENTAL value)
Sulpiride	O=C(NCC1N(CC)CCC1)c2cc(ccc2(OC))S(=O)(=O)N	Possible NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)	NON-Carcinogen (moderate reliability)	Carcinogen (low reliability)
Tramadol	OC2(c1cccc(OC)c1)(CCCCC2(CN(C)C))	NON-Carcinogen (EXPERIMENTAL value)	Carcinogen (low reliability)	NON-Carcinogen (low reliability)	Possible NON-Carcinogen (low reliability)
Tyrosine	O=C(O)C(N)Cc1ccc(O)cc1	Possible NON-Carcinogen (good reliability)	NON-Carcinogen (moderate reliability)	NON-Carcinogen (good reliability)	Possible NON-Carcinogen (good reliability)

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Chapter 2

Proposal of a new, fast, cheap, and easy method using DLLME for extraction and preconcentration of diazepam and its transformation products generated by a solar photo-Fenton process

Introduction

Diazepam (DZP), a pharmaceutical used as an anticonvulsant, sedative, muscle relaxant, hypnotic, and anxiolytic, has been detected in different aqueous matrices [73]. DZP was one of the pharmaceuticals identified in all the samples analyzed in the study detailed in Chapter 1, although there have been only a few studies concerning its degradation. The degree of metabolization of DZP can vary between 50 and 95%, and the compound presents recalcitrance to photodegradation [74]. Since DZP is not completely removed by conventional treatment processes in wastewater treatment plants, it is necessary to study the use of additional processes, such as AOPs, to improve its removal [75]. Among the AOPs, SPFP offers some advances, especially the use of solar UV radiation [43]. However, when AOPs such as SPFP are employed, it is essential to be able to identify the main TPs generated during the treatment, because some TPs may be more toxic and less biodegradable, compared to the original compound [76]. For this purpose, in addition to sensitive analytical techniques, it is necessary to use extraction and preconcentration steps to enable the detection of TPs formed at lower concentrations during degradation processes. The commonest method employed for the preconcentration of TPs is undoubtedly SPE, despite having a number of disadvantages. Another option is to use DLLME, which is especially attractive in terms of time and cost [77]. In addition to compound identification, an increasingly accepted approach to evaluate toxicity is by *in silico* predictions using (Q)SAR methods, which can provide information about the biological activity and physicochemical properties of TPs, by considering their chemical structures [78].

Objectives

The aims of the present study were as follows: i) Firstly, to degrade DZP by SPFP, with identification of the TPs formed during the process, and to use (Q)SAR tools to predict the toxicological parameters of DZP and the TPs; ii) Secondly, to develop, optimize, and apply a DLLME extraction methodology for DZP and its TPs present in ultrapure water (UPW), simulated wastewater (SW), and raw hospital wastewater (RHW).

Main results

During the degradation of DZP by SPFP, it was possible to identify six TPs. The identification of TPs is a very difficult and complex process, since no analytical standards are generally available. The strategy adopted in this work was based on the appearance and disappearance of chromatographic peaks, followed by tentative proposal of the structures and fragmentation profiles of the TPs. This kind of work requires in-depth knowledge of high resolution mass spectrometry, as well as the many possible compounds that could be formed, given the nonspecific nature of the SPFP reaction. Considering this, excellent results were achieved in identifying and elucidating six TPs, especially because three of them have never been described previously in the literature.

For all the TPs proposed in this work, the criteria adopted included determination of the exact mass, elemental composition with mass errors below 5 ppm for the molecular ion and all the proposed fragments, and double bond equivalence (DBE). This involved a more laborious process, but ensured greater reliability of the results. From the study of the appearance of the TPs during the process, it was possible to propose a degradation pathway, a very important result that has never been reported previously for the degradation of DZP using SPFP.

In a degradation study where the goal is to identify the TPs formed, it is essential to ensure that all the TPs are detected. For this, two possible strategies can be adopted. One is to start with a high concentration of the study compound, although this precludes any similarity with environmental levels of contamination. Another option is to work at environmental concentrations, although an extraction step is necessary. Different initial concentrations lead to different responses using the same SPFP, since lower concentrations mean that there is less competition for reaction with the available hydroxyl radicals [79]. In order to study the process at near-environmental concentrations, lower concentrations of DZP were tested, in association with different extraction methods.

A comparison was performed between classical SPE and a DLLME method for the extraction of DZP and its TPs. The DLLME method was optimized, validated, and applied using three different aqueous matrices (ultrapure water (UPW), simulated wastewater (SW), and raw hospital wastewater (RHW)). Although some differences were noted between the DLLME method and the traditional SPE method, the same TPs were

identified in both cases, so the results were the same for the two methodologies. However, DLLME was much less expensive (~32-fold lower cost), compared to SPE. The DLLME method was also at least four times faster than SPE, as well as being easier and requiring only small amounts of organic solvent. Given these characteristics, DLLME is an attractive method that may be used to concentrate samples degraded by SPFP, since without this step it would not be possible to identify all the TPs formed during the treatment process. The most important achievement in this work was the development of a cheap and fast extraction method for TPs, especially envisaging its use in routine analyses where time and cost considerations are paramount. The use of the technique can also make the difference between whether or not a TP formed is actually detected. Until the publication of this study, there were no reports in the literature concerning a DLLME extraction method validated for TPs, further demonstrating the importance of innovation and searching for new analytical approaches to identify TPs formed during SPFP.

The *in silico* predictions showed mutagenicity alerts for three TPs that might therefore present greater environmental risk than DZP. This result reinforced the importance of the study and the need to develop efficient methodologies for the assessment of compound degradation, considering not only the parent compound, but also all the TPs formed during the process. The only way to do this is to develop methodologies for the monitoring of TPs during degradation process, until achieving the maximum rates of degradation of the analyte and all the TPs formed during the process, especially when high mineralization rates are not achieved.

All the methods, results, and conclusions are presented in **Paper II** and **Supplementary Material II**.

PAPER II

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Proposal of a new, fast, cheap, and easy method using DLLME for extraction and preconcentration of diazepam and its transformation products generated by a solar photo-Fenton process

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This work evaluated the formation of transformation products (TPs) during the degradation of diazepam (DZP) by a solar photo-Fenton process. Six TPs were identified, three of them for the first time. After elucidation of the TPs, a new, cheap, fast, and easy method was employed to extract and preconcentrate DZP and its TPs, using dispersive liquid-liquid microextraction (DLLME). The method was optimized us- ing factorial and Doehlert designs, with the best results obtained using acetonitrile as disperser solvent and chloroform as extraction solvent, with volumes of 1000 and 650 μL, respectively. When DZP degra- dation was performed in ultrapure water, the extraction/preconcentration of DZP and its TPs by DLLME was very similar to the results obtained using a traditional SPE method. However, when hospital wastew- ater was used as the matrix, more limited extraction efficiency was obtained using DLLME, compared to SPE. Meanwhile, all the TPs extracted by SPE were also extracted by the DLLME technique. Furthermore, DLLME was much less expensive than SPE, besides being faster, easier, and requiring only small amounts of organic solvents. This work reports a new and very important tool for the extraction and preconcen- tration of TPs formed during degradation using techniques such as advanced oxidation processes (AOPs), since without this step it would not be possible to identify all the TPs formed in some complex wastew-ater matrices.

1. Introduction

Neurological disorders, such as epilepsy, among others, are some of the most important classes of diseases affecting the global population. The treatment of these illnesses frequently involves the prescription of benzodiazepines, with diazepam (DZP) being one of the most important of these compounds. DZP has anticonvulsant, anxiolytic, sedative, and muscle relaxant properties (Dudley et al., 2019; Sulaiman, 2017). It has been estimated that following inges- tion, 5–50% of the DZP dose is excreted unchanged, consequently entering the environment (West and Rowland, 2012). In a previous study, Becker et al. (2020) reported that DZP is commonly found in hospital wastewater (RHW). In aquatic environments, DZP has been detected in a wide range of concentrations, ranging from 20 ng L^{-1} (river water) to 600 ng L^{-1} (hospital wastewater) (Almeida et al., 2015; Khan et al., 2018).

The widespread global consumption of DZP and its constant re- lease into the environment have stimulated studies of the degra- dation of this pharmaceutical. Conventional wastewater treatments are unable to completely eliminate this type of compound, so the development and application of advanced oxidation processes (AOPs) may offer a solution, since they can provide highly effective and rapid degradation of organic compounds (Bosio et al., 2019). The efficiency of AOPs is due to the formation of hydroxyl rad- icals (HO•), which are highly oxidizing, very reactive, and non- selective (Bautitz and Nogueira, 2010). The degradation of DZP has been studied using different AOPs including heterogeneous pho- tocatalysis (Bosio et al., 2019; Sulaiman, 2017) and the heteroge- neous photo-Fenton process (Valcárcel et al., 2012). Although these processes are attractive options for use in DZP degradation, they employ artificial sources of UV irradiation. As a more environmen- tally friendly approach, the use of solar radiation can considerably reduce the cost of the treatment, making the solar photo-Fenton (SPF) process one of the most economic and efficient techniques (Bautitz and Nogueira, 2010; Giménez et al., 2015).

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The use of the SPF process for degradation of DZP has been shown to be a viable option (Bautitz and Nogueira, 2010; Cuervo Lumbaque et al., 2019), despite DZP being considered a recalcitrant compound (Banaschik et al., 2018; Kosjek et al., 2012). Nonetheless, it is very important to investigate the formation of transformation products (TPs) that may be more toxic than the parent compound (Lambropoulou and Nollet, 2014; Postigo and Richardson, 2014). Evaluation of the formation of TPs requires sensitive techniques such as UHPLC-QTOF MS for their identification, using exact masses, since commercial standards are not usually available. In addition, predictions of toxicity, biodegradability, mutagenicity, and carcinogenicity provide valuable information about TPs. For this purpose, computational methods such as QSAR (quantitative structure-activity relationships), VEGA, and EPI Suite, among other methodologies, are increasingly used for assessment of the environmental risk of different compounds, including TPs (Della-Flora et al., 2020; Jentzsch et al., 2016; Westphal et al., 2020; Yuval et al., 2017).

It is important to be able to detect the maximum possible number of TPs formed during a degradation process because, sometimes, the less abundant TP could be the most toxic/mutagenic/carcinogenic intermediate. To do this, it is necessary to use methodologies for the extraction and preconcentration of the TPs formed, since they may be present at very low concentrations. Currently, solid-phase extraction (SPE) is the technique most widely used for this purpose (Campos-Mañas et al., 2019; Senta et al., 2019). However, although this methodology is very efficient and a large number of different solid-phase extractants are commercially available, it requires large volumes of solvents and can involve lengthy procedures. As an alternative, microextraction using dispersive liquid-liquid microextraction (DLLME) is very fast, simple, environmentally friendly, and less expensive. Thistechnique, which was proposed in 2006 (Rezaee et al., 2006), uses only a few microliters of extraction and disperser solvents and can be applied for different organic compounds present in a variety of matrices (Carasek et al., 2018).

The present work has two aspects. Firstly, identification was made of the TPs formed during an SPF process for the degradation of DZP, together with a predictive risk assessment of these compounds. Secondly, an optimized methodology for the extraction of DZP and its TPs by DLLME was developed, which was applied for the SPF degradation of DZP in three different aqueous matrices: ultrapure water (UPW), simulated wastewater (SW), and raw hospital wastewater (RHW).

2. Experimental

2.1. Chemicals

Diazepam 98% and Diazepam-D5 (Sigma Aldrich, USA) were used. To chromatographic analyses, methanol LC-MS grade from Merck (Germany), ultrapure water (18.2 MΩ-cm, Millipore, USA) and formic acid (98–100%) from Sigma Aldrich (USA) were used. SPF experiments were performed using iron solution ($FeSO_4 \cdot 7H_2O$, P.A), hydrogen peroxide (35% w/v), sodium hydroxide and sulfuric acid (P.A.) for pH adjustment. To DLLME performance, acetonitrile (HPLC), ethanol (P.A.), methanol (P.A.), chloroform (P.A.), trichlorethylene (P.A.) and dichloromethane (P.A.) were tested. To compare DLLME to a traditional preconcentration method (SPE), Oasis HLB® (6 mL, 200 mg) cartridges were used.

2.2. Instrumentation

All analysis to identified DZP degradation and its TPs generated by SPF process were performed using a Shimadzu Nexera X2 UHPLC system connected to an Impact II QTOF mass spectrometer (BrukerDaltonics). QTOF operation parameters were: capillary voltage, 4000 V; end plate offset, 500 V; nebulizer pressure, 4 bar (N_2); dry gas, 8 $Lmin^{-1}$ (N_2) and dry temperature, 200 °C. The QTOF MS system operated in broadband collision–induced dissociation (bbCID) acquisition mode, which provided MS and MS/MS spectra at the same time. The chromatographic system was equipped with a reversed phase Hypersyl GOLD analytical column (150 mm x 2.1 mm x 3 μm), thermostated at 35 °C. QTOF MS system was equipped with an electrospray ionization source (ESI), operating in positive ionization mode. Mobile phase was (A) MeOH acidified with 0.1% (v/v) formic acid and (B) H_2O acidified with 0.1% (v/v) formic acid , at a flow rate of 0.5 mL min^{-1}. The gradient elution program was: 0 min, 95% B; 1 min, 95% B; 11 min, 5% B; 14 min, 5% B; 16 min, 95% B; 20 min, 95% B. All the MS information was recorded over the m/z range from 50 to 1200, using a scan rate of 2 Hz. The bbCID mode allows operation with two different collision energies: low collision energy of 10 eV (MS) and high collision energy variation from 20 eV to 40 eV (MS/MS). All data were processed using DataAnalysis 4.2 software and TargetAnalysis 1.3, both from BrukerDaltonics.

2.3. Primary elimination of diazepam and TPs identification

The SPF process for the degradation of DZP, prior to identification of the TPs, was carried out using a solar batch photoreactor (1 L) equipped with a magnetic stirrer. Before the experiments, the initial pH of the DZP solution was adjusted to 5.0 using H_2SO_4 (0.05 mol L^{-1}). After initial tests, this pH was selected because use of the classical photo-Fenton pH (2.8–3.0) (Bassam et al., 2012) would imply higher reagent consumption for acidification of the medium. After pH adjustment, addition was made of 5 mg L^{-1} Fe^{2+} (using a 10 g L^{-1} Fe^{2+} stock solution) and hydrogen peroxide at an initial concentration of 50 mg L^{-1} (using a 35% w/v solution), in this order, followed by exposure to solar radiation for initiation of the treatment process. The solar UV radiation was measured with a solar energy meter (SP-2000, ICEL), which provided data in terms of incident UV (W m^{-2}). These data were used to calculate the t_{30W} values (Nogueira et al., 2005). An initial solution of DZP at a concentration of 1000 μg L^{-1}, prepared in ultrapure water (UPW), was used to identify TPs formed during the degradation process. Subsequently, different water matrices and initial DZP concentrations were employed in the experimental assays (detailedin Section 2.7).

The reaction time was initially obtained as the clock time, with subsequent transformation to t_{30W} after the experiment. During the treatment process, eight samples were collected at different times, filtered using a 0.22 µm PVDF syringe filter, and analyzedby LC-QTOF MS to identify the formation of possible TPs, considering the appearance of new chromatographic peaks during the process. Data from the TP analyses were processed using Data Analysis 4.2 software, selecting the elemental composition and double-bond equivalency (DBE) options. In most cases, possible elemental compositions for the ions were assigned with a maximum error of ±5 ppm. After the identification of TPs, a DZP degradation pathway was proposed.

2.4. In silico *quantitative structure-activity relationship (QSAR) models used for prediction and risk assessment*

The structure of the DZP and its TPs were transformed into SMILES strings by means of ChemBioDraw Ultra (v.12) and subjected to *in silico* predictions by freely available and different software and models. The log K_{OW} and log BCF values were predicted by EPI Suite™ KOWWIN v1.68 and BCFBAF v3.01 programs, respectively. The persistence and biodegradability were predicted by

EPI Suite™ BIOWIN v4.10. The predicted ready biodegradability of the combined BIOWIN 1–7 models (Boethling and Costanza, 2010; Pavan and Worth, 2008) is pointed out as "yes" or "no" based on the following criteria: "yes" if the BIOWIN 3 result is "weeks", "days" or "days to weeks" and in the case of BIOWIN 5 the probability is $\geq$ 0.5. If these criteria were not met the prediction is "no" (US EPA, 2012).

The Prometheus software (Pizzo et al., 2016) was used for predicting and ranking the DZP and TPs depending on its persistence (P), bioaccumulation (B), and toxicity (T), *i.e.* PBT compounds, for the hazard assessment according to Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (REACH Commission regulation, 2011).

Moreover, the mutagenicity activity was predicted by means of VEGA QSAR v.1.1.4 software (Benfenati et al., 2013) and CASE Ultra (Chakravarti et al., 2012; Saiakhov et al., 2014, 2013). A consensus approach was used by applying the Mutagenicity (Ames test) CONSENSUS model (v1.0.2). It performs an analysis among the CAESAR model (v.2.1.13), ISS model (v.1.0.2), KNN/Read-Across model (v.1.0.0), and SarPy/IRFMN model (v.1.0.7) to predict the mutagenicity by a consensus score from 0 to 1 (as the consen-sus score approach to 1 more reliable is the prediction). Concern- ing carcinogenicity activity, VEGA QSAR software (Benfenati et al., 2013) was applied for the predictions. It provides the resultsin four different models for carcinogenicity: (i) Carcinogenicity model (IRFMN/Antares) (version 1.0.0), (ii) Carcinogenicity model (CAESAR) (version 2.1.9), (iii) Carcinogenicity model (ISS) (version 1.0.2), and (iii) Carcinogenicity model (IRFMN/ISSCAN-CGX) (version 1.0.0). In general, VEGA QSAR provides an applicability do- main index (ADI). If the ADI is lower than 0.75, it might indicate that there are differences in the target compound compared to thesimilar one found in the database. CASE Ultra modes (GT_Expert, GT1_BMUT and PHARMA_MUT) statistical and ruled-based system according to ICH M7 guidelines for mutagenicity was applied as well (ICH, 2017).

2.5. Analytical measurements during the SPF treatment process

During all the SPF treatments, the total iron and hydro- gen peroxide concentrations were monitored. The total iron con- tent was determined by a colorimetric method employing 1,10-phenanthroline, according to ISO (1988), using a Cary 50 UV– Vis spectrophotometer. Hydrogen peroxide was determined by a spectrophotometric method employing ammonium metavanadate (Nogueira et al., 2005).

2.6. DLLME method

Preconcentration using the DLLME technique was performed to improve the detection of TPs during DZP degradation. In preliminary tests, the best combination of extraction solvent (ES) and disperser solvent (DS) was selected for maximizing analyte extraction efficiency. For this, nine ES/DS pairs were eval- uated: chloroform/acetonitrile, chloroform/methanol, chloroform/ ethanol, trichloroethylene/acetonitrile, trichloroethylene/ methanol, trichloroethylene/ethanol, dichloromethane/acetonitrile, dichloromethane/methanol, and dichloromethane/ethanol. In these tests, a SPF degradation process was performed under the same conditions as the experiment for identification of the TPs, with treatment for 120 min. The volumes of ES and DS were initially set at 100 and 400 μL, respectively. A 7.5 mL aliquot of the sample was transferred to a glass centrifugation tube with a conical base. The solvent mixture was rapidly dispersed in the sample, resulting in the instantaneous formation of an emulsion (microdrops). The emulsion was centrifuged at 2000 rpm for 5 min, after which the organic phase was removed using a microsyringe and transferred to a 2 mL chromatography vial. The solvent was evaporated under a gentle stream of nitrogen and the extract was reconstituted with 500 µL of water:methanol (90:10 v/v), prior to analysis using UHPLC-QTOF MS. The DS/ES combination that provided the best extraction of DZP and its TPs was selected and a 2^3 factorial design with a central point was used to evaluate the effects of the DS and ES volumes, as well as the ionic strength (Na_2SO_4 concentration). An optimization of the experimental DLLME conditions was then performed according to a Doehlert design, with the volumes of DS and ES as the variables.

2.7. DLLME method for extraction of TPs in different matrices

The optimized DLLME variables for extraction of the TPs were tested in new SPF assays performed using the different matrices (UPW, SW, and RHW), under the same SPF conditions described above, but with an initial DZP concentration of 500 µg L^{-1}. The SW composition was adapted from OECD (OECD, 2001), simulating the organic content of real hospital wastewater, as follows: 160 mg L^{-1} of peptone, 110 mg L^{-1} of beef extract, 30 mg L^{-1} of urea, 2 mg L^{-1} of $Mg_2SO_4 \cdot 7H_2O$, and 4 mg L^{-1} of $CaCl_2 \cdot 2H_2O$. For the analysis using RHW, a sample of wastewater was collected at a hospital in the city of Porto Alegre (Rio Grande do Sul, Brazil). All the degradation experiments using the different matrices were carried out in duplicate.

2.8. Cost and time evaluation of the DLLME technique

The cost and time requirements of the DLLME method were obtained considering the value of the reagents and the ex- traction time, and were compared to a standard SPE method (Hernández et al., 2015).

3. Results and discussion

3.1. Elucidation of proposed transformation products

Since TPs may be generated in low concentrations during a degradation process, an initial experiment was performed to iden-tify the TPs using a solution of DZP at 1000 µg L^{-1} in UPW, with- out application of any extraction or preconcentration method. Be- fore addition of ferrous iron and hydrogen peroxide, the pH of the solution was adjusted to 5. The SPF process was carried out for 120 min, with aliquots collected at 0, 15, 30, 45, 60, 90, and 120 min. In order to stop the degradation process, the hydrogen peroxide was quenched with excess of sodium bisulfite. The results obtained for the total iron concentration, hydrogen peroxide con- sumption, and DZP degradation are provided in Section S1 (*Supple- mentary Material*).

After the SPF process, the collected samples were filtered and analyzed by LC-QTOF MS for identification of possible TPs, based on the appearance of new chromatographic peaks. This resulted inthe identification of five proposed TPs (TP1-TP5). After optimiza- tion of the DLLME process (Section 3.3), additional samples were collected for LC-QTOF MS analyses, in order to discover any pos- sible TPs that had not been identified in the first experiment, but were detectable after sample preconcentration by DLLME. This re- sulted in the identification and proposal of another new TP (TP6), showing the importance of using a suitable extraction method for the analysis of TPs during treatment processes.

Elucidation of the structures of TPs is a complex task that re-quires consideration of many parameters in order to propose the most representative structure. Section S2 (*Supplementary Material*) shows the results provided by the analytical software for all the TPs identified, including the experimental and calculated masses

(protonated molecules), the best formula proposed for each *m/z*, the error (in ppm), and DBE (double-bond equivalence) information, besides de MS spectra of each TP, with the fragments proposed.

For all the data presented, low errors (below 5 ppm) indicated that the proposed elemental composition was in agreement with the exact experimental mass. In order to ensure a reliable fragmentation pattern for each TP, collision energies between 20 and 40 eV were applied in broadband collision-induced dissociation (bbCID) acquisition mode.

The SPF degradation of DZP proceeds according to different pathways (Fig. 1). In one of these pathways (Fig. 1A), the degrada-

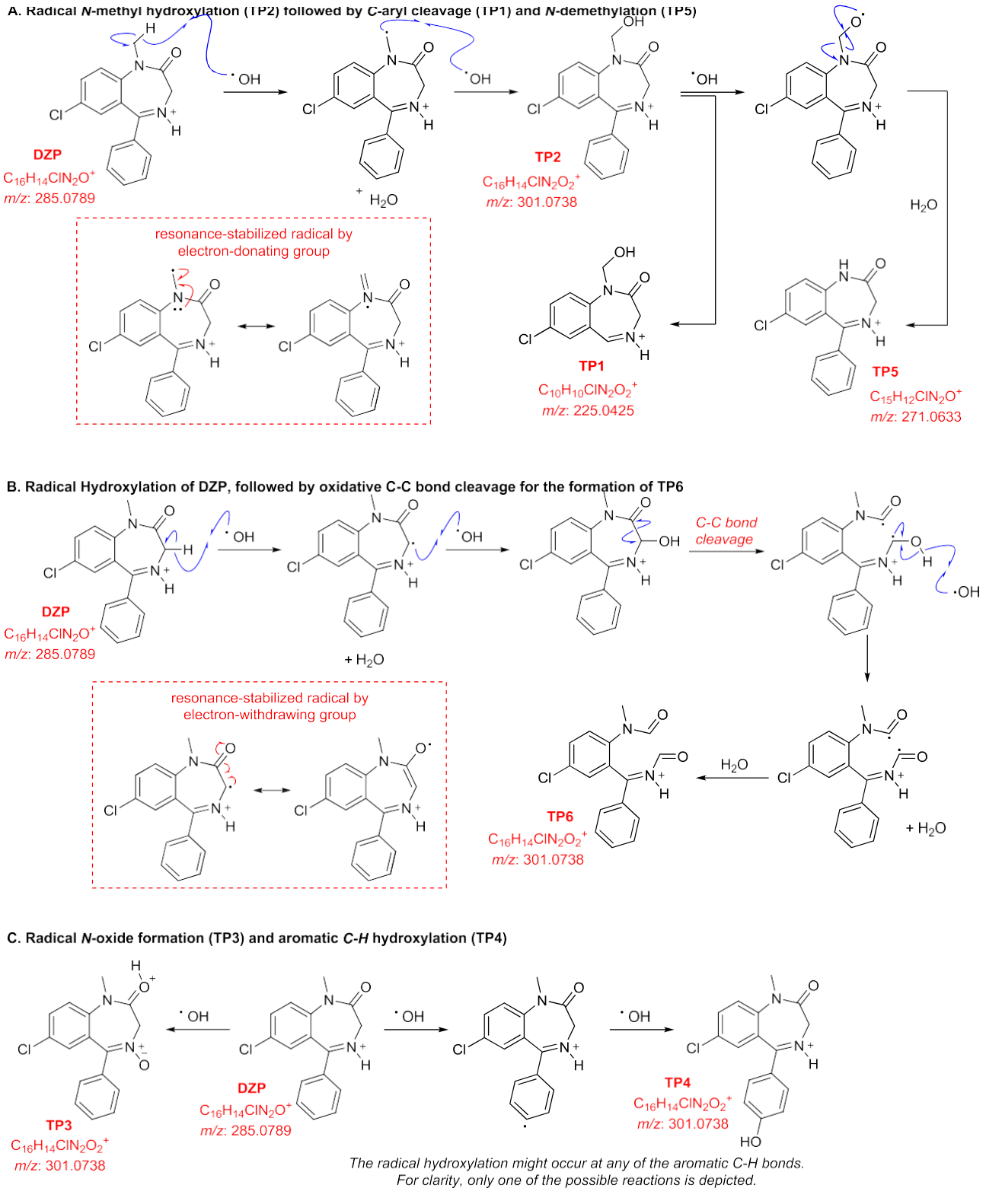

Fig. 1. Proposal pathway to DZP degradation during SPF treatment.

tion starts with the reaction of a hydroxy radical with the N–CH_3 group leading to a resonance-stabilized radical with an electron-donating group. Further reaction of this radical leads to hydroxylation and formation of TP2 (*m/z* 301.0738), which can undergo radical C-aryl cleavage to generate TP1 (*m/z* 225.0425) or radical *N*-demethylation to form TP5 (nordiazepam; *m/z* 271.0633). TP5 is known to be a major metabolite of DZP (Greenblatt et al., 1980) and has been identified in different water matrices (Aymerich et al., 2016; López-Serna et al., 2012; Wang et al., 2017). Both DZP and nordiazepam are pharmacologically-active compounds (Greenblatt et al., 1981). A second degradation pathway is triggered by the radical hydroxylation of DZP at the α-carbonyl position, generating a radical which is stabilized by resonance with an electron-withdrawing group (Fig. 1B). The resulting radical is trapped by another hydroxyl radical to yield the highly reactive α-hydroxy ketone, which under oxidative conditions undergoes carbon-carbon bond cleavage, ultimately leading to formation of the oxidized product TP6 (*m/z* 301.0738). Finally, Fig. 1C shows the direct oxidation pathway of the sp^2 nitrogen of aryl C–H bonds, resulting in formation of the corresponding *N*-oxide, TP3 (*m/z* 301.0738). In addition, hydroxylation at any of the aromatic C–H bonds leads to the formation of TP4 (*m/z* 301.0738), which can exist as several oxidized positional isomers (for clarity, only one is shown).

Although the transformation of DZP to TP5 is very well established, the aromatic hydroxylation is not clearly reported in the current literature. Only a few studies describing the degradation of DZP have tried to elucidate its TPs. Some proposals for TP4 were published by Kosjek et al., al.(2012) and Helbling et al., al.(2010). However, these authors were unable to indicate which aromatic ring was hydroxylated, since the MS spectra showed common fragments and several isomeric products were possible (see Section S2, *Supplementary Material*). Other TPs proposed here (TP1, TP2, TP3, and TP6) have not been previously reported in the literature. Only one study (Bautitz et al., 2012) reported four structural isomers with *m/z* 301, which could correspond to the addition of one hydroxyl radical in different positions of DZP, but no structure was proposed.

Besides all the data provided by the Data Analysis software, another parameter used in proposal of the structures was the log K_{OW} of each structure. Given the use of a nonpolar chromatographic separation column, the log K_{OW} value should increase with increase of the retention time. The proposed structures were in agreement with this parameter, since the log K_{OW} values predicted by the EPI SuiteKOWWIN v.1.68 software were 2.82, 0.41, 1.23, 1.70, 2.22, 2.87, and 3.40 for DZP, TP1, TP2, TP3, TP4, TP5, and TP6, respectively.

3.2. In silico *risk assessment of DZP and its TPs*

The persistence of DZP and its TPs was assessed by *in silico* predictions provided by the BIOWIN models of the EPI Suite software. The predicted values are shown in Table S2 (Section S3, *Supplementary Material*). Fig. 2A shows the BIOWIN 5 MITI (Japanese Ministry of International Trade and Industry biodegradation database) predictions for the analyzed compounds. Since values higher than 0.5 indicate possible biodegradability, the predictions suggested that DZP and all the TPs were persistent.

All the TPs were predicted to be non-readily biodegradable using both the BIOWIN 5 model and the VEGA IRFM model. Such behavior could be explained according to the generalized “rules of thumb” for biodegradation, where the presence of chlorine in the molecule increases the recalcitrance.

Assessment of the potential mutagenicity and carcinogenicity of DZP and the TPs was performed using Prometheus software to obtain the PBT values. The predicted *in silico* values are shown in Table S3 (Section S3, *Supplementary Material*). A total score threshold of 0.5 was used to discriminate non-PBT (<0.5) and potentially PBT or vPvB (≥0.5) compounds (Pizzo et al., 2016). Fig. 2B shows the predicted PBT values for DZP and its TPs. All the structures were ranked below the threshold value of 0.5, with the exception of TP6 (PBT = 0.514). However, the TPs could be PBT compounds, considering the uncertainty of the predictions (Pizzo et al., 2016) and the fact that the values were close to the threshold value.

The results obtained using the VEGA QSAR toolbox and CASE Ultra models for the prediction of carcinogenicity and mutagenicity are summarized in Table S4 (Section S3, *Supplementary Material*). According to the VEGA QSAR models for Mutagenicity (Ames test) CONSENSUS (A), none of the molecules evaluated gave a positive alert. The predictions provide a consensus score from 0 to 1, where a score of 1 usually corresponds to structures inside the ADI, with the presence of the experimental value in at least one of the four CONSENSUS models. A consensus score below 0.75 indicates structural differences among the predicted compounds, with the structures present in the predictive models and the predictionswith low consensus scores being classified as having moderate or low reliability. In the present case, the structures studied were not similar to the models, so these results could not be considered reliable. The VEGA carcinogenicity models IRFMN/Antares (B), CAESAR (C), ISS (D), and IRFMN/ISSCAN-CGX (E) showed no carcinogenicity alerts for DZP, with all of the models having experimental values available. Evaluation of the TPs using the different models resulted in no positive alerts with good reliability. There were some positive alerts with moderate reliability, but divergences were observed between the models, so no reliable conclusions could be reached.

Considering the QSAR Toolbox results, no alerts were reported considering DNA alerts for AMES by OASIS (A) and DNA alerts for CA and MNT by OASIS (B). However, *in vitro* mutagenicity (Ames test) alerts by ISS (C) and *in vitro* mutagenicity (micronucleus) alerts by ISS (D) identified two types of alerts. N-methylol derivative alerts for TP1 and TP2 are shown in Fig. 2C, while H-acceptor-path3-H-acceptor alerts found for all the molecules are provided in Section S4 (*Supplementary Material*). An aromatic N-acyl amine alert was obtained for TP6. CASE Ultra analysis of DZP and the TPs was performed using three models: i) the CASE ULTRA GT_EXPERT model, which identified mutagenicity alerts for TP1 and TP2, due to the N-methylol derivative group; ii) the CASE ULTRA GT1_BMUT model, which identified two more alerts for TP1,while four more alerts were obtained for TP2 (in addition to the mutagenicity alerts for TP1 and TP2 due to the N-methylol derivative, also predicted by QSAR Toolbox) and iii) PHARM_BMUT model, which identified N-methylol derivative alert for TP1.

The risk assessment analysis results predicted possible risks of mutagenicity associated with TP1 and TP2, especially due to the N-methylol derivative group present in these TPs. The potential toxicity of this class of compounds is probably associated with the generation of formaldehyde by hydrolysis, since formaldehyde is a highly reactive genotoxic agent (Ashby and Tennant, 1988; Benigni and Bossa, 2008). These results showed the importance of TPs evaluation and the need for a preconcentration/extraction method that enables the identification of TPs during treatment processes.

3.3. *DLLME method*

The development of a DLLME preconcentration/extraction method for the TPs generated during DZP degradation started with selection of the best solvent pair (DS/ES). Nine pairs of solvents

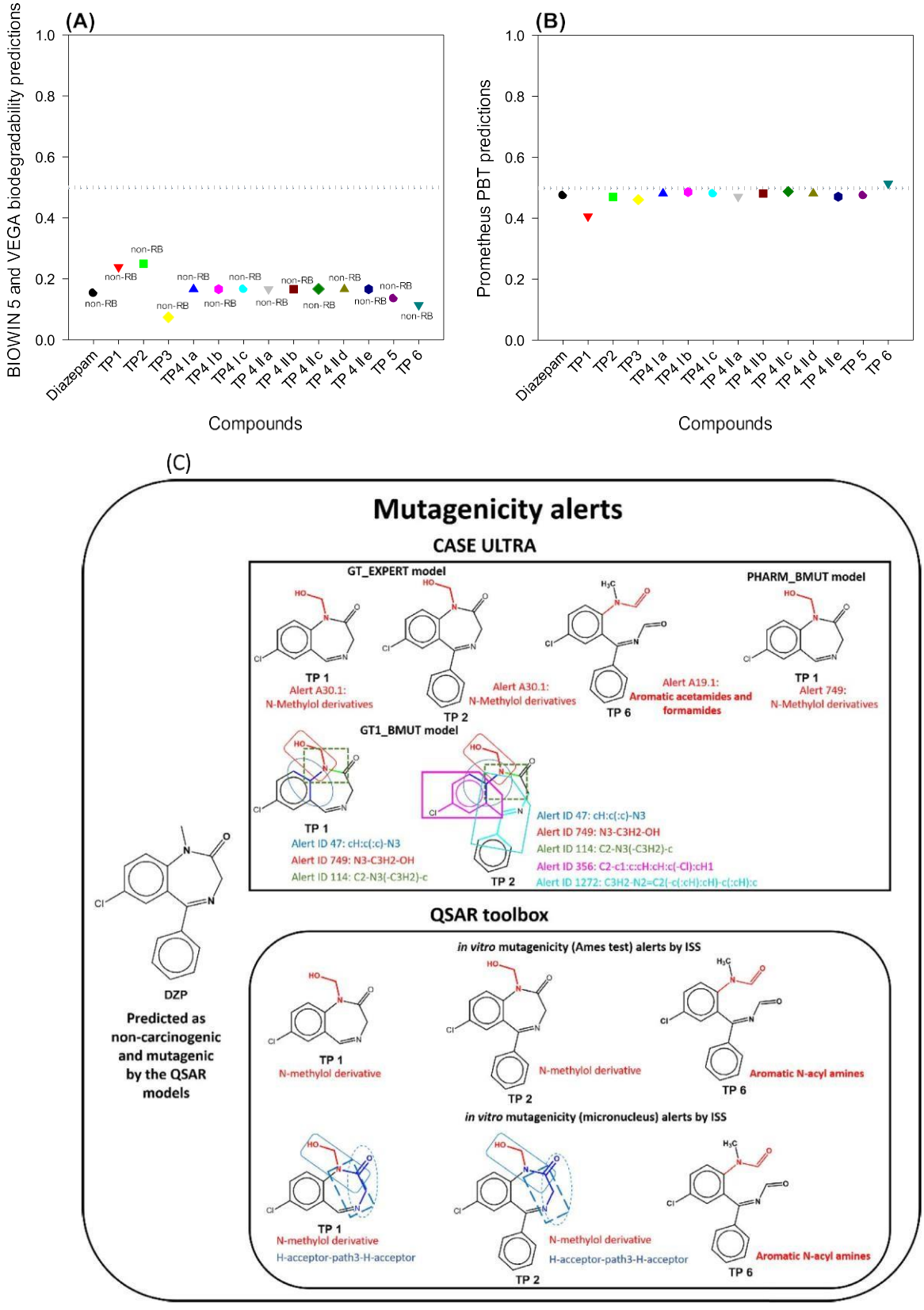

Fig. 2. *In silico* risk assessment by QSAR for DZP and TPs, (A) Biodegradability prediction; (B) PBT prediction; (C) Mutagenicity alert predicted for TP1, TP2, and TP6.

(A)

Codified value	Variable 1 (levels)				
	-1	-0.5	0	0.5	1
Experimental value DS (µL)	400	550	700	850	1000
	Variable 2 (levels)				
Codified value		-0.866	0	0.866	
Experimental value ES (µL)		100	300	500	

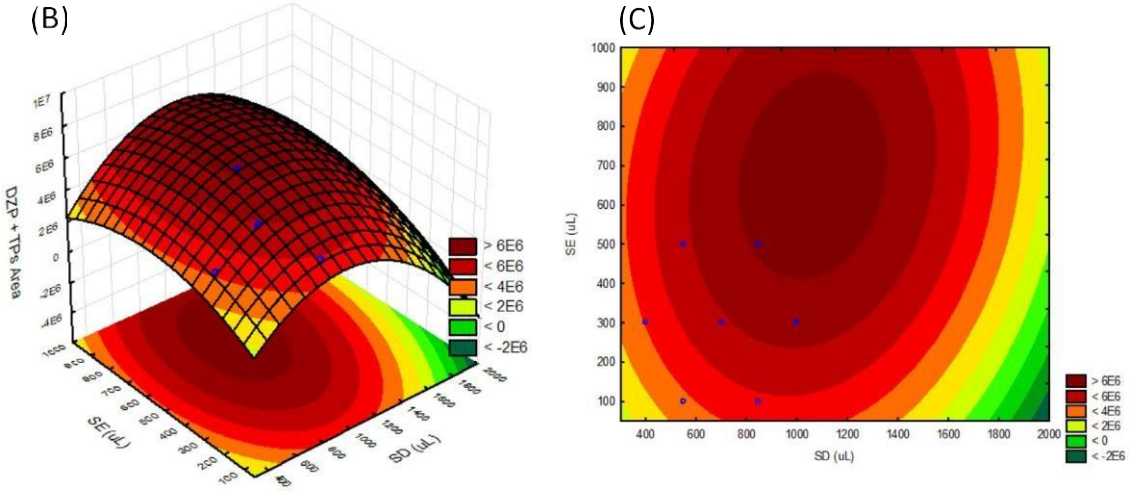

Fig. 3. Doehlert design level tests for the two variables studied and results obtained in Doehlert desing that allowed to represent (A) levels of the variables; (B) response surface; and (C) contour plot for ES volume *vs.* DS volume, on DZP and TPs area, after extraction by DLLME.

were tested and the best response was obtained when acetonitrile was used as DS and chloroform was used as ES. Throughout the optimization of the preconcentration/extraction method, the analytical response adopted was the summation of the peak areas of DZP and the TPs.

After identifying the best solvent pair for extraction of the TPs, evaluation was made of the effects of several variables that could influence the extraction efficiency. Three main variables were selected for investigation: i) DS volume (µL), ii) ES volume (µL), and (iii) ionic strength (mol L^{-1} Na_2SO_4). The centrifugation was set at 2000 rpm for 5 min, this parameter was not studied since literature studies shows that centrifugation is responsible for the phases separation and should not be much longer, once this could lead to a degradation of the analytes (Ahmad et al., 2015; Andruch et al., 2013; Nowak et al., 2020). Although the pH of the solution during the dispersion process could have an influence on the extraction efficiency, the pH was not a variable studied here. The pH was set at 5, because slightly acidic conditions favor the photo-Fenton reaction, while pH above 6 can cause iron precipitation (Cuervo Lumbaque et al., 2019).

A 2^3 factorial design was used with the three defined variables, in order to provide an indication of their effects. After analysis of the results, a more refined design was applied to obtain the best condition. The variable values and the response surface of the 2^3 factorial design are provided in Section S5 (*Supplementary Material*). The results indicated that the extraction was favored at themaximum DS and ES volumes, although this experimental design did not define an optimum point. The ionic strength of the solution, in the range studied, showed no statistically significant effect on the analytical response, and was not considered for the followed design. Given these results, a new experiment was proposed, based on a Doehlert design, to determine the best condition for the extraction of DZP and its TPs, considering only two variables, namely the volumes of DS and ES. It is important to highlight that, to identification of the best conditions for each variable, in both experimental designs, the desirability function, proposed by Derringer and Suich in 1980 (Derringer and Suich, 1980) was used. A very usual tool frequently applied in response surface methodology, desirability function brings efficiency, economy, and objectivity in the optimization process (Bezerra et al., 2008) and was applied for different variables and their responses. Desirability scale ranges between $d = 0$ (for a non-desirable response value) and $d = 1$ (for a more desirable value) and it was calculated for individual values. The desirability function D is calculated by combining the individual desirability values with the geometric mean: $D = (d1 \times d2 \times \ldots dm)^{1/m}$ (Ferreira et al., 2007). The desirability graphics of each experimental design can be seen in sections S5 and S6 (*Supplementary Material*).

Fig. 3A shows the different levels of the two variables selected in the Doehlert design. The ranges tested were 400–1000 µL of DS and 100–500 µL of ES, with a total of thirteen experiments (further details are provided in Section S6, *Supplementary Material*). The results of the Doehlert design experiments were used to construct the response surface graph shown in Fig. 3B and C, according Eq. (1).

$$z = -2057159,08 + 12608,57 * x - 6,70 * x^2 + 8750,05 * y - 8,36 * y^2 + 2,38 * x * y + 0 \quad (1)$$

where $z = \Sigma$ (DZP area + TPs area); $x =$ DS volume and $y =$ ES volume.

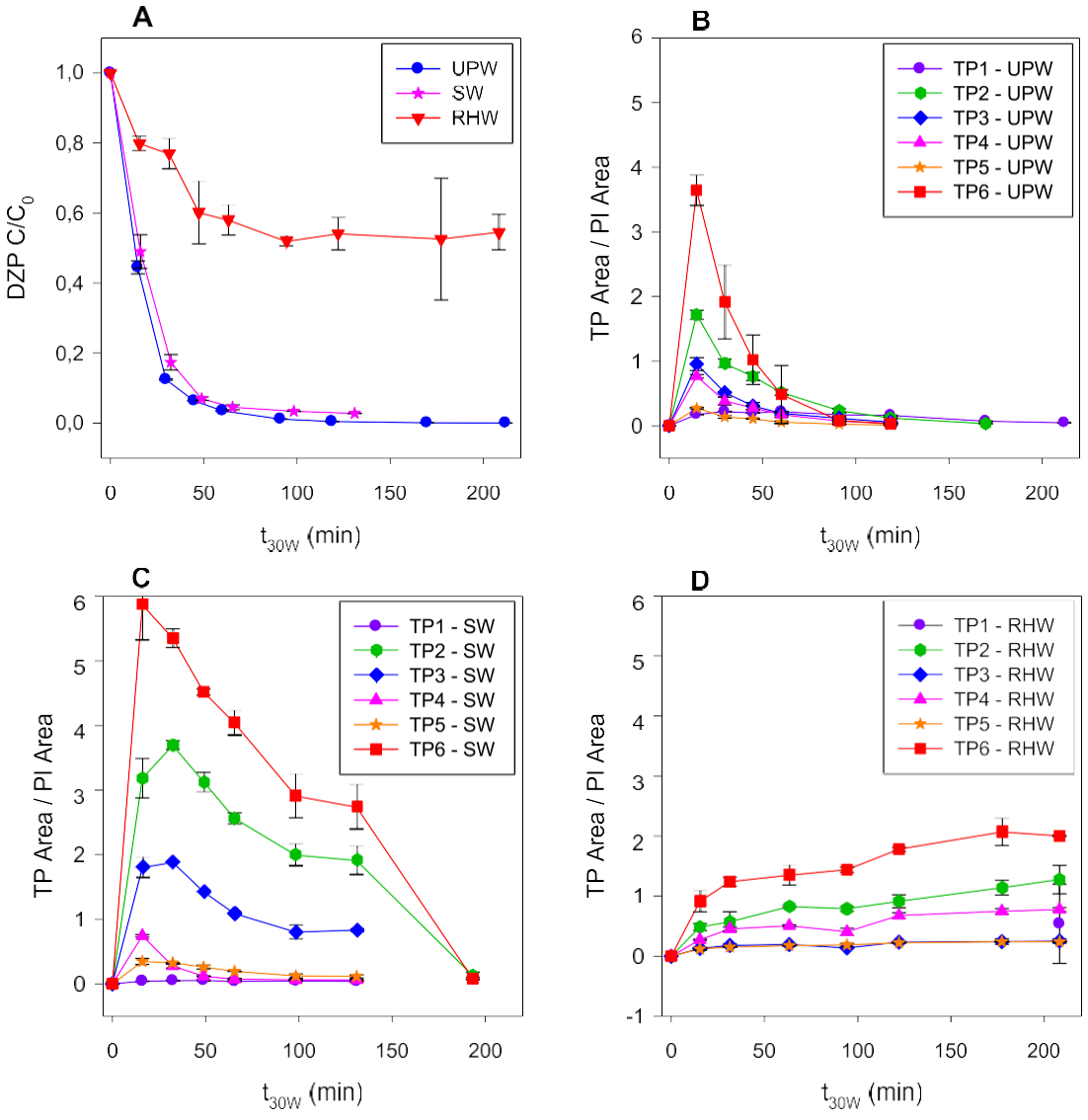

Fig. 4. DZP degradation (A) in different matrices; (B) TPs formation in ultrapure water; (C) simulated wastewater; and (D) hospital wastewater.

Considering the response only around the experimental conditions (blue points), the best response was obtained when the maximum values of DS and ES were used (1000 µL of DS and 500 µL of ES).

All the statistical parameters of the Doehlert design are shownin Section S6 (*Supplementary Material*). The maximum explainable variation of the model was 94% ($r_{adjusted}$ = 0.94), demonstrating that the model presented a good fit, enabling prediction of the best extraction condition. The maximum predicted response was obtained using around 1000 µL of DS and 650 µL of ES. A new ex- periment was then performed to test the best condition predictedby the model, resulting in a difference of 7.9% between the pre- dicted and experimental values. Consequently, the optimized con- dition for extraction of DZP and its TPs was defined as the use of 1000 µL of DS and 650 µL of ES, dispersed in 7.5 mL of the sample at pH 5, with centrifugation of the mixture for 5 min at 2000 rpm, followed by collection of the lower phase with a microsyringe. The extract was evaporated, the sample was reconstituted with 500 µL of H_2O:methanol (90:10 v/v), and analysis was performed by LC- QTOF MS.

3.4. Use of DLLME for preconcentration/extraction of DZP and its TPs present in different matrices

The method developed to extract DZP and its TPs in the SPF degradation experiments was applied using three different matrices: UPW, SW, and RHW. An initial solution of DZP at 500 µg L^{-1} was used in these experiments. The SPF experimental parameters are provided in Fig. S18 (Section S7, *Supplementary Material*).

Fig. 4A shows the results for degradation of DZP in the three different matrices, obtained using the optimized DLLME extrac- tion procedure. The analytical response employed was the ratio between the observed and initial DZP concentrations (C/C_0). The analytical curve presented an r^2 value of 0.9987 and the limits of detection and quantification were 2.8 and 8.6 µg L^{-1}, respectively. As expected, the nature of the matrix had a significant influence on the degradation process. In the UPW, only DZP was initially present in the solution, so the hydroxyl radicals only acted in the degrada- tion of DZP and the TPs. Very fast degradation of DZP was observed in the SW, despite the presence of other organic compounds. How- ever, very slow degradation occurred in the RHW, which could be

attributed to the highly complex nature of the matrix, with the presence of many organic compounds, including other pharmaceuticals and human metabolites, as reported in a previous study employing RHW (Becker et al., 2020). In the case of RHW, the hydroxyl radicals generated during the solar photo-Fenton process were not selective towards DZP and its TPs, so competition involving other molecules led to slower degradation of DZP.

The experimental data for the degradation of DZP at an initial concentration of 500 μg L^{-1} in the different matrices were fitted using a pseudo-first order model, performed with SigmaPlot 10 software (Systat Software, USA). The degradation of DZP in UPW followed pseudo-first order kinetics, according to Eq. (2):

$$[C] = [C_0] \cdot e^{-k_{obs} \cdot t} \quad (2)$$

where, C is the concentration of DZP at time t, C_0 is the initial concentration of DZP, and k_{obs} is the degradation rate constant. The half-life ($t_{½}$) for DZP degradation was calculated according to Eq. (3):

$$t_{1/2} = \ln 2 / k_{obs} \quad (3)$$

For DZP degradation in UPW, the value of k_{obs} was 6.07 × 10^{-2} min^{-1} t_{30W}, with r^2 of 0.9967 ($p < 0.0001$), C_0 of 1.0064, and a half-life time ($t_{½}$) of t_{30W} = 11.42 min.

DZP degradation in SW also followed pseudo-first order kinetics, with the same equations Eqs. (2) and (3). The k_{obs} value was 4.93 × 10^{-2} min^{-1} t_{30W}, with r^2 of 0.9950 ($p < 0.0001$), C_0 of 1.0080, and a half-life time ($t_{½}$) of t_{30W} = 14.06 min.

The degradation of DZP in RHW presented different order kinetics, with the process being biphasic (Langdon et al., 2011). In the first stage, the degradation followed pseudo-first order kinetics, while no degradation was observed in the second stage (Eq. (4)). This was indicative of the existence of one degradable fraction and another recalcitrant fraction, shown by the y-intercept (y_0).

$$[C] = [C_0] \cdot e^{-k_{obs} \cdot t} + y_0 \quad (4)$$

In Eq. (4), C is the concentration of DZP at time t, C_0 is the initial DZP concentration, k_{obs} is the degradation rate constant, and $y_0$represents the recalcitrant fraction. The half-life ($t_{½}$) of DZP degradation was calculated according to Eq. (3). For the degradation of DZP in RHW, k_{obs} was 3.09 × 10^{-2} min^{-1} t_{30W}, with r^2 of 0.9672 ($p < 0.0001$), a half-life time ($t_{½}$) of t_{30W} = 22.43 min, and y_0 = 0.5226.

After analysis of the degradation of DZP in the different matrices, investigation was made of the formation and degradation of the TPs (Fig. 4). Since the most of TPs are new and unknown compounds, analytical standards are not available. Concerning the DPZ TPs, only TP5 (a DZP metabolite known as nordiazepam) has commercially analytical standard available. Accordingly, no quantitative analysis was developed for the TPs and the evaluation of formation and further degradation of TPs was based on the ratio between the TP and internal standard (PI) peak area as analytical response. Although the values obtained for DZP degradation in UPW and SW were very similar, the formation and degradation of the TPs differed in these two matrices. In UPW, DZP degradation by the SPF process resulted in very fast formation of TPs, with maximum formation of TP2, TP3, TP4, TP5, and TP6 at t_{30W} of 14.7 min, after which these TPs were considered degraded. An exception was TP1, which showed maximum formation at t_{30W} of 29.7 min, which was reasonable, considering that TP1 was formed by degradation of TP2. After t_{30W} of 118.7 min, it is not possible to detect TP3, TP4, TP5, and TP6, while it was possible to detect TP2 and TP1 until t_{30W} of 169.7 and 211.8 min, respectively. This was of concern, since TP1 and TP2 presented mutagenic characteristics and were the most persistent TPs.

In the treatment of the SW, there was rapid formation of the TPs, while their initial degradation rates were slower, compared to the UPW treatment. The formation of TP1 was lower, compared to the UPW treatment, and after t_{30W} of 131 min, it was not possible to detect TP1, TP3, TP4, and TP5. After t_{30W} of 193.2 min, TP2 and TP6 could still be detected in low amounts, while no TPs were detectable after t_{30W} of 246.9 min.

The degradation of DZP in the RHW was significantly slower than in the other matrices, which was directly reflected in the formation and degradation of the TPs. Even after t_{30W} of 208.2 min, all the TPs were identified in the degraded samples. During this treatment process, it was only possible to detect TP1 after t_{30W} of 208.2 min, which could be explained by the fact that there was less formation of TP2, so consequently there was lower formationof TP1, since the latter was formed from TP2.

In addition to evaluation of the formation of the TPs in the different matrices, it was possible to determine the preconcentration factor of the method for these matrices. Consistent with the effect of the matrix on DZP degradation, the preconcentration factor also differed according to the matrix. This could be explained by the fact that the extraction was not exclusive for the compounds identified, but could also have involved other substances present, particularly in the case of a complex real matrix such as RHW. Considering DZP and all the TPs, the preconcentration factor values for the UPW matrix ranged between 5.9 and 13. For the SW matrix, the preconcentration factor values were in the range from 4.3 to 8.6, while for the RHW matrix, lower values of between 1.9 and 7.1 were obtained. More details are presented in Table S12 (Section 7, *Supplementary Material*).

The importance of preconcentration was shown by the fact that TP1 was not detected in samples analyzed without extraction and preconcentration, but could be identified after application of the extraction methods (SPE and DLLME). Considering the predicted mutagenicity of TP1 (as an example), it is clearly important that an extraction method should be available for the extraction and concentration of TPs during solar photo-Fenton degradation processes.

Table 1
Time demand and cost evaluation for DZP and its TPs extraction by DLLME and SPE.

Extraction technique	Materials and reagents	Cost Value per sample extraction[a] USD	Value for 01 sample extraction USD	Temporary Demand Time for 01 sample extraction (min)
DLLME	Chloroform	0.07	0.43	15
	Acetonitrile	0.21		
	Methanol PA*	0.08		
	Dichloromethane PA*	0.06		
SPE	Methanol HPLC/UV	1.03	13.72	70
	Oasis® HLB Cartridge	12.69		

[a] Value of material and reagents used for one sample.
* Solvents used to clean the microsyringe.

3.5. Time demand and cost evaluation of DLLME

Cost and time evaluations were performed by comparing the DLLME technique and a standard SPE method described in the literature for extraction of pharmaceuticals and TPs (Hernández et al., 2015). For this comparison, the values of con- sumables, solvents, and reagents were considered. Permanent ma- terials (such as the manifold used in SPE and the microsyringe used in DLLME), the cost of LC-QTOF MS analysis, the working hours of the analyst, and equipment maintenance costs were not considered.

The results (Table 1) showed that SPE was much more expen- sive than DLLME. The cost of SPE was US$ 13.44 per extraction, dueto the high cost of the extraction cartridge. Furthermore, a long time of 70 min was required for the extraction and resuspension of one sample in the SPE procedure. In contrast, the DLLME method presented a cost of US$ 0.43 and a time requirement of 15 min per sample extraction.

The development of an accessible preconcentration methodol- ogy is very important, since it allows analyses to be readily per- formed during a degradation process, without concern about ad- ditional cost implications, enabling significant improvement in theability to detect and identify TPs.

4. Conclusions

In this work, it was possible to elucidate six transformation products generated during SPF degradation of DZP, four of which had not been reported previously in the literature. In risk as- sessment of the TPs, two of the compounds (TP1 and TP2) pre- sented mutagenicity alerts, indicating that they could present a greater environmental risk, compared to the parent compound. This demonstrated the need to understand the mechanisms of DZP degradation, as well as to use an effective method capable of de- grading both DZP and its TPs, especially given that DZP is consid- ered a recalcitrant compound.

The proposed DLLME method for extraction and preconcentra- tion of DZP and its TPs was shown to be fast, cheap, and sim- ple. Its use enabled the elucidation of a TP that could not bedetected without preconcentration. When the SPF treatment was performed in UPW, the proposed DLLME method provided a pre- concentration rate that was very similar to that of SPE. When the SPF treatment was performed in RHW, the DLLME method showed a lower preconcentration efficiency, compared to SPE, but nonetheless achieved the same goal, since it succeeded in extract- ing/preconcentrating all the TPs also found by the SPE method. In terms of the cost and time of the method, DLLME was much cheaper and faster than SPE, with a significant reduction of the quantity of solvents used in the process.

Declaration of Competing Interest

he authors declare that they have no known competing finan- cial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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SUPPLEMENTARY MATERIAL II

SUPPLEMENTARY MATERIAL

Proposal of a new, fast, cheap, and easy method using DLLME for extraction and preconcentration of diazepam and its transformation products generated by a solar photo-Fenton process

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Section S1. DZP degradation

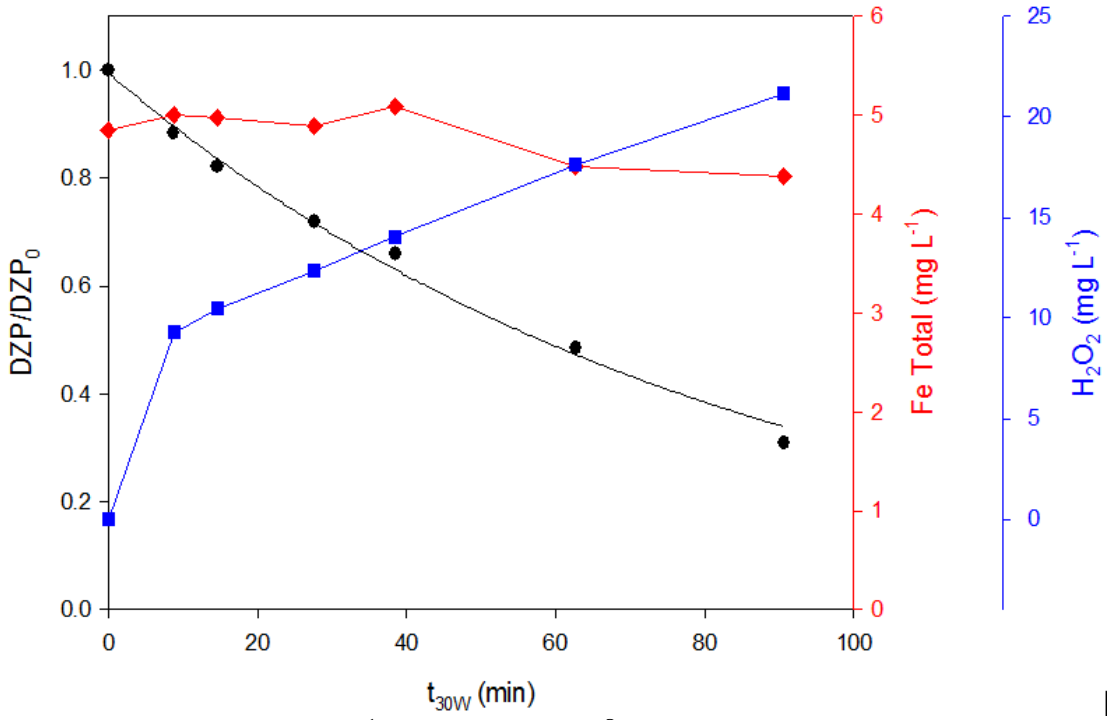


Figure S1. DZP (1000 µg L^{-1}) degradation; Fe^{2+} and H_2O_2 consumption during SPF process in ultrapure water matrix to initial TPs identification.

In this case, DZP degradation follows a pseudo-first order kinetic (according Equation 1).

$$[C] = [C_0] \cdot e^{-k_{obs} \cdot t} \quad (1)$$

where C is the concentration of DZP at time t, C_0 is the initial concentration of the DZP and k_{obs} is the degradation rate constant.

The half-life ($t_{½}$) of DZP degradation was calculated according to Equation 2.

$$t_{1/2} = {\ln 2}/{k_{obs}} \quad (2)$$

The experimental data and the determination of the degradation rate constant were fitted with the software SigmaPlot 10 (Systat Software, USA). To this experiment, that was performed with a 1000 µg L^{-1} DZP solution, the k was determined 1.19×10^{-2} min^{-1} t_{30W} with an r^2 of 0.9929 ($p < 0.0001$), C_0 of 0.9946 and a half-life time ($t_{½}$) of t_{30W} = 58.25min.

Section S2. TPs elucidation during DZP degradation

Table S1. Experimental data of DZP and its TPs generated by SPF treatment.

Comp.	Ion formula $[M+H]^+$	Experimental mass	Calculated mass	Error (ppm)	mSigma	DBE*	R_t# (min)
DZP	**$C_{16}H_{14}ClN_2O$**	**285.0792**	**285.0789**	**-0.9**	**2.2**	**10.5**	**8.8**
DZP F1	$C_{16}H_{13}ClN_2NaO$	307.0613	307.0609	-1.4	1.4	10.5	
DZP F2	$C_{15}H_{14}ClN_2$	257.0837	257.084	1	9.6	9.5	
DZP F3	$C_{14}H_{10}ClN_2$	241.0528	241.0527	-0.4	167	10.5	
DZP F4	$C_{14}H_{11}ClN$	228.0571	228.0575	1.5	17.5	9.5	
DZP F5	$C_{15}H_{14}N_2$	222.1147	222.1151	1.9	9.8	10	
DZP F6	$C_{14}H_{11}N$	193.0883	193.0886	1.5	12	10	
DZP F7	C_9H_9ClNO	182.0363	182.0367	2.2	9.9	5.5	
DZP F8	$C_{10}H_8N_2O$	172.0628	172.0631	1.8	5	8	
DZP F9	C_8H_9ClN	154.0417	154.0418	0.6	7.2	4.5	
TP1 DZP	**$C_{10}H_{10}ClN_2O_2$**	**225.0425**	**225.0425**	**0.2**	**9.6**	**6.5**	**5.7**
TP1 DZP F1	$C_{10}H_9ClN_2NaO_2$	247.0247	247.0245	-1.1	177	6.5	
TP1 DZP F2	C_8H_7ClNO	168.0209	168.0211	0.7	6.8	5.5	
TP2 DZP	**$C_{16}H_{14}ClN_2O_2$**	**301.0746**	**301.0738**	**-2.7**	**4**	**10.5**	**6.7**
TP2 DZP F1	$C_{16}H_{13}ClN_2NaO_2$	323.0567	323.0558	-2.8	104.3	10.5	
TP2 DZP F2	$C_{15}H_{14}ClN_2O$	273.0796	273.0789	-2.6	6.4	9.5	
TP2 DZP F3	$C_{14}H_{11}ClNO$	244.0526	244.0524	-1.1	31.8	9.5	
TP2 DZP F4	$C_{15}H_{14}N_2O$	238.1106	238.1101	-2.3	2.1	10	
TP2 DZP F5	$C_{11}H_{14}ClN_2$	209.0835	209.084	2.5	165.5	5.5	
TP2 DZP F6	C_9H_9ClNO	182.0367	182.0367	0.1	34.4	5.5	
TP2 DZP F7	$C_{10}H_8N_2O$	172.0631	172.0631	0.1	n.a.	8	
TP2 DZP F8	C_8H_9ClN	154.0417	154.0418	0.9	11.9	4.5	
TP3 DZP	**$C_{16}H_{14}ClN_2O_2$**	**301.0745**	**301.0738**	**-2.3**	**8.8**	**10.5**	**7.2**
TP3 DZP F1	$C_{15}H_{14}ClN_2O$	273.0795	273.0789	-2.1	39.2	9.5	
TP3 DZP F2	$C_{14}H_{11}ClNO$	244.0529	244.0524	-2.3	44.7	9.5	
TP3 DZP F3	$C_{14}H_{11}NO$	209.0842	209.0835	-3.2	41.9	10	
TP3 DZP F4	$C_9H_9ClNO_2$	198.0317	198.0316	-0.2	18.6	5.5	
TP3 DZP F5	C_8H_9ClNO	170.0366	170.0367	0.8	16.6	4.5	
TP4 DZP	**$C_{16}H_{14}ClN_2O_2$**	**301.0743**	**301.0738**	**-1.4**	**3.9**	**10.5**	**8.0**
TP4 DZP F1	$C_{16}H_{13}ClN_2NaO_2$	323.056	323.0558	-0.8	78.8	10.5	
TP4 DZP F2	$C_{15}H_{14}ClN_2O$	273.0791	273.0789	-0.5	10.3	9.5	
TP4 DZP F3	$C_{14}H_{11}ClNO$	244.0526	244.0524	-1.1	20.5	9.5	
TP4 DZP F4	$C_{15}H_{14}N_2O$	238.1104	238.1101	-1.4	33.8	10	
TP4 DZP F5	$C_{11}H_{14}ClN_2$	209.0833	209.084	3.1	181.9	5.5	
TP4 DZP F6	C_9H_9ClNO	182.0365	182.0367	0.9	32.2	5.5	
TP4 DZP F7	$C_{10}H_8N_2O$	172.0631	172.0631	-0.1	n.a.	8	
TP4 DZP F8	C_8H_9ClN	154.0415	154.0418	1.7	20.7	4.5	
TP5 DZP (Nordiazepam)	**$C_{15}H_{12}ClN_2O$**	**271.0633**	**271.0633**	**-0.3**	**15.9**	**10.5**	**8.5**

TP5 DZP F1	$C_{14}H_{12}ClN_2$	243.0677	243.0684	2.8	57.5	9.5	
TP5 DZP F2	$C_{14}H_9ClN$	226.0431	226.0418	-5.9	198.7	10.5	
TP5 DZP F3	$C_{14}H_{12}N_2$	208.0989	208.0995	3	24	10	
TP5 DZP F4	$C_8H_6ClN_2$	165.0216	165.0214	-1.2	77.7	6.5	
TP5 DZP F5	C_7H_7ClN	140.026	140.0262	1.4	15	4.5	
TP6 DZP	**$C_{16}H_{14}ClN_2O_2$**	**301.0739**	**301.0738**	**-0.4**	**16.3**	**10.5**	**9.2**
TP6 DZP F1	$C_{15}H_{14}ClN_2O$	273.079	273.0789	-0.4	66.7	9.5	
TP6 DZP F2	$C_{14}H_{11}ClNO$	244.0524	244.0524	0	35.4	9.5	
TP6 DZP F3	$C_{15}H_{14}N_2O$	238.1102	238.1101	-0.7	25.1	10	
TP6 DZP F4	$C_{11}H_{14}ClN_2$	209.0834	209.084	2.8	165	5.5	
TP6 DZP F5	C_9H_9ClNO	182.0365	182.0367	1.5	9.2	5.5	
TP6 DZP F6	$C_{10}H_8N_2O$	172.0631	172.0631	0	7.8	8	
TP6 DZP F7	C_8H_9ClN	154.0416	154.0418	1.5	33.6	4.5	

*DBE = double-bond equivalency; #R_t = retention time

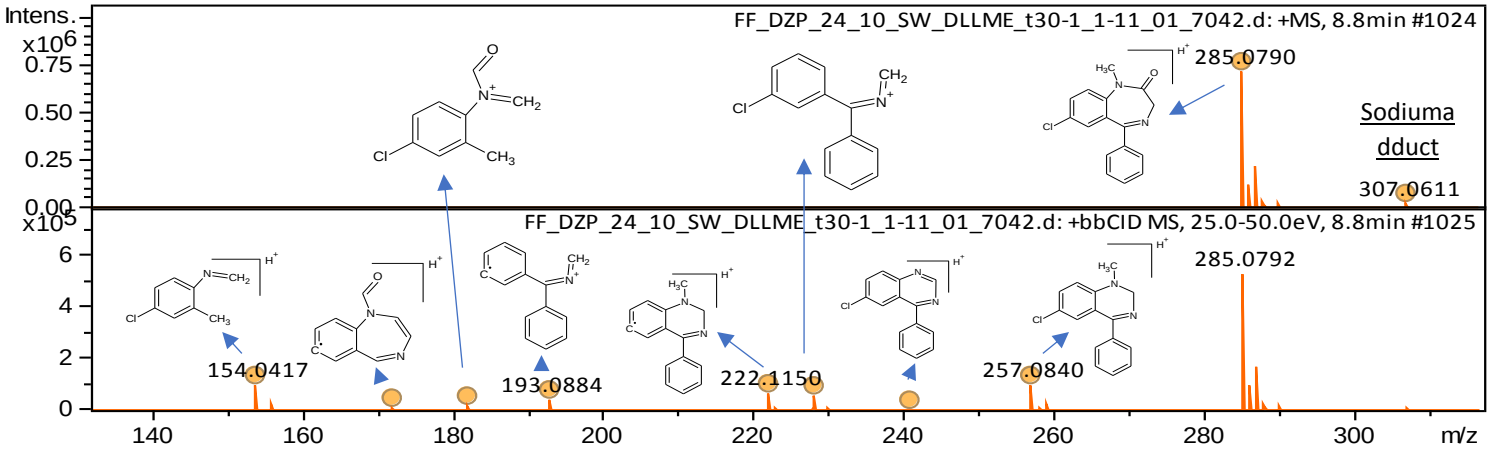


Figure S2. Mass spectrum and fragments proposed for DZP.

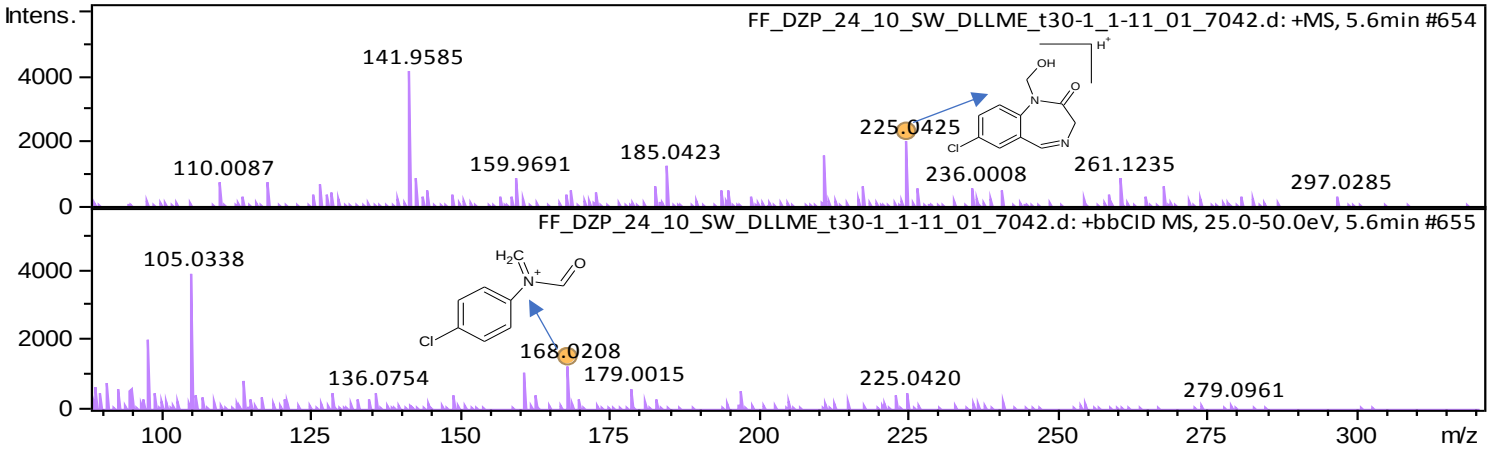


Figure S3. Mass spectrum and fragments proposed for TP1.

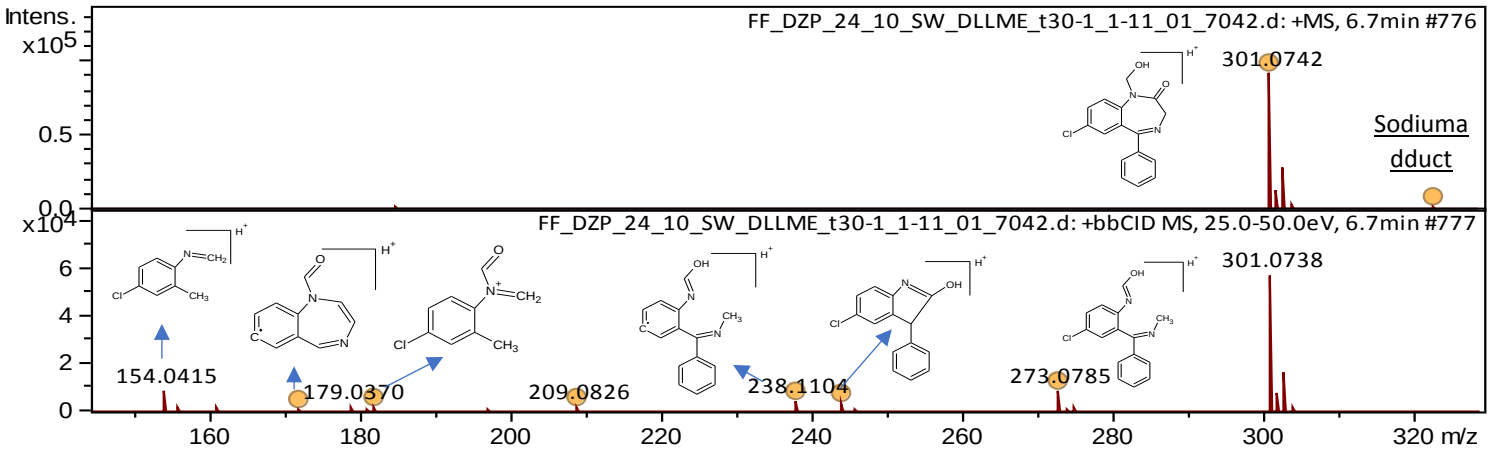


Figure S4. Mass spectrum and fragments proposed for TP2.

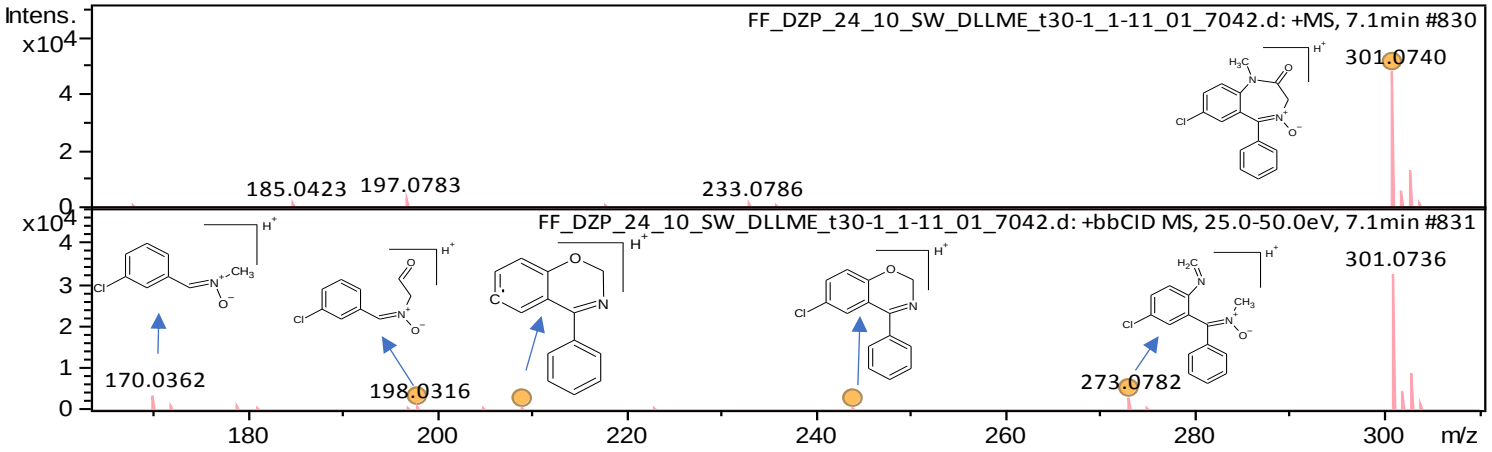


Figure S5. Mass spectrum and fragments proposed for TP3.

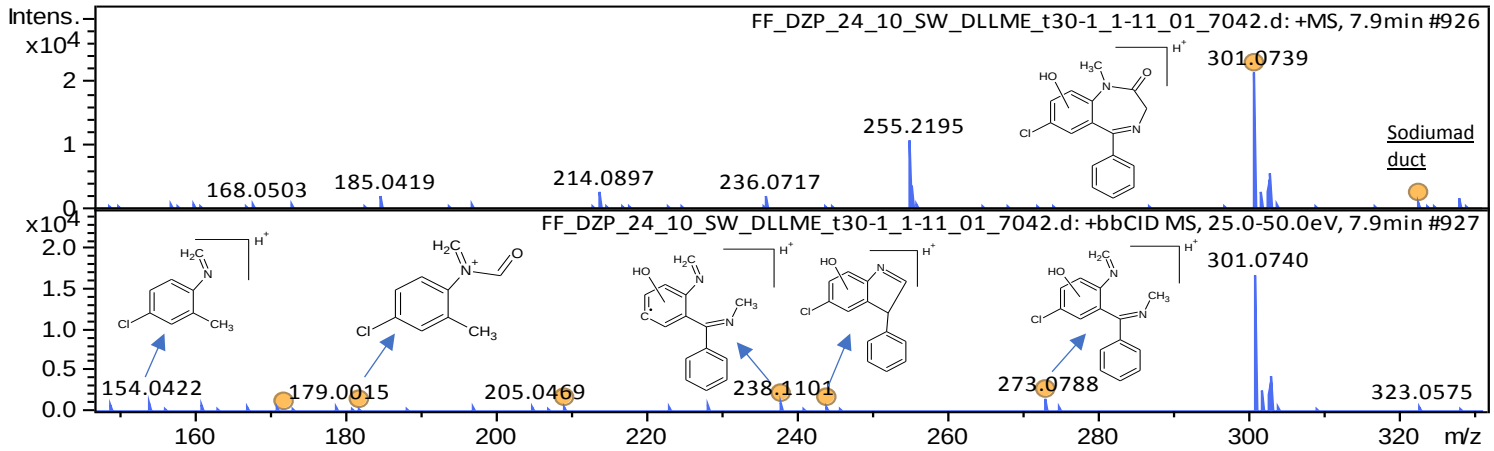


Figure S6. Mass spectrum and fragments proposed for TP4-1.

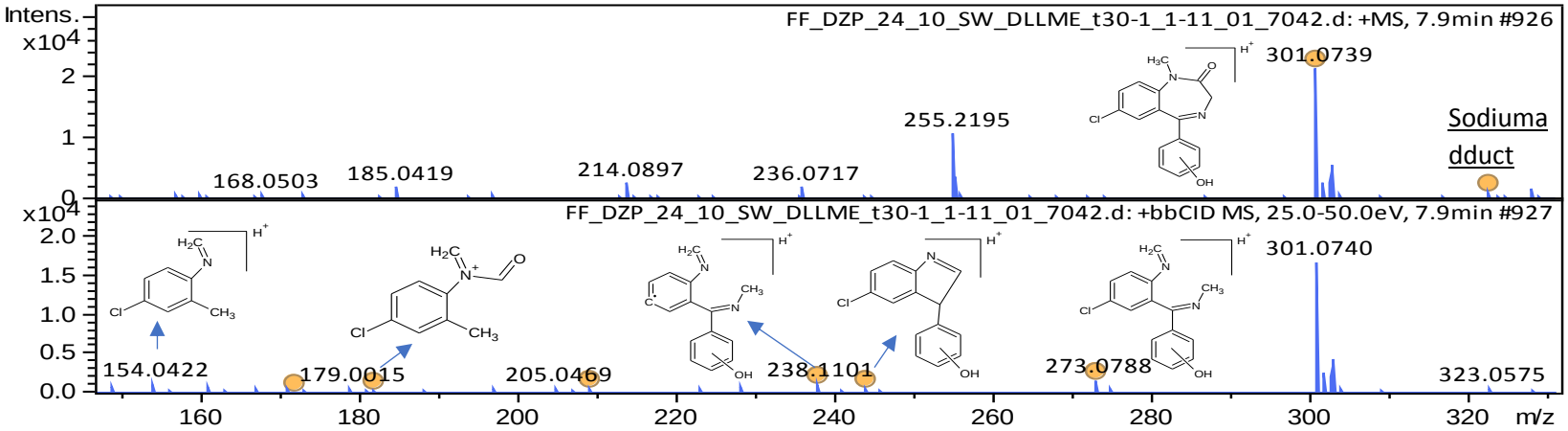


Figure S7. Mass spectrum and fragments proposed for TP4-2.

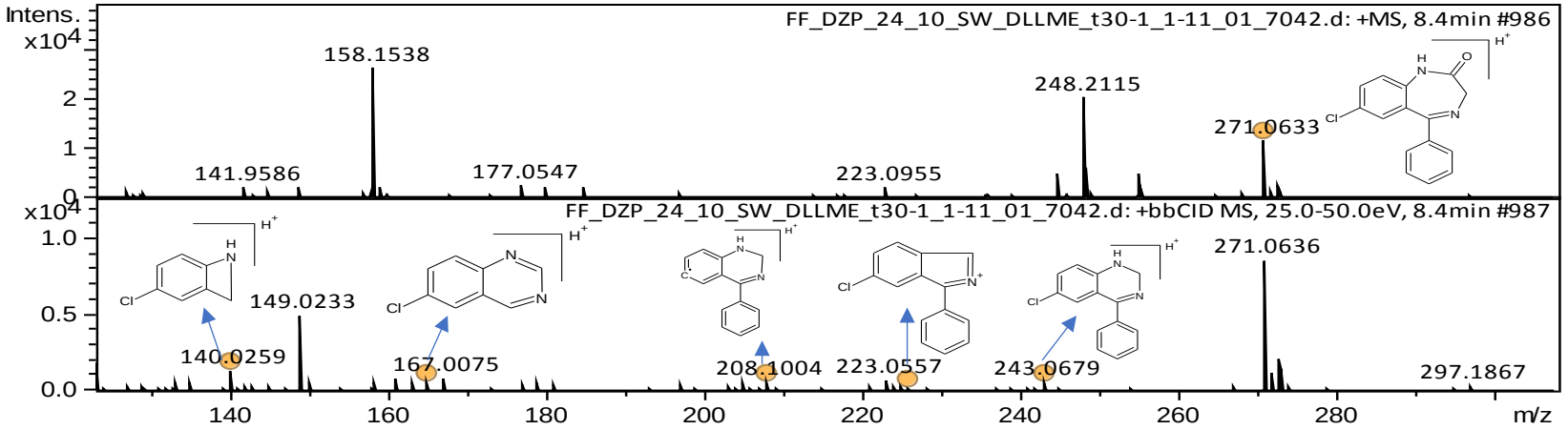


Figure S8. Mass spectrum and fragments proposed for TP5.

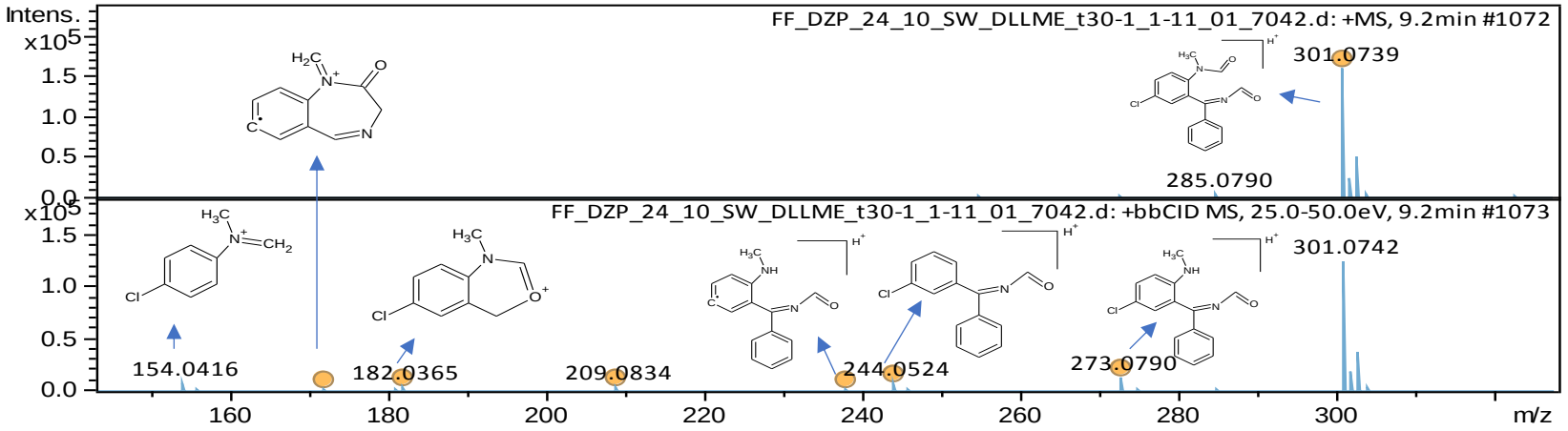


Figure S9. Mass spectrum and fragments proposed for TP6.

Section S3. *In silico* risk assessment of DZP and its TPs

Table S2. *In silico* QSAR predictions for ready biodegradability (0 means not biodegradable and 1 biodegradable) of DZP and its TPs.

Compound	CAS	log BCF (regression)	log Kow Theo	log Kow Exp	BIOWIN 1	BIOWIN 2	BIOWIN 3	BIOWIN 4	BIOWIN 5	BIOWIN 6	BIOWIN 7	Biodegradability Predictions	VEGA IRFM model
Diazepam	439-14-5	1.528	2.7	2.82	0.7678	0.8085	2.3311	3.4819	0.1532	0.0338	-0.8789	NO	NON Readily Biodegradable (low reliability)
TP1		0.5	0.41		0.8271	0.8337	2.6019	3.6932	0.238	0.0890	-0.3485	NO	NON Readily Biodegradable (low reliability)
TP2		0.226	1.23		0.9189	0.9114	2.4557	3.5883	0.2494	0.0514	-0.6406	NO	NON Readily Biodegradable (low reliability)
TP3		0.5	1.7		0.7602	0.7708	2.2958	3.4588	0.0726	0.0202	-1.3166	NO	NON Readily Biodegradable (low reliability)
TP4 I a		1.131	2.22		0.876	0.893	2.3521	3.4985	0.1658	0.0326	-0.7028	NO	NON Readily Biodegradable (low reliability)
TP4 I b		1.131	2.22		0.876	0.893	2.3521	3.4985	0.1658	0.0326	-0.7028	NO	NON Readily Biodegradable (low reliability)
TP4 I c		0.909	2.22		0.876	0.893	2.3521	3.4985	0.1658	0.0326	-0.7028	NO	NON Readily Biodegradable (low reliability)
TP 4 II a		0.541	1.66		0.7479	0.5799	2.3301	3.4936	0.1658	0.0326	-0.9209	NO	NON Readily Biodegradable (low reliability)
TP 4 II b		1.131	2.22		0.7479	0.5799	2.3301	3.4936	0.1658	0.0326	-0.9209	NO	NON Readily Biodegradable (low reliability)
TP 4 II c		1.131	2.22		0.7479	0.5799	2.3301	3.4936	0.1658	0.0326	-0.9209	NO	NON Readily Biodegradable (low reliability)
TP 4 II d		1.131	2.22		0.7479	0.5799	2.3301	3.4936	0.1658	0.0326	-0.9209	NO	NON Readily Biodegradable (low reliability)
TP 4 II e		0.541	1.66		0.7479	0.5799	2.3301	3.4936	0.1658	0.0326	-0.9209	NO	NON Readily Biodegradable (low reliability)
TP 5		1.6	2.87	2.93	0.7745	0.8374	2.3621	3.5021	0.1354	0.0340	-0.7993	NO	NON Readily Biodegradable (low reliability)
TP-6		1.908	3.4		0.7602	0.7708	2.2958	3.4588	0.1138	0.0230	-0.7589	NO	NON Readily Biodegradable (low reliability)

Table S3. *In silico* QSAR prediction of PBT values by the Prometheus software for DZP and its TPs.

Compound	LogP	LogP rel.	P	P rel.	B	B rel.	T	T rel.	Score P	Score B	Score T	**PBT**	PB
Diazepam	2.82	1	P/vP	0.5	1.57	0.4	0.087	0.3	0.712	0.306	0.509	**0.475**	0.467
TP 1	1.17	0.2	P/vP	0.5	0.66	0.4	0.876	0.3	0.712	0.239	0.38	**0.406**	0.413
TP 2	2.73	0.4	P/vP	0.5	1.49	0.4	0.085	0.3	0.712	0.298	0.51	**0.47**	0.461
TP 3	1.1	0.4	P/vP	0.5	1.33	0.4	0.084	0.3	0.712	0.284	0.51	**0.461**	0.45
TP 4 I a	2.98	0.8	P/vP	0.5	1.6	0.4	0.057	0.3	0.712	0.309	0.533	**0.481**	0.469
TP 4 I b	2.98	0.8	P/vP	0.5	1.68	0.4	0.057	0.3	0.712	0.317	0.533	**0.486**	0.475
TP 4 I c	2.98	0.8	P/vP	0.5	1.66	0.4	0.084	0.4	0.712	0.315	0.512	**0.481**	0.474
TP 4 II a	2.98	0.8	P/vP	0.5	1.57	0.4	0.12	0.4	0.712	0.306	0.487	**0.471**	0.467
TP 4 II b	2.98	0.8	P/vP	0.5	1.6	0.4	0.057	0.3	0.712	0.309	0.533	**0.481**	0.469
TP 4 II c	2.98	0.8	P/vP	0.5	1.71	0.4	0.057	0.3	0.712	0.32	0.533	**0.488**	0.478
TP 4 II d	2.98	0.8	P/vP	0.5	1.6	0.4	0.057	0.3	0.712	0.309	0.533	**0.481**	0.469
TP 4 II e	2.98	0.8	P/vP	0.5	1.57	0.4	0.12	0.4	0.712	0.306	0.487	**0.471**	0.467
TP 5	2.93	1	P/vP	0.5	1.57	0.4	0.085	0.3	0.712	0.306	0.51	**0.475**	0.467
TP-6	3.51	0.4	P/vP	0.5	2.15	0.4	0.085	0.3	0.712	0.373	0.51	**0.514**	0.515

Table S4. Carcinogenicity and mutagenicity prediction for DZP and its TPs using VEGA, QSAR Toolbox and CASE Ultra models.

Comp.	(Q)SAR predictions											
	VEGA[a]					QSAR Toolbox[b]				CASE Ultra		
	A	B	C	D	E	A	B	C	D	A	B	C
DZP	– (0)	– (Exp.)	– (Exp.)	– (Exp.)	– (Exp.)	NA	NA	NA	H	–	–	–
TP 1	– (0.1)	+ (lr)	– (lr)	+ (mr)	+ (lr)	NA	NA	N-meth der	H/ N-meth der	+	+	+
TP 2	– (0.1)	+ (lr)	– (gr)	+ (mr)	+ (mr)	NA	NA	N-meth der	H/ N-meth der	–	+	–
TP 3	– (0.05)	+ (lr)	– (lr)	– (lr)	+ (lr)	NA	NA	NA	H	OD	OD	OD
TP 4 I a	– (0.05)	+ (lr)	+ (lr)	– (lr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 I b	– (0.05)	+ (lr)	– (gr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 I c	– (0.05)	+ (lr)	– (gr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 II a	– (0.05)	+ (lr)	– (lr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 II b	– (0.05)	+ (lr)	+ (lr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 II c	– (0.05)	+ (lr)	– (lr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 II d	– (0.05)	+ (lr)	+ (lr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 4 II e	– (0.05)	+ (lr)	– (lr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 5	– (0)	+ (lr)	– (gr)	– (mr)	+ (mr)	NA	NA	NA	H	–	–	–
TP 6	+ (0.05)	– (lr)	+ (mr)	+ (mr)	– (mr)	NA	NA	Ar. N-Ac. Am.	Ar. N-Ac. Am.	OD	+	OD

+: positive alert; – negative alert; OD: out of domain

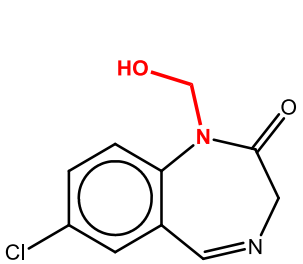
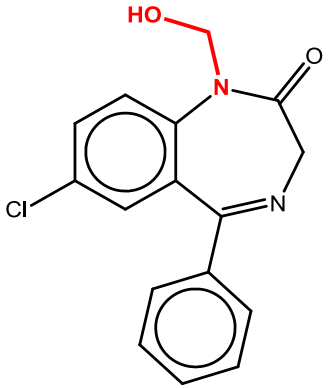
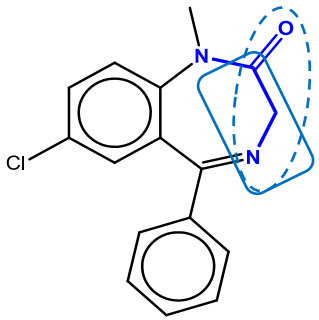
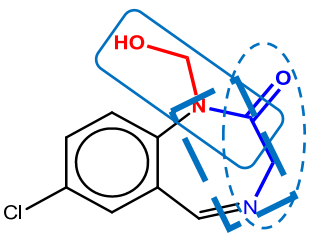
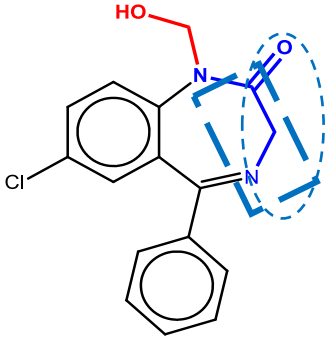
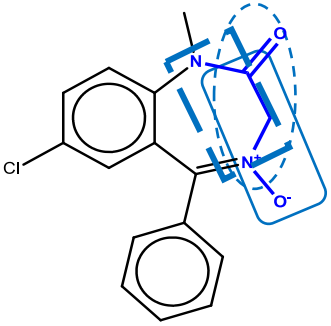
VEGA models: Mutagenicity: (A)Ames test CONSENSUS model; Carcinogenicity models: (B) IRFMN/Antares; (C) CAESAR; (D) ISS; (E) IRFMN/ISSCAN-CGX. Exp (Experimental value); lr (low reliability); mr (moderate reliability); gr (good reliability);

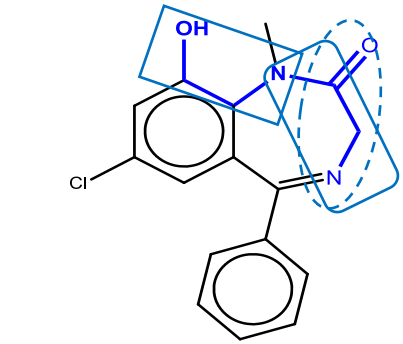
QSAR toolbox models: (A) DNA alerts for AMES by OASIS; (B) DNA alerts for CA and MNT by OASIS; (C) *in vitro* mutagenicity (Ames test) alerts by ISS; (D) *in vitro* mutagenicity (micronucleus) alerts by ISS;

QSAR toolbox models type of alerts: N-meth der: N-methylol derivative; H: H-acceptor-path3-H-acceptor; Ar. N-Ac. Am.: Aromatic N-acyl Amine;

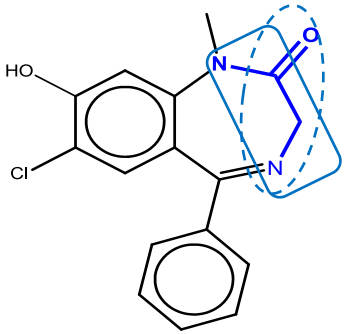
CASE Ultra models: (A) GT1_BMUT, (B) GT_EXPERT and (C) PHARM_BMUT;

Section S4. Mutagenicity Alerts by QSAR Toolbox for DZP and its TPs

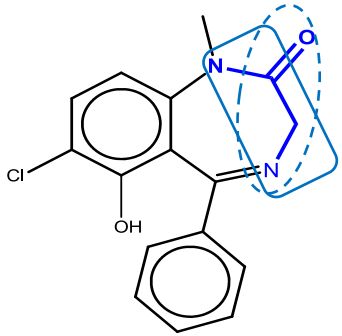
Model	Alerts
in vitro mutagenicity (Ames test) alerts by ISS	 TP 1  TP 2 N-methylol derivative
in vitro mutagenicity (micronucleus) alerts by ISS	 DZP  TP 1  TP 2  TP 3



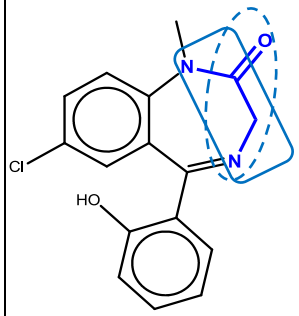
TP 4 I a



TP 4 I b



TP 4 I c



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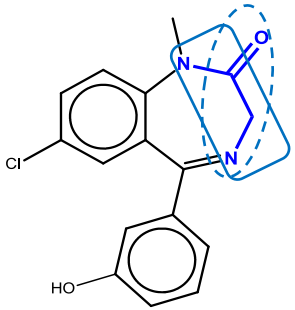
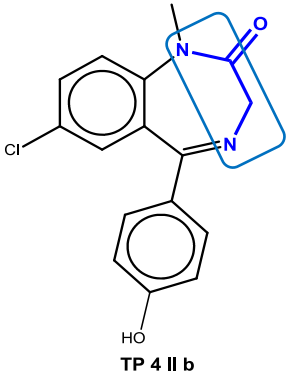
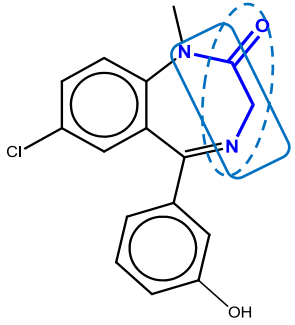


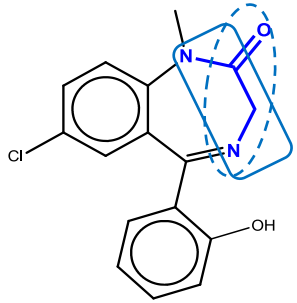
TP 4 II b



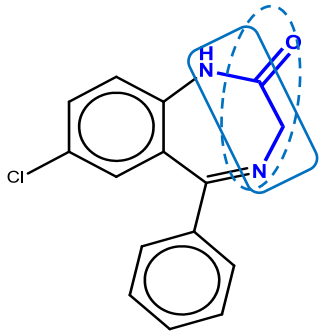
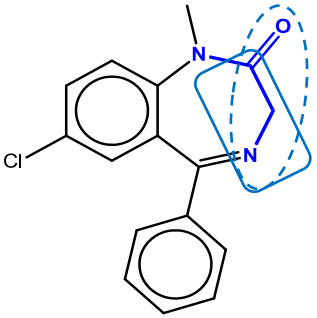
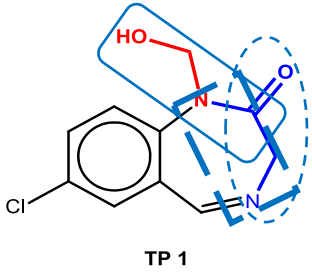
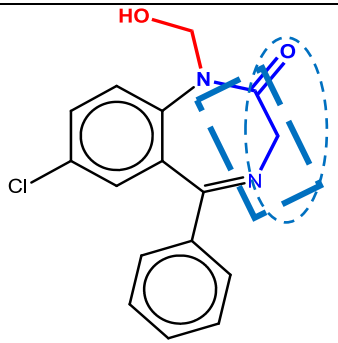
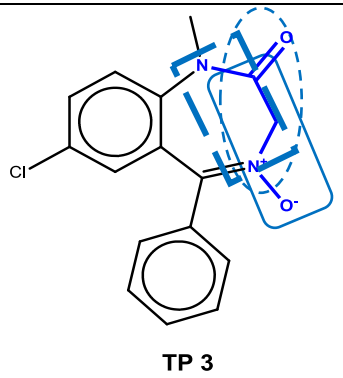
TP 4 II b



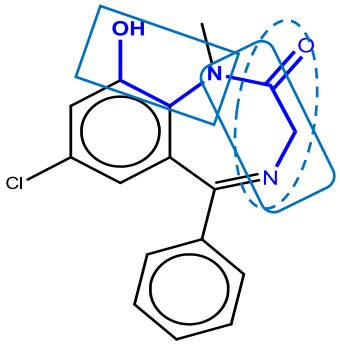
TP 4 II d



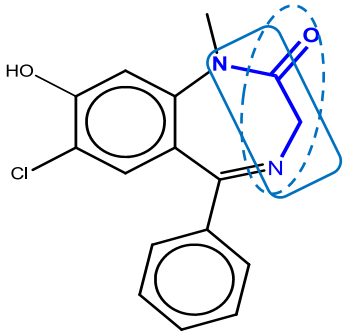
TP 4 II e

	H-acceptor-path3-H-acceptor N-methylol derivative  TP 5
in vitro mutagenicity (micronucleus) alerts by ISS	 DZP  TP 1  TP 2  TP 3

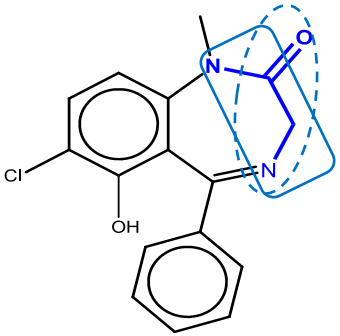
H-acceptor-path3-H-acceptor



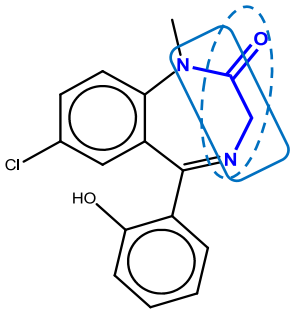
TP 4 I a



TP 4 I b



TP 4 I c



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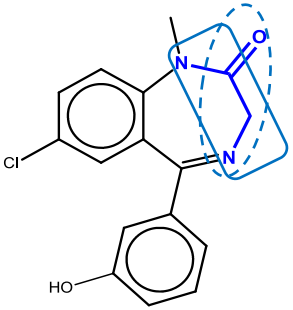
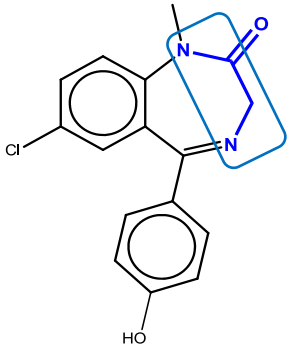


TP 4 II b



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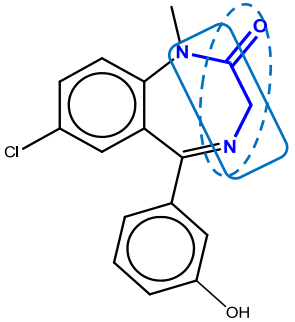
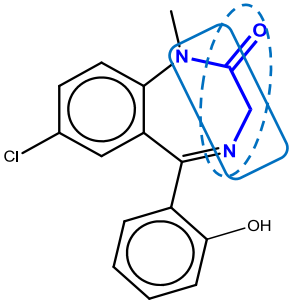
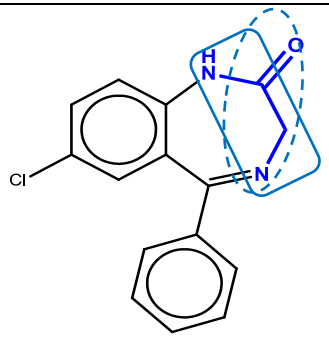
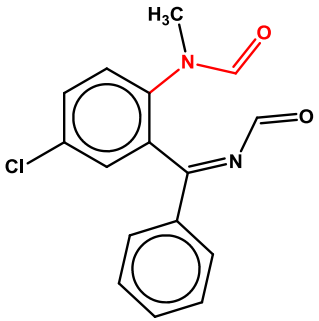


TP 4 II d



TP 4 II e

	 TP 5  TP 6	H-acceptor-path3-H-acceptor **Aromatic N-acyl amines**

S5. Factorial design planning and results

Table S5. Factorial design (2^3) variables, experimental range and levels of the variables.

	Variable	-1	0	+1
X1	DS - Acetonitrile (µL)	400	550	700
X2	ES - Chloroform (µL)	100	200	400
X3	Ionic strength Na_2SO_4 (mol L^{-1})	0	0.1	0.2

Table S6. Matrix for Factorial design (2^3), considering variables and its levels.

Experiment	X1	X2	X3
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+
9	0	0	0
10	0	0	0
11	0	0	0

Table S7. Effects estimation for the independent variables and interaction between them.

Factor	Effect Estimates; Var.:DZP + TPs Area; R-sqr=.9729; Adj:.90966 (DLLME fatorial) 3 factors, 1 Blocks, 11 Runs; MS Residual=112345E4									
	Effect	**Std.Err.**	**t(3)**	**p**	**-95.%**	**+95.%**	**Coeff.**	**Std.Err.**	**-95.%**	**+95.%**
Mean/Interc.	560306,3	19351,55	28,95407	0,000090	498721	621891,6	560306,3	19351,55	498721,1	621891,6
(1) DS (L)	166181,6	23700,72	7,01167	0,005958	90755	241607,9	83090,8	11850,36	45377,7	120803,9
DS (Q)	19011,2	45383,42	0,41890	0,703472	-125419	163441,5	9505,6	22691,71	-62709,5	81720,8
(2) ES (L)	155369,6	23700,72	6,55548	0,007218	79943	230795,9	77684,8	11850,36	39971,7	115397,9
(3) IS (L)	52134,4	23700,72	2,19970	0,115205	-23292	127560,7	26067,2	11850,36	-11645,9	63780,3
1L by 2L	33705,9	23700,72	1,42215	0,250125	-41720	109132,2	16852,9	11850,36	-20860,2	54566,1
1L by 3L	68453,1	23700,72	2,88823	0,063104	-6973	143879,4	34226,5	11850,36	-3486,6	71939,7
2L by 3L	-10042,9	23700,72	-0,42374	0,700303	-85469	65383,4	-5021,4	11850,36	-42734,6	32691,7

PS: highlighted in red color, variables that present significant variance ($p < 0.05$).

Table S8. ANOVA of the result for factorial design tested.

Factor	ANOVA; Var.:DZP + TPs Area; R-sqr=.9729; Adj:.90966 (DLLME fatorial) 3 factors, 1 Blocks, 11 Runs; MS Residual=112345E4				
	SS	df	MS	F	p
(1)DS (L)	5,52 E+10	1	5,52 E+10	49,16	0,006
DS (Q)	1,97 E+08	1	1,97 E+08	0,18	0,703
(2)ES (L)	4,83 E+10	1	4,83 E+10	42,97	0,007
(3)IS (L)	5,44 E+09	1	5,44 E+09	4,84	0,115
1L by 2L	2,27 E+09	1	2,27 E+09	2,02	0,250
1L by 3L	9,37 E+09	1	9,37 E+09	8,34	0,063
2L by 3L	2,02 E+08	1	2,02 E+08	0,18	0,700
Error	3,37 E+09	3	1,12 E+09		
Total SS	1,24 E+11	10			

PS: highlighted in red color, variables that present significant variance ($p < 0.05$).

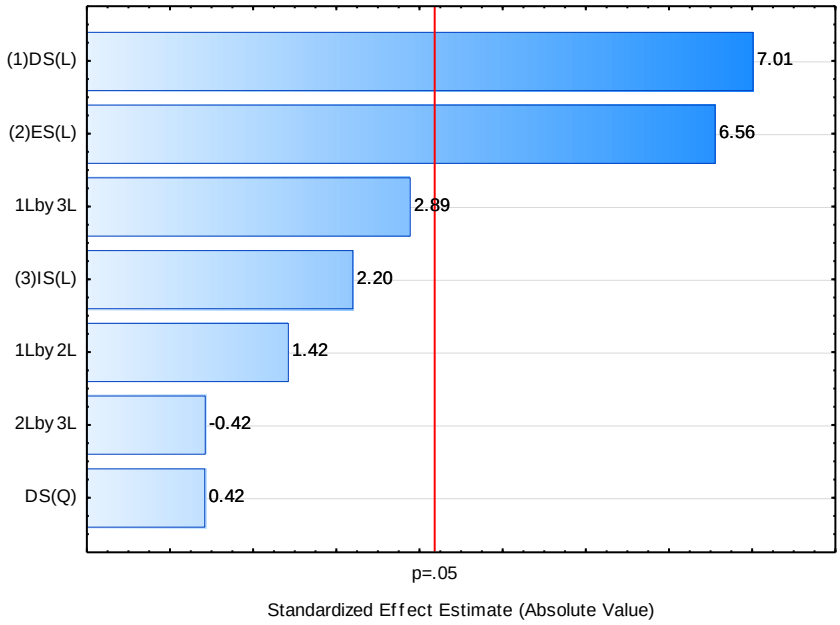


Figure S10. Pareto Chart of Effects for factorial design. Significant effect is observed when values are higher than p value, represented by the red line, with 95% of confidence.

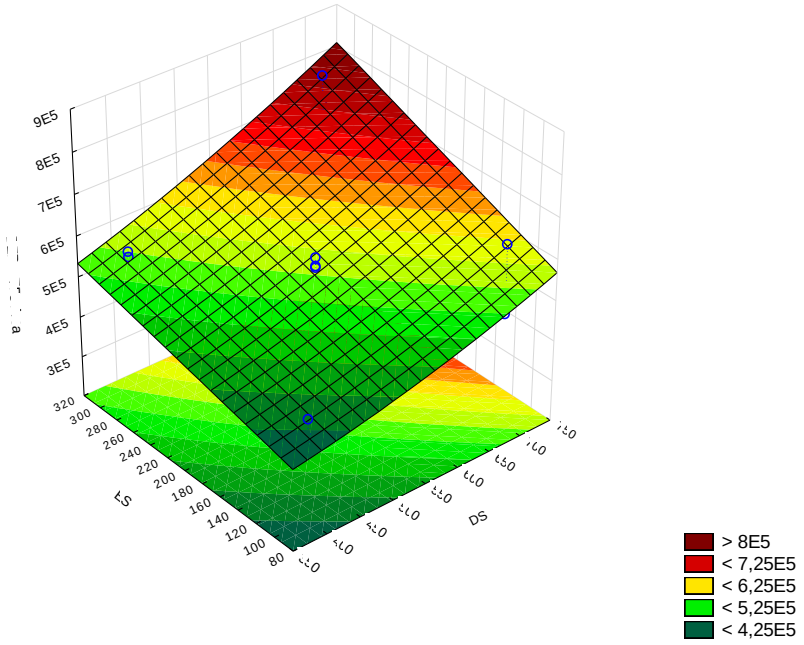


Figure S11. Response surface of ES volume vs. DS volume on DZP and TPs area after extraction by DLLME.

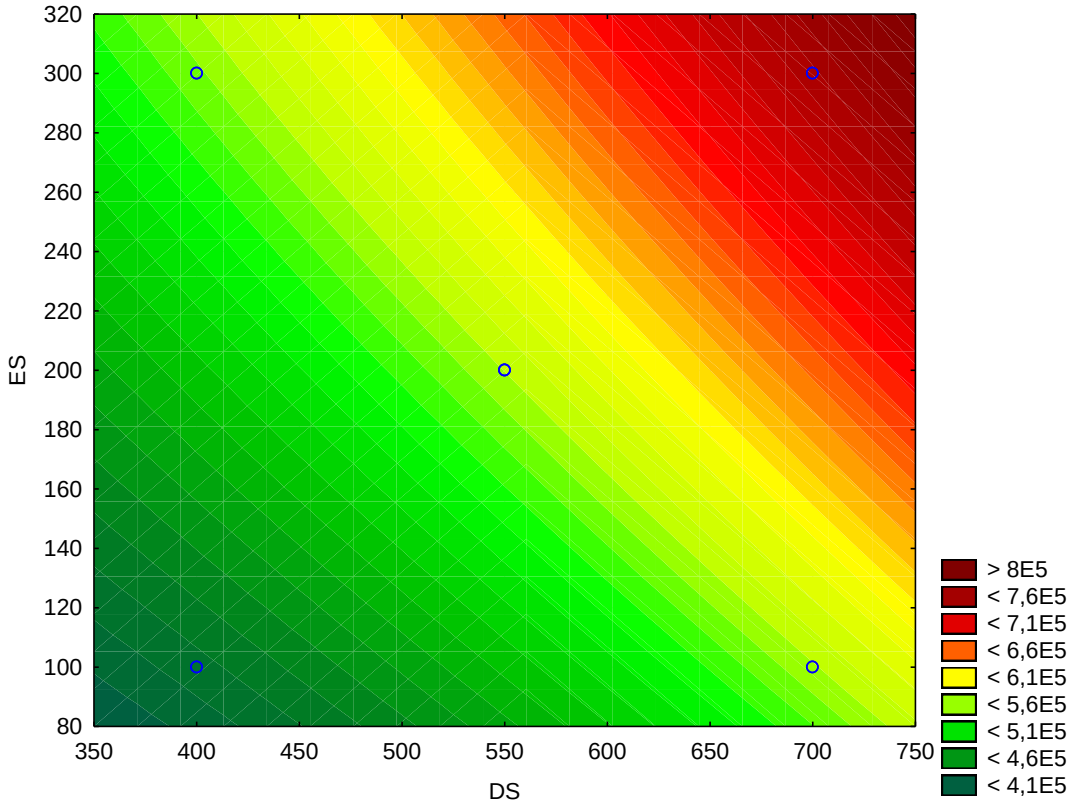


Figure S12. Contour plot for ES volume vs. DS volume on DZP and TPs area after extraction by DLLME.

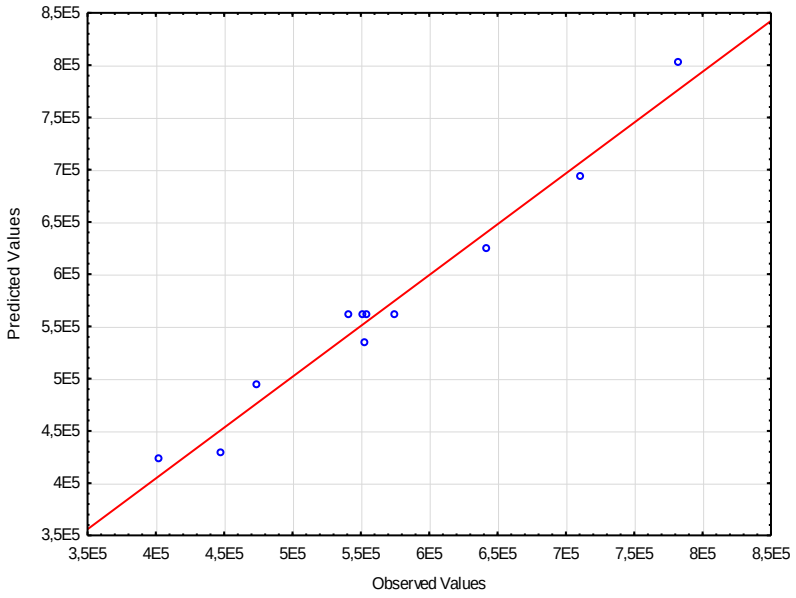


Figure S13. Parity plot showing the distribution of observed vs. predicted values of DZP and its TPs area for the factorial design experiments.

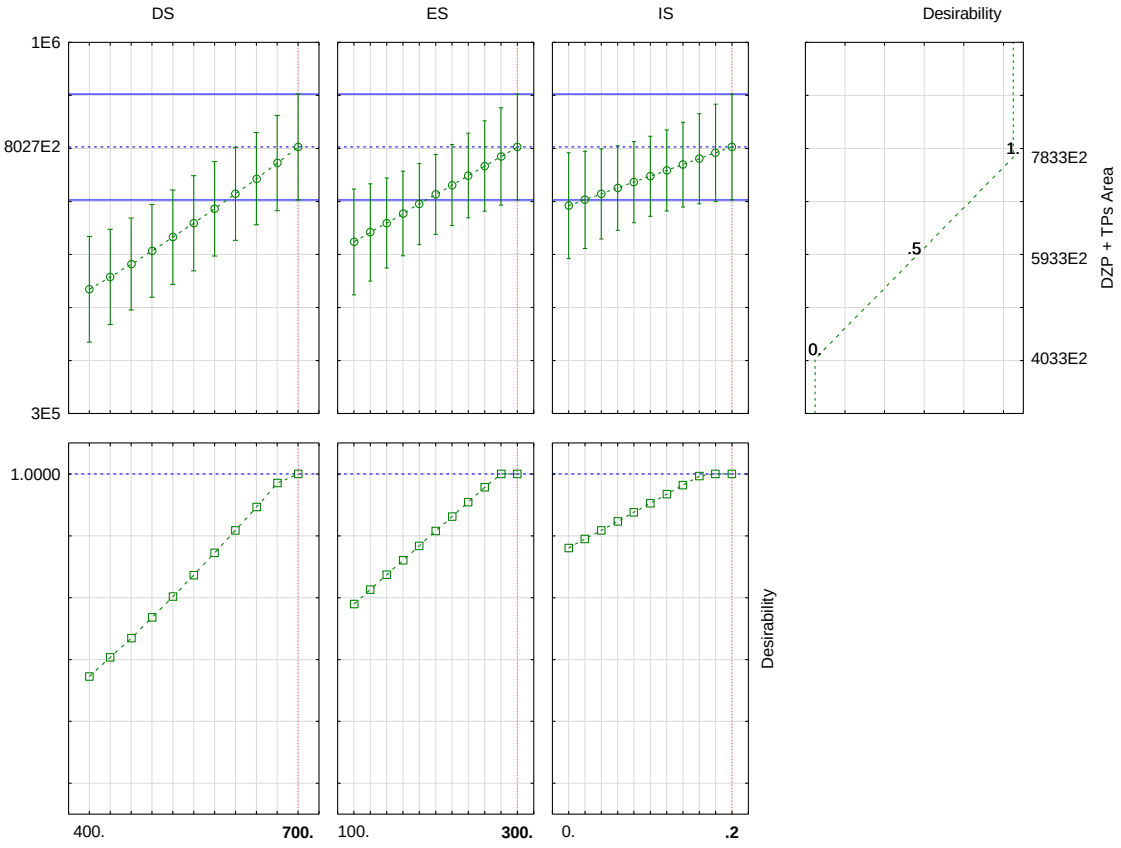


Figure S14. Desirability profile for variables studied in factorial design experiments from 0 (undesirable) to 1 (very desirable).

S6. Doehlert design planning and results

Table S9. Matrix Doehlert design, considering two variables and its levels tested.

Doehlert Design		
Experiment	**Var 1**	**Var 2**
1	1	0
2	0.5	0.866
3	-1	0
4	-0.5	-0.866
5	0.5	-0.866
6	-0.5	0.866
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0

Table S10. Effects estimation for the independent variables and interaction between them.

Factor	Effect Estimates; Var.:DZP + TPs Area; R-sqr=.96267; Adj:.936 (DLLME Doehlert) 2 factors, 1 Blocks, 13 Runs; MS Residual=542966E5									
	Effect	Std.Err.	t(7)	p	-95.%	+95.%	Coeff.	Std.Err.	-95.%	+95.%
Mean/Interc.	5859404	88071,9	66,52	0,00000	5651147	6067660	5859404	88071,9	5651147	6067660
(1)DS (uL)(L)	1183103	134532,0	8,79	0,00005	864985	1501220	591551	67266,0	432493	750610
DS (uL)(Q)	-301508	93414,3	-3,23	0,01450	-522398	-80619	-150754	46707,2	-261199	-40309
(2)ES (uL)(L)	2162050	233016,2	9,28	0,00004	1611054	2713045	1081025	116508,1	805527	1356523
ES (uL)(Q)	-668547	280242,9	-2,39	0,04848	-1331217	-5878	-334274	140121,5	-665608	-2939
1L by 2L	143072	233016,2	0,61	0,55863	-407923	694068	71536	116508,1	-203962	347034

PS: highlighted in red color, variables that present significant variance ($p < 0.05$).

Table S11. ANOVA of the result for Doehlert design tested.

Factor	ANOVA; Var.:DZP + TPs Area; R-sqr=.96267; Adj:.936 (DLLME Doehlert) 2 factors, 1 Blocks, 13 Runs; MS Residual=542966E5				
	SS	df	MS	F	p
(1)DS (uL)(L)	4,20 E+12	1	4,20 E+12	77,34	0,00005
DS (uL)(Q)	5,66 E+11	1	5,66 E+11	10,42	0,01450
(2)ES (uL)(L)	4,67 E+12	1	4,67 E+12	86,09	0,00004
ES (uL)(Q)	3,09 E+11	1	3,09 E+11	5,69	0,04848
1L by 2L	2,05 E+10	1	2,05 E+10	0,38	0,55863
Error	3,80 E+11	7	5,43 E+10		
Total SS	1,02 6E+13	12			

PS: highlighted in red color, variables that present significant variance ($p < 0.05$).

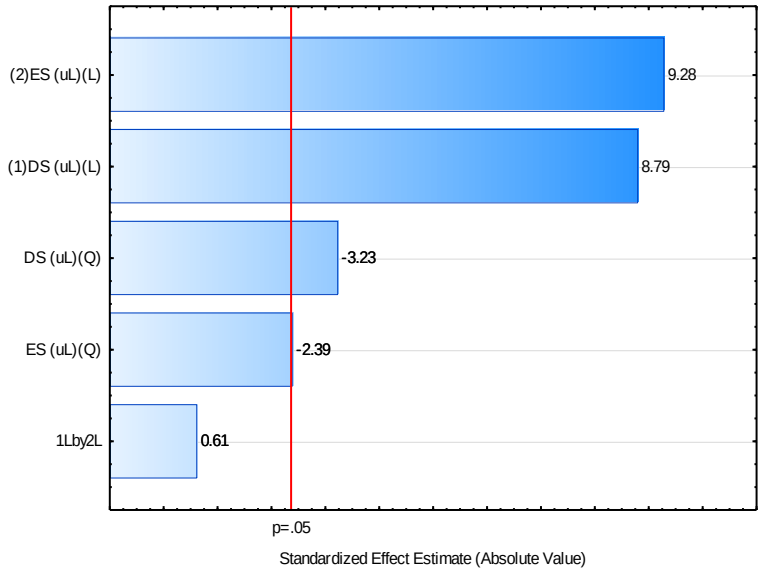


Figure S15. Pareto Chart of Effects for Doehlert design. Significant effect is observed when values are higher than p value, represented by the red line, with 95% of confiance.

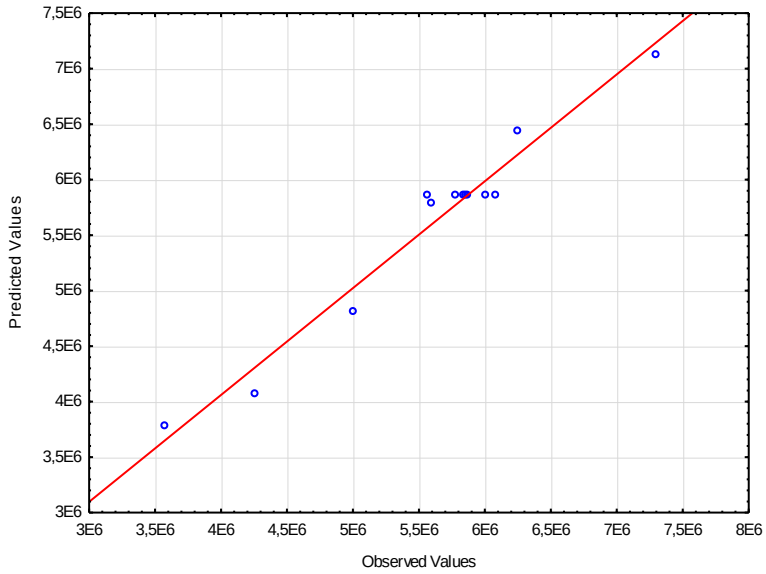


Figure S16. Parity plot showing the distribution of observed vs. predicted values of DZP and its TPs area for the Doehlert design experiments

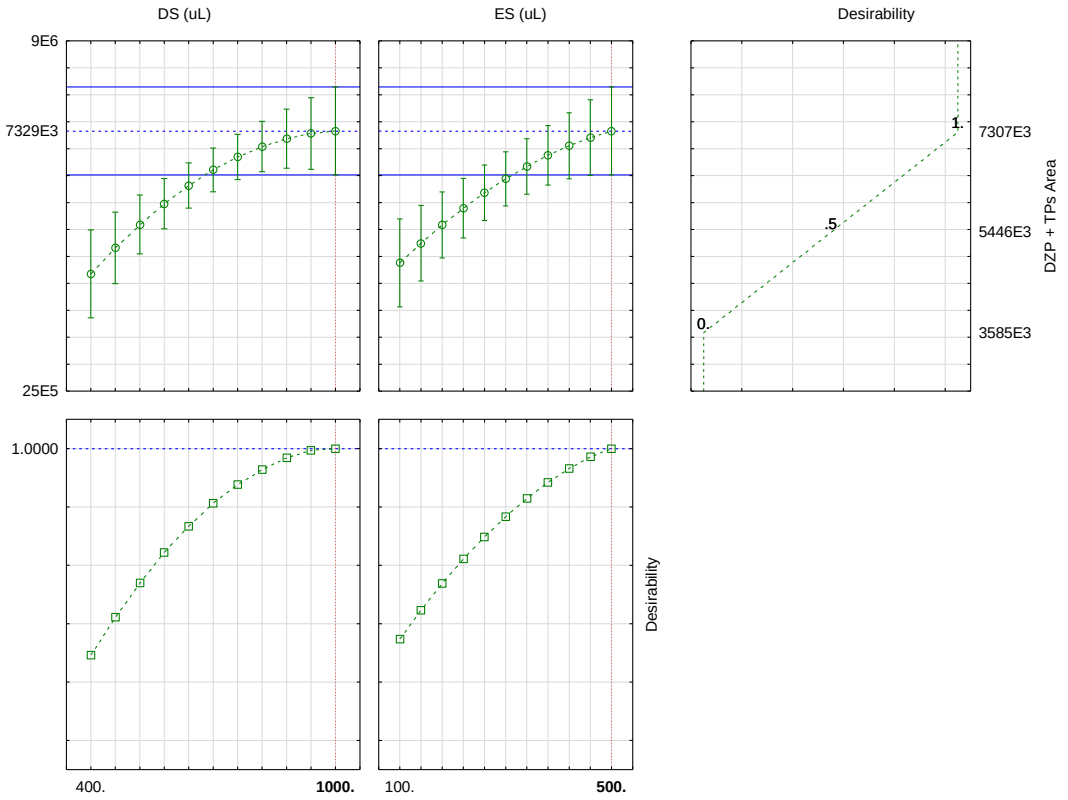


Figure S17. Desirability profile for all variables studied in Doehlert design experiments.

S7. DZP degradation in different matrices

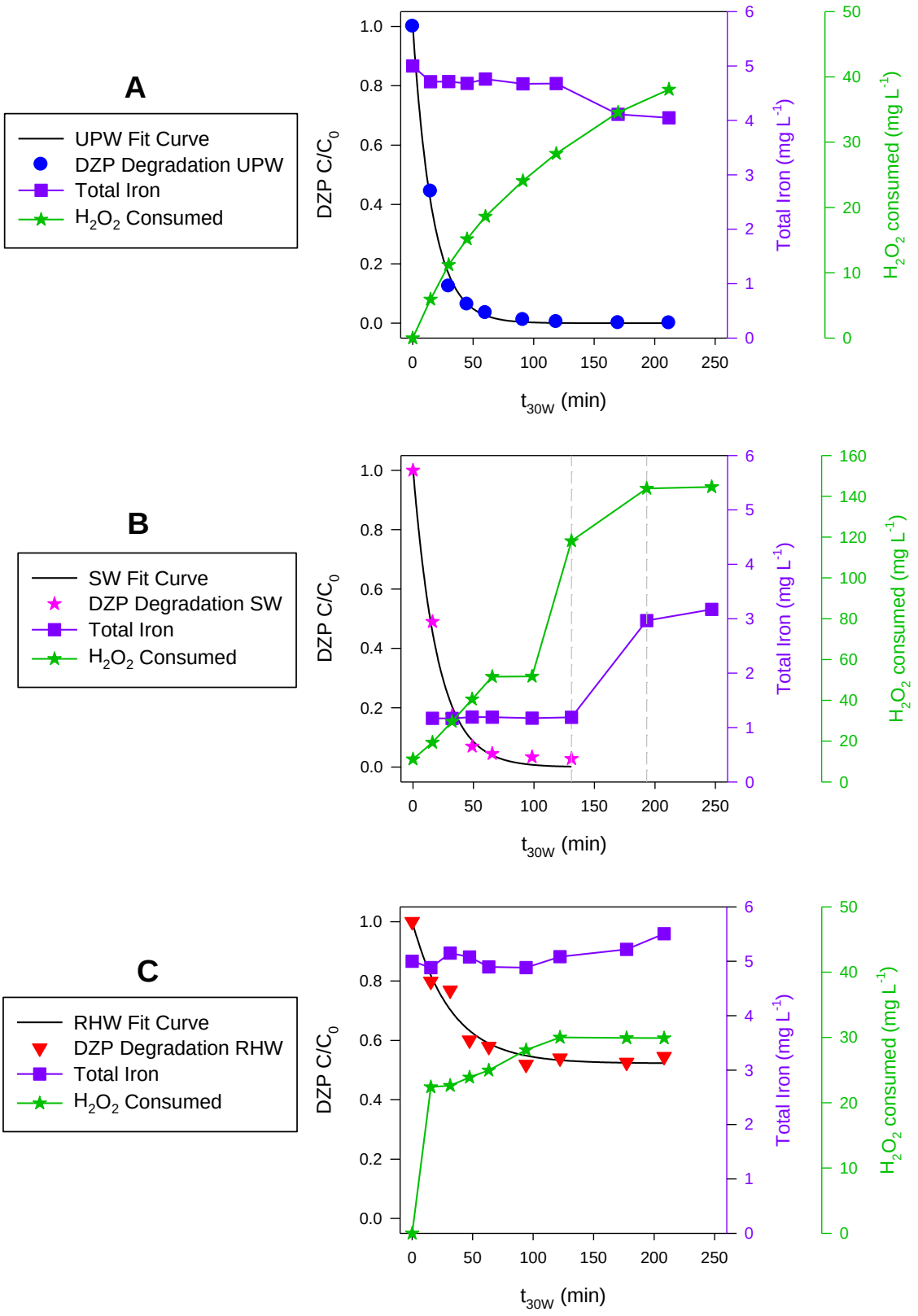


Figure S18. Analytical determinations during the DZP (500 µg L^{-1}) treatment processes in different matrices: A) UPW; B) SW and C) RHW.

Table S12. Pre-concentration rate of DZP and its TPs for SPE and DLLME in different matrices.

Compound	UPW				RHW			
	DLLME		SPE		DLLME		SPE	
	pre concentration Factor	StDev (%)	pre concentration Factor	StDev (%)	pre concentration Factor	StDev (%)	pre concentration Factor	StDev (%)
DZP	9.4	5.1	10.5	8.4	7.3	8.3	7.2	21.7
TP1	8.4	30	9.5	11.2	-	-	-	-
TP2	6.2	6.9	8.3	4.1	1.9	11	7.7	5.1
TP3	5.9	0.5	7.8	4.7	2.8	18.6	7.6	3.6
TP4	11	11.9	7.4	21.7	2.5	1.1	8.6	15.6
TP5	6.8	6.7	5.9	1.3	1.9	7.7	8.5	11
TP6	13	1.4	4.6	14.2	2.5	7.8	5.1	2.4

Chapter 3

Multi-criteria decision-making techniques associated with (Q)SAR risk assessment for ranking surface water microcontaminants identified using LC-QTOF MS

Introduction

Different studies around the world show that the contamination of aquatic environments includes the presence of different classes of CECs [80–82], including pharmaceuticals, metabolites, and TPs. The huge number of chemicals marketed globally (in the region of 100,000 compounds) can lead to the contamination of surface water by different routes [83]. Evaluation of CECs in environmental compartments needs to be carefully planned, in order to try to cover as many microcontaminants as possible. For this purpose, the adoption of more embracing strategies for the screening of suspect compounds, using extensive databases containing information about thousands of compounds from different classes, can be one very promising way to improve understanding of the contamination of aquatic systems [84]. The study presented here is an evolution of the work developed in Chapter 1, with the aim of expanding the monitoring of contaminants to other levels. As discussed in the other chapters, screening for microcontaminants, on its own, is unable to provide a full understanding of the potential risks of the contamination to humans and the environment. However, the association of screening methodologies with *in silico* predictions can enable a more representative understanding of the risks.

Interaction between different areas of knowledge is crucial for developing more realistic and applicable investigations. In the present work, the integration of techniques in the areas of environmental analytical science and computer engineering raised the work to another level, with multi-criteria decision-making (MCDM) techniques being used to prioritize microcontaminants present in the aquatic environment [85]. This combination of different methods and expertise, based on LC-QTOF MS screening → *in silico* (Q)SAR predictions → MCDM (ToxPi and TOPSIS ranking), has never been published before and seems to be a very promising strategy to assist in the development of more directed quantitative methodologies, as well as actions for the monitoring and remediation of contaminated aquatic systems.

Objectives

The aim of this study was to apply a very extensive suspect screening analysis to surface water, using a purpose-built database associated with MCDM techniques to rank

the identified microcontaminants, according to *in silico* (Q)SAR predictions. Statistical sensitivity tests were performed to evaluate the robustness of the MCDM techniques employed.

Main results

The screening methodology applied in this work, using a very large customized database containing information for 3250 CECs from different classes, enabled the identification of 150 suspected and confirmed contaminants in surface water from a 5th order river located in the northwest of Rio Grande do Sul State, Brazil. This is the first investigation of microcontaminants present in surface waters in the study area.

The large number of compounds detected made it almost impossible to understand all the *in silico* (Q)SAR prediction data. Therefore, eight different endpoints were carefully selected, in order to compare the new data treatment approaches with a previously published study [85]. After the predictions, the endpoints were normalized and a simple multi-attribute rating technique (SMART) was used to apply different weights. The two MCMD methods ToxPi and TOPSIS were used for ranking of the detected compounds, according to their risk. ToxPi ranking considered a simple weighted sum as an aggregation function to calculate the score for ranking the compounds. TOPSIS calculated the weighted Euclidean distance between each compound and the previously established positive-ideal and negative-ideal solutions. The ToxPi and TOPSIS ranking techniques provided similar results, especially for the 20 highest priority compounds, but evaluation of the sensitivity of these two methods showed greater robustness of TOPSIS.

The most detected class of contaminants was pesticides, as expected considering the high level of agricultural activity in the region. The second most detected class was pharmaceuticals. This result suggested the possibility of developing a quantitative methodology for pesticides. However, analysis of the ranking results indicated that despite being the second most detected class of contaminants, pharmaceuticals presented high possible risks to the environment. This showed the importance of associate screening with (Q)SAR and MCMD methods to guide the development of a more appropriate quantitative methodology. The findings of this study demonstrated the suitability of the adopted techniques for analytical environmental research, reinforcing the need to develop

new ideas and new multidisciplinary approaches for risk assessment and the mitigation and prevention of environmental contamination.

All the methods, results, and conclusions are provided in **Paper III** and **Supplementary Material III**.

PAPER III

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Multi-criteria decision-making techniques associated with (Q)SAR risk assessment for ranking surface water microcontaminants identified using LC-QTOF MS

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HIGHLIGHTS

- Qualitative monitoring of 3250 microcontaminants in surface waters
- *In silico* risk assessment of 150 identified microcontaminants
- TOPSIS was used for the first time to rank microcontaminant risk.
- Comparison of ranking performance and robustness of ToxPi and TOPSIS
- Hybrid multicriteria decision-making tools help in guiding future chemical analysis.

GRAPHICAL ABSTRACT

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abstract

Contaminants of emerging concern (CECs) have been a focus of study for years, with investigations revealing the contamination of different environmental matrices (surface water, soil, air, and sediment) by diverse classes of microcontaminants. Understanding the contamination profiles requires identification and risk assessment of the microcontaminants. In the present work, analysis was made of the presence of 3250 compounds in 27 samples from the Conceição River (Rio Grande do Sul State, Brazil), using an SPE-LC-QTOF MS method. In total, 150 microcontaminants (confirmed and suspected) of different classes, especially pesticides and pharmaceuticals, were identified by an initial qualitative analysis. Subsequently, *in silico* predictions of eight endpoints, using quantitative structure-activity relationship ((Q)SAR) models, were employed to determine the risk of each previously screened microcontaminant. This large amount of (Q)SAR data, frequently with conflicting information in relation to the responses of the different endpoints, makes it difficult to define which microcontaminants should be prioritized for analysis. Therefore, in order to rank the identified microcontaminants by risk assessment, two multi-criteria decision-making (MCDM) ranking techniques (ToxPi and TOPSIS), associated with a weighting method, were performed to establish the order of priority for further quantitative analysis of the most hazardous microcontaminants. The two rankings were statistically similar, especially for the 20 highest priority microcontaminants. Nonetheless, sensitivity tests carried out for the ToxPi and TOPSIS outputs showed higher performance robustness of TOPSIS, compared to ToxPi. This is the first time that such an approach (screening/

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(Q)SAR/MCDM methods) has been performed in the context of microcontaminant environmental risk evaluation and demonstrated to be an available strategy to help rank the most concern microcontaminants identified in aqueous environment samples.

1. Introduction

Water quality is a worldwide concern, since water is indispensable for life. Studies have reported the presence of different classes of microcontaminants in the aquatic environment (Kandie et al., 2020; Park and Jeon, 2021; Picó et al., 2021). Depending on their chemical structures and concentration levels, these compounds can affect biological systems (Dong et al., 2015; Bradley et al., 2020). Important sources of contamination of the aquatic environment include agricultural activities, industrial wastes, raw domestic and hospital wastewaters, and wastewater treatment plant effluents that are not efficiently treated (Čelić et al., 2021). These various sources may contain thousands of microcontaminants of emerging concern (CECs), encompassing different classes of substances including pesticides, pharmaceuticals, personal care products, and stimulants, as well as their metabolites and transformation products (TPs).

Since there are so many CECs that may be present in the aquatic environment, multi-residue methodologies that combine diverse identification strategies are essential. Generally, a first step is to perform a qualitative analysis for screening of microcontaminants. This initial evaluation is crucial for understanding the environmental contamination, providing a guide for a second step involving the development of quantification methodologies for at least the most commonly occurring compounds (Becker et al., 2020; Čelić et al., 2021). For both qualitative and quantitative analyses, previous sample preparation steps are essential for preconcentration and extraction of the analytes. The technique most widely used for this purpose is solid phase extraction (SPE) (Senta et al., 2019; Campos-Mañas et al., 2019).

Many published studies concerning multi-residue methodologies are based on ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The use of LC-MS/MS instrumentation typically provides highly sensitive, selective, and robust analytical methods. However, its use depends on the availability of analytical standards for all the analytes of interest. Therefore, the broader quantitative methods imply high financial outlays for the purchase of analytical standards and for maintenance of the equipment. Consequently, most of the published studies have evaluated a limited number of substances, generally not exceeding a few hundred compounds (Aalizadeh et al., 2016; García-Galán et al., 2016; Beccaria and Cabooter, 2020; Campos-Mañas et al., 2020; Styszko et al., 2021).

Qualitative methodologies based on high-resolution mass spectrometry (HRMS) allow the screening of a large number of analytes, potentially as many as thousands of compounds (Alygizakis et al., 2020; Menger et al., 2020; da Silva et al., 2021; Guardian et al., 2021; Liu et al., 2021). The use of extensive databases containing information about the molecular ions and fragmentation profiles of thousands of compounds supports the use of these screening methodologies (Ibáñez et al., 2017; Becker et al., 2020; Čelić et al., 2021). The main advantage of this technique is the possibility of identifying unknown compounds by nontarget screening, as well as by suspect screening when database information is available (Styszko et al., 2021). HRMS may be used to identify suspect compounds with high reliability, without the mandatory use of analytical standards, considering the valuable information provided by accurate full-spectrum mass data (Hernández et al., 2019a; Hernández et al., 2019b; Čelić et al., 2021).

Analysis to identify and/or quantify microcontaminants in the aquatic environment is essential. However, it is also vital to undertake risk assessments of these compounds, which can indicate the potential contribution of each microcontaminant to the environmental pollution. Computational modeling (*in silico*) methods based on the (quantitative) structure-activity relationship ((Q)SAR) approach are powerful tools for risk assessment (Roos et al., 2012; Mansour et al., 2016; Thomas et al., 2019). These prediction assays can be very fast to perform and are much less expensive than experimental studies, providing various endpoints related to parameters such as toxicity, carcinogenicity, mutagenicity, PBT (persistence-bioaccumulation-toxicity), K_{OC}, and half-life, among others. (Q)SAR methods can be used to correlate structural characteristics and other features of the compounds to chemical properties and biological effects (Abramenko et al., 2020), employing computer-based models based on mathematical relationships generated from information for similar structures (Sanabria et al., 2021).

Nonetheless, when many compounds are evaluated using instrumental methods of analysis and (Q)SAR tools, it can be difficult to identify the compounds that should be considered more or less environmentally hazardous, given the large quantity of data available. One solution is to use tools that allow the ranking of compounds according to different defined endpoints. One of these tools is ToxPi GUI (Toxicological Priority Index Graphical User Interface) software. ToxPi is a flexible decision-support tool that allows the integration of different metrics established for each compound (K_{OC}, half-life, carcinogenicity, and others), providing a ranking of compounds by transforming the data into a dimensionless score index, with different scales and attributing a different weight to each endpoint (Reif et al., 2013; Marvel et al., 2018). A ToxPi model may be described as the recombination of individual data sources, which may be components or metrics, into explicitly-weighted slices that represent one or more endpoints (Reif et al., 2013).

Tools such as ToxPi are based on multi-criteria decision-making (MCDM) techniques that treat endpoints and their relative importance as criteria and weights, respectively, and the compounds as alternatives to be compared for ordering or selection. Among the techniques used in various areas of knowledge, one of the most widespread is TOPSIS (Technique for Order Preference by Similarity to Ideal Solution). TOPSIS is an MCDM technique that ranks alternatives in relation to the positive-ideal or the negative-ideal, defined from previously established criteria and weights, considering the characteristics assessed (Hwang and Yoon, 1981). Evaluation of similarity and ranking of all the options of interest is performed using the combination of closest proximity to the ideal solution and greatest distance from the negative-ideal, considering Euclidean distances and weighted criteria (Triantaphyllou, 2000; Ruiz-Padillo et al., 2016). In the environmental sciences field, TOPSIS was used by Li et al. (2016) to assess the degree of groundwater pollution. However, until now, no studies have used TOPSIS to rank microcontaminants with higher environmental risk. In addition, no reports were found in the literature that have compared screening results obtained using different MCDM methods.

Therefore, the aim of the present study was to use suspect screening analyses to evaluate 3250 microcontaminants in surface waters, associating (Q)SAR tools and multi-criteria decision-making techniques to rank the identified microcontaminants according to assessment of their risk. Statistical comparisons and sensitivity tests were performed in order to evaluate the performance and robustness of the MCDM techniques employed.

2. Materials and methods

2.1. Chemicals and materials

The solvents used in the LC-QTOF MS analyses were acetonitrile and methanol (LC-MS grade), purchased from Merck. Analytical standards

used for the confirmation of certain compounds were purchased from various suppliers. A list of the standards is provided in Section I (*Supplementary Material*). Formic acid (98%) was from Merck and ultrapure water (18.2 MΩ.cm) was from a Millipore purification system. Sample preparation was performed using Oasis HLB cartridges (500 mg, 6 mL), purchased from Waters Corporation. Extracts were filtered through 0.22 μm PVDF syringe filters obtained from Allcrom.

2.2. Study area and sampling

Twenty-seven samples were collected at three different sampling points in the Conceição River (Rio Grande do Sul State, Brazil). In addition, one sample was collected in a tributary of the river, near the other sampling points. The coordinates of the sampling sites are provided in Section SII (*Supplementary Material*), together with a figure indicating their locations.

Sampling was carried out at the selected points during the 2018–2019 summer harvest, considering the agricultural calendar of the Conceição River region. Nine samplings were performed in two campaigns: i) the first campaign, with three samplings during the period of the year corresponding to preparation of the soil for planting of soybeans and maize (between October 21st and November 16th 2018); ii) the second campaign, with six samplings during the period of the year corresponding to pest and weed control of soybean and maize crops (March 3rd to 16th 2019). The sample collected in the river tributary, protected by riparian forest, was used as a blank. Samples were collected biweekly, always at around the same time (about 9 a.m.). Nearly 2 L of sample were collected in plastic bottles, around 30 cm depth and 1 m from the riverside. The samples were refrigerated until arrival at the laboratory, where they were maintained at −4 °C until preparation.

2.3. Screening analysis

The solid phase extraction (SPE) procedure was adapted from Diaz et al. (2013). A complete description of the SPE procedure steps is provided in Section SIII (*Supplementary Material*), together with the conditions of the chromatographic separation and details of the instrumental conditions of the LC and QTOF MS systems.

For screening analysis, an extended purpose-built database was constructed with data from the literature and from open access platforms such as mzCloud (www.mzcloud.org). The raw LC-HRMS data were processed using TASQ v. 2.2 (Bruker Daltonics). Compounds were positively identified by the presence of the molecular ion, using the low energy (LE) function, and the presence of at least two fragments, using the high energy (HE) function. The isotopic profile was also evaluated, especially when chlorine and bromine atoms were present in the molecule. Compounds that presented molecular ions and at least two characteristic fragments with error below 5 ppm, but for which analytical standards were not available, were designated as "suspected" (S), while compounds that presented molecular ions and at least two characteristic fragments with error below 5 ppm, and for which analytical standards were available, were designated as "confirmed" (C).

The database included information about the molecular formula, *m/z*, and fragmentation profile of each compound. A total of 3250 compounds were added to the database and were subsequently analyzed. A total of 2592 compounds were analyzed in positive ionization mode, 238 in negative ionization mode, and 420 compounds in both ionization modes.

2.4. In silico quantitative structure-activity relationship ((Q)SAR) predictions

The evaluation of (Q)SAR predictions started with transformation of all the identified compounds in SMILES, and by searching in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Eight endpoints were predicted using different free access software packages, according to Table 1. For some endpoints, the data were successively treated before obtaining the final endpoint value.

The mutagenicity score was predicted using VEGA, with values higher than 0.75 being considered indicative of mutagenicity. Predicted mutagenic analytes were then confirmed by means of alerts using *in vivo* and *in vitro* (Q)SAR methods. Four different models were used to calculate carcinogenicity. Evaluation of the combined models was performed according to the classification shown in Table S2 (Section IV, *Supplementary Material*). Compounds were considered carcinogenic if the final score was ≥0.75, because lower values indicated differences between the database compounds and the target compounds. Compounds were classified as non-carcinogenic if the score was ≤0.25. Estrogen receptor binding is similar to protein binding and can cause endocrine disruption. The estrogen receptor binding profiler classifies chemicals as non-binders or binders, depending on their molecular weight and structural characteristics (Schultz et al., 2002).

BIOWIN 5 was used for initial biodegradability evaluation, with values lower and higher than 0.5 considered indicative of non-biodegradable and biodegradable compounds, respectively. After this selection, another criterion was applied, only for compounds presenting BIOWIN 5 scores higher than 0.5, with predictive classification of

Table 1
(Q)SAR software and data used for each endpoint calculated.

Data	Software	Endpoint
log K_{OC} and log K_{OW}	QSAR Toolbox (v. 4.4.1)	Mobility
Half-life (days)	QSAR Toolbox (v. 4.4.1)	Persistence
Estrogen receptor binding	QSAR Toolbox (v. 4.4.1)	Estrogen receptor binding
STP total removal	QSAR Toolbox (v. 4.4.1)	STP (Sewage Treatment Plant) total removal
BIOWIN 5	QSAR Toolbox (v. 4.4.1)	Biodegradability
Predicted ready biodegradability (model (IRFMN) 1.0.9)	VEGA QSAR (v.1.1.5)	
PBT	Prometheus (v. 1.0) (REACH Commission regulation, 2011).	PBT (persistence, bio-concentration, toxicity)
Mutagenicity score	Mutagenicity (Ames test) CONSENSUS model (v1.0.2) by VEGA QSAR (v.1.1.5) (Benfenati et al., 2013) (consensus of models: Mutagenicity (Ames Test) model (CAESAR) 2.1.13, Mutagenicity (Ames Test) model (SARpy/IRFMN) 1.0.7, Mutagenicity (Ames Test) model (ISS) 1.0.2 and Mutagenicity (Ames Test) model (KNN/Read-Across) 1.0.0)	Mutagenicity
Mutagenicity alerts	*In vitro* mutagenicity (Ames test) alerts by ISS (IstitutoSuperiore di Sanità) And *In vivo* mutagenicity (Micronucleus) alerts by ISS QSAR Toolbox (v. 4.4.1)	
Carcinogenicity score	(i) Carcinogenicity model (IRFMN/Antares) (version 1.0.0), (ii) Carcinogenicity model (CAESAR) (version 2.1.9), (iii) Carcinogenicity model (ISS) (version 1.0.2), and (iv) Carcinogenicity model (IRFMN/ISSCAN-CGX) (version 1.0.0) Models by VEGA QSAR (v.1.1.5) (Benfenati et al., 2013)	Carcinogenicity

compounds into readily biodegradable or biodegradable ones. All other results were considered indicative of non-biodegradability.

2.5. Multi-criteria analysis for ranking identified microcontaminants: ToxPi and TOPSIS tools

Predicted endpoints were used to rank the identified compounds in order of environmental concern. Prior to the ranking, all the endpoints were normalized and adjusted using different scales to obtain linear distributions of the values, following maximization or minimization effects, as shown in Table S3(Section SV, *Supplementary Material*). In addition to these normalized endpoints, ToxPi and TOPSIS received weights for each endpoint as input data. These weights were defined by the Simple Multi-Attribute Rating Technique (SMART), because ToxPi uses this weighting method, so comparison with the TOPSIS technique was possible. SMART calculates weights for criteria (w_i) from direct values (x_i) assigned to them according to a preset scale regarding their relative importance, using Eq. (1):

$$w_i = \frac{x_i}{\sum x_i} \times 100\% \quad (1)$$

Therefore, the endpoints were firstly ranked and related to the following x_i values: i) 1 for physicochemical properties (half-life, mobility, PBT, and total removal); ii) 2 for *in vitro* measurements (biodegradability) and possible endocrine disruption (estrogen receptor binding); iii) 3 for properties that involve *in vivo* tests (carcinogenicity and mutagenicity). Higher values represented higher toxicity of the endpoint, adapting the values proposed by dos Santos and Nardocci (2019).

ToxPi uses a simple weighted sum as an aggregation function to calculate the score for ranking, employing the normalized endpoints and weights obtained by SMART. The ToxPi score was then calculated using Eq. (2):

$$S_{ToxPi,j} = \sum_{i=1}^{n} w_i \times y_{ij} \quad (2)$$

where, $S_{ToxPi,j}$ is the ToxPi score of the j-th compound (j = 1, …, m, with m being the total number of compounds tentatively identified to be ranked), w_i is the weight of the i-th endpoint (i = 1, …, n, with n = 8 being the number of endpoints evaluated in this study), and y_{ij} is the normalized value of the endpoint for each compound.

From the input data, TOPSIS calculates the weighted Euclidean distance between each compound and the previously established positive-ideal solution (the compound with the ideal best environmental value) and negative-ideal solution (the compound with the ideal worst environmental value). The ideal and anti-ideal solutions, denoted PIS and NIS, respectively, were constructed by taking the best and worst normalized values, respectively, of all the compounds for each endpoint, using Eqs. (3) and (4):

$$PIS = \left\{\left(\min y_{ij}\right)\right\}, i = 1, \ldots, n \quad (3)$$

$$NIS = \left\{\left(\max y_{ij}\right)\right\}, i = 1, \ldots, n \quad (4)$$

Euclidean distances were then calculated between the evaluated compounds and the PIS and NIS values, denoted D_j^+ and D_j^-, respectively, using Eqs. (5) and (6):

$$D_j^+ = \sqrt{\sum_{i=1}^{n}\left(PIS - y_{ij}\right)^2}, j = 1, \ldots, m \quad (5)$$

$$D_j^- = \sqrt{\sum_{i=1}^{n}\left(NIS - y_{ij}\right)^2}, j = 1, \ldots, m \quad (6)$$

From these Euclidean distances, TOPSIS obtained the so-called similarity ratio, denoted $S_{TOPSIS,j}$, as the score used to rank the identified compounds according to their toxic effects towards humans and the environment (Eq. (7)):

$$S_{TOPSIS,j} = \frac{D_j^-}{D_j^+ + D_j^-} \quad (7)$$

The scores obtained from ToxPi and TOPSIS were used to rank the identified compounds. In order to compare the methods, the raw score values were standardized to z-scores by subtracting the population mean (μ) from the individual raw score (S) and then dividing the difference by the population standard deviation (σ), as shown in Eq. (8):

$$z = \frac{S - \mu}{\sigma} \quad (8)$$

In order to measure the sensitivity of the results obtained and the robustness of the two MCDM methods, sensitivity tests were performed by changing the endpoint weights from SMART, relative to the initial values, as follows: i) $x_i + 1$, ii) $x_i + 2$, and iii) $x_i - 0.5$, where x_i is the initially defined value of the relative importance of the i-th endpoint. In addition, the rankings obtained from the ToxPi and TOPSIS tools and the sensitivity tests results were compared using a statistical hypothesis test, performed with Action statistical software. The Levene and Shapiro-Wilk tests were performed to identify non-homogeneity and non-normality of the data, respectively, so that the nonparametric Friedman test could be used to detect differences in the rankings. The null hypothesis (H_0) in the Friedman tests was that there were no differences between the rankings according to tool or weights set. If the H_0 hypothesis was rejected, it was assumed that the compared rankings had significant differences, so post-hoc paired comparison tests could be applied to identify differences. Otherwise, it was assumed that there was no evidence to suggest differences.

3. Results and discussion

3.1. Identification of suspected and confirmed microcontaminants byLC-QTOF MS: screening analysis

The qualitative analysis performed in this study evaluated 3250 compounds of various classes, including pesticides, human and veterinary pharmaceuticals, and illicit drugs, as well as some metabolites. Previous studies have reported the successful use of customized databases for qualitative screening analysis, where the availability of analytical standards is not a critical requirement (Ibáñez et al., 2017; Becker et al., 2020). This strategy allows the evaluation of thousands of analytes in the same analysis, besides the possibility of retrospective analysis. Conventionally, such screening can assist in guiding the selection of analytes, prior to the subsequent development of quantitative methods.

In this study, the screening analysis of the samples collected from the Conceição River and the tributary (blank sample) enabled the identification of a total of 150 microcontaminants, of which 17 were "confirmed compounds" (C) and 133 were "suspected compounds" (S). Table S4 (Section SVI, *Supplementary Material*) shows the classification of all the identified microcontaminants in the samples analyzed. The tentatively identified analytes belonged to 12 different classes, with pesticides being the most representative, with 60 compounds (40%), followed by pharmaceuticals, with 55 compounds (36%), and metabolites of pesticides and pharmaceuticals, with 17 compounds identified (12%) (Fig. S2, Section SVI, *Supplementary Material*). The other microcontaminants included veterinary pharmaceuticals, illicit drugs and their metabolites, and plasticizers, among others.

In addition to division according to class, the compounds were separated using subclasses. The pesticide subclasses most frequently identified included herbicides, fungicides, and insecticides, with 22, 18, and 11 compounds, respectively. A total of 55 pharmaceuticals used in humans were detected, belonging to 18 different subclasses, with

psychoactive substances, hormones, and antihypertensives being most frequently identified, with 13, 8, and 6 analytes, respectively. Two groups with no extended compounds identification, but that merit concern, were a subclass of three antibiotics (isoniazid, lincomycin, and the natural antibiotic 3-phenyl-2-propenal(cinnamaldehyde)), which could be responsible for progressive increases of antibiotic-resistant bacteria and antibiotic-resistant genes (Li et al., 2021), and two antineoplastic agents (exemestane and flutamide). The demand for chemotherapy has increased in recent years, resulting in increased occurrence of antineoplastic agents in the aquatic environment. This is of particular concern, because antineoplastic agents may present potential mutagenic, carcinogenic, and genotoxic effects, even at trace levels (Tousova et al., 2017; Henry et al., 2020). Figs. S3 and S4 (Section SVI, *Supplementary Material*) show the subclasses of pharmaceuticals and pesticides identified by screening.

The frequencies of detection of the microcontaminants identified at each sampling point are shown in Table S5 (Section SVI, *Supplementary Material*). The identifications included 2-aminonicotinic acid, an anti-inflammatory veterinary, in 26 samples; triphenylphosphate, a plasticizer, in 21 samples; dodecyl benzenosulfonic acid, a saponification agent, in 25 samples; alpha-PVP (α-pyrrolidinovalerophenone), an illicit drug, in 20 samples; and caffeine, a natural alkaloid found in coffee, products containing cocoa or chocolate, dietary supplements, and some pharmaceuticals such as analgesics (Gracia-Lor et al., 2017), in 27 samples.

The class of pharmaceuticals deserves special attention, especially subclasses with high environmental risk, such as antineoplastic and antibiotic substances. Eight pharmaceuticals and two metabolites were detected more frequently in the samples analyzed. The compounds 4-acetamidoantipyrine and 4-methylamino-antipyrine, two metabolites of dipyrone, were present in 21 and 20 samples, respectively. Another highly recurrent subclass was antihypertensives, with the compounds acebutolol, atenolol, and propranolol identified in 25, 19, and 21 samples, respectively. Two pharmaceuticals with psychoactive properties were detected: levetiracetam, in 22 samples, and memantine, in 18 samples. Benzododecinium, an antiseptic, was identified in 24 samples. One antibiotic, isoniazid, was identified in 23 samples, while one antineoplastic agent, flutamide, was identified in all the samples analyzed, including the blank sample.

The pesticides class presented the greatest number of compounds, as well as the highest number of detections in the samples analyzed. The area around the Conceição River is mainly agricultural and some analytes were identified according to the agricultural calendar, especially during the summer soybean harvest. According to an IPEA (Institute of Applied Economic Research, Brazil) technical note published in 2016, 72% of the family farms in south Brazil use pesticides (Valadares et al., 2020). The identification of pesticides was expected, since it is well known that the use of pesticides in Brazilian agriculture constitutes a public health problem (Pignati et al., 2017), due to contamination of the environment, food, and humans associated with the use of increasing quantities of these substances.

In the biocide subclass, the compounds BAC 12 and benzisothiazolone were detected in 24 and 25 samples, respectively. Linoleic acid, used as a formicide, was detected in 23 samples. Uniconazole, a growth regulator, was identified in 24 samples. The insect repellents DEET (diethyltoluamide) and icaridin were identified in 27 and 26 samples, respectively. The insecticide, herbicide, and fungicide subclasses of pesticides were frequently found, since these substances are widely used in the management of soybean and other crops. Large quantities of pesticides are applied throughout the summer harvest period, with different compounds used at different times, as shown by the analytes identified in the samples (Ávila et al., 2013).

Six insecticides were identified in both sampling campaigns: anabasine (22 samples), chlorantraniliprole (21 samples), flubendiamide (27 samples), imidacloprid (25 samples), nicotine (27 samples), and fipronil (27 samples). Also present were the fipronil metabolites fipronildesulfinyl, fipronil sulfide, and fipronilsulfone, formed by photolysis, reduction, and oxidation of fipronil, respectively. These compounds have been detected in matrices including the aquatic environment and food (Li et al., 2020).

The second pesticide subclass widely used in agriculture during the summer is fungicides. Six analytes and three metabolites were identified. Azoxystrobin, carbendazim, and cyproconazole were identified in both campaigns, with 27, 26, and 22 samples, respectively. CGA 321113 (trifloxystrobin metabolite) and azoxystrobin acid (azoxystrobin metabolite) were identified in 27 and 26 samples, respectively. Fluxapyroxad was identified in 24 samples, especially in the second campaign. Picoxystrobin and prothioconazole-desthio were identified in 19 and 17 samples, respectively, notably in the second sampling period. Propiconazole was identified in 19 samples, notably in the first sampling campaign.

The most frequent pesticides subclass was herbicides, used in agriculture to control weeds that could compete with the main crop for nutrients and water present in the soil (Ávila et al., 2013). Herbicides were identified in both sampling campaigns, divided into three different groups. The first consisted of compounds mainly identified in the first campaign: 2,4-D (11 samples), chlorimuronethyl (9 samples, only in the first campaign), imazethapyr (11 samples), simazine (9 samples, only in the first campaign), and deisopropylatrazine (DIA), an atrazine metabolite (10 samples). The second group consisted of herbicides mainly identified in the second campaign: isouron (17 samples) and molinate (19 samples). The third herbicide group consisted of compounds identified equally in both campaigns: 2-hydroxyatrazine, atrazine, and deethylatrazine (DEA), with all three compounds identified in 27 samples, and icaridin, identified in 26 samples.

Considering all the samples analyzed, except the blank, an average of 53 analytes were identified in each sample, with the major compounds being pesticides, in accordance with the types of land use and occupation close to the river. The activities in the study region were predominantly soybean cultivation, other crops, and cattle ranching, so it was expected to identify many more pesticides than pharmaceuticals, despite the river traversing nearby urban zones. The Brazilian Institute of Geography and Statistics (IBGE) reported that in 2019, over 3 million hectares were used for soybean production in the northwest of Rio Grande do Sul State, the region of this study (IBGE, 2021).

3.2. In silico predictions for tentatively identified microcontaminants

After identification of the CECs in the samples analyzed, *in silico* prediction of risk was performed considering eight endpoints: half-life, mobility, carcinogenicity, mutagenicity, estrogen receptor binding, biodegradability, PBT, and STP total removal. The results for each endpoint are provided in Table S6 (Section SVII, *Supplementary Material*).

For some endpoints, data treatment was performed until a final value was reached. For example, compounds were considered biodegradable when BIOWIN 5 values were higher than 0.5 and ready biodegradability predictions were 1. Others cases were considered as non-biodegradable. Considering the BIOWIN 5 criteria, 23 biodegradable compounds were identified. When ready biodegradability prediction was applied, only 14 compounds were considered as biodegradable, corresponding to only 9% of the total microcontaminants identified. The biodegradable compounds were3-phenyl-2-propenal, L-glutamic acid, divalproate, dodecylbenzenesulfonic acid, ecgonine methyl ester, gabapentin, linoleic acid, L-lysine, methyl *p*-hydroxybenzoate, *N*-methyl pregabalin, norcocaine, *p*-hydroxybenzoic acid, and pyridoxine. The other 136 compounds were considered non-biodegradable. Data crossing between persistence and biodegradability showed that all the compounds considered biodegradable were also non-persistent and belonged to different classes. The biodegradability distribution is shown in Fig. 1A.

Persistence, calculated as half-life in water, followed the criteria of dos Santos and Nardocci (2019): half-life < 30, non-persistent; 30 ≤half-life < 60, moderately persistent; 60 ≤ half-life < 180, persistent; and half-life ≥ 180, very persistent (Fig. 1B). Considering the criteria values, 42 compounds were classified as "non-persistent", 49 as "moderately persistent",

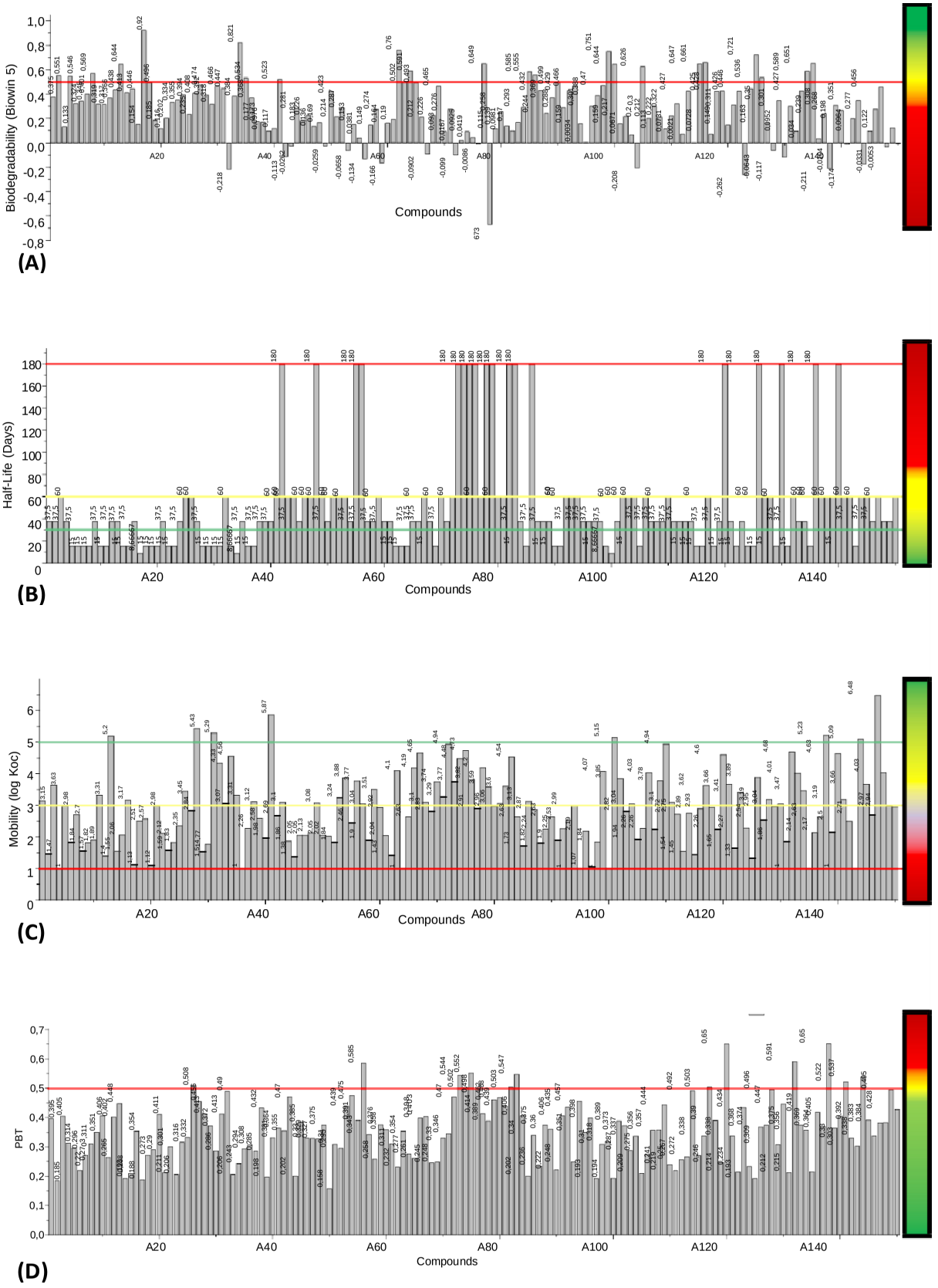


Fig. 1. (Q)SAR predictions for identified compounds: (A) biodegradability; (B) half-life; (C) mobility and (D) PBT. Values of each endpoint can be seen with detail in Table S7 (Section SVII, *Supplementary Material*). Red, yellow and green thresholds represent the classification of each endpoint: higher concern (red), lower concern (green) and values that could suffer with the uncertain of the method (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

40 as "persistent", and 19 as "very persistent". Some concerning results were observed from association of the persistence data with the screening results. Out of 19 very persistent compounds, 8 were identified very frequently (>19 samples), with all being classified as pesticides: 5 insecticides (chlorantraniliprole, fipronil, fipronildesulfinyl, fipronilsulfone, and flubendiamide) and 3 fungicides (fluxapyroxad, picoxystrobin, and prothioconazole-desthio). The classification of very persistent microcontaminants included 17 halogenated pesticides, 1 halogenated pharmaceutical, and 1 antineoplastic agent. Only one very persistent compound was not halogenated. It is known that halogenated compounds can cause cancer, immunotoxicity, and neurotoxicity, and that they can affect the reproduction and development of humans (Davidsen et al., 2021), in addition to effects in the environment.

The mobility of the analytes was investigated using the soil organic adsorption coefficient (K_{OC}) classification method proposed by McCall et al., which is considered the best approach for non-ionic compounds (Navarro et al., 2012; Fenoll et al., 2015). The mobility scale values were adapted from dos Santos and Nardocci (2019). Values of log K_{OC} (L kg^{-1}) calculated by the MCI method, using QSAR Toolbox (v. 4.4.1), were assigned to 4 levels of mobility: log $K_{OC} \geq 5$ (immobile); $3 < \log K_{OC} < 5$ (slightly mobile); $1 < \log K_{OC} < 3$ (moderately mobile); and $\log K_{OC} \leq 1$ (highly mobile). Smaller values indicate greater mobility and higher possibility of the CECs leaching into groundwater. The results showed that few of the microcontaminants could be considered immobile or highly mobile, with most being classified as slightly mobile (54 compounds) or moderately mobile (81 compounds). The immobile compounds identified were acequinocyl, BAC 12, benzododecinium, losartan, sertraline, thioridazine, trifloxystrobin, and CGA 321113 (trifloxystrobin metabolite), representing pharmaceuticals and pesticides with lower risk of leaching. Compounds identified as being highly mobile were 2-aminonicotinic acid, caffeine, ecgonine methyl ester, isoniazid, L-lysine, pyridoxine, and theobromine, representing compounds of greater environmental concern, especially considering groundwater contamination. Some of these compounds of higher concern have low risk for humans, including caffeine (stimulant), L-lysine (amino acid), pyridoxine (vitamin), and theobromine (principle alkaloid present in the cocoa bean). The veterinary anti-inflammatory 2-aminonicotinic acid was also predicted to be highly mobile. Two compounds of high concern that were predicted to be highly mobile were ecgonine methyl ester (analogue of cocaine), which affects the central nervous system, and isoniazid (antibiotic), which can promote bacterial resistance. The mobility behaviors of the microcontaminants are shown in Fig. 1C.

The microcontaminants identified in this study were also assessed using their PBT values. A threshold of 0.5 was used to distinguish non-PBT (<0.5) from potentially PBT compounds (≥0.5) (Pizzo et al., 2016). Despite this established threshold, there was a degree of uncertainty in the prediction, so a range from 0.475 to 0.525 could be considered as PBT or non-PBT. Eight compounds were identified as PBT (difenoconazole, fipronil, fipronil sulfide, fluxapyroxad, oxyfluorfen, pyraclostrobin, sertraline, and thioridazine). Eleven compounds were in the uncertainty zone, and 131 compounds were identified as non-PBT. Out of the eight PBT compounds, six were halogenated pesticides and two were psychoactive pharmaceuticals (Fig. 1D).

Another physicochemical parameter predicted using the QSAR Toolbox software was STP Total Removal, based on the original model developed by Clark et al. (1995). This model predicts the behavior of a compound in an activated sludge sewage treatment plant, with some variation depending on the fate of the chemical in a specific plant. The results showed that only eight compounds presented removal expectancy higher than 90% (bis(2-ethylhexyl) adipate, cholecalciferol, acequinocyl, linoleic acid, CGA 321113 (trifloxystrobin metabolite), pyrethrin 1, thioridazine, and spirodiclofen). Predicted removal lower than 10% was found for 102 compounds, indicating that conventional activated sludge sewage treatment was not effective for these microcontaminants, so more efficient techniques would be required for their removal (Bosio et al., 2019).

Estrogen receptor binding is a property predicted using different databases and approaches. The criteria used for estrogen receptor binding classification can be found on the website of the Laboratory of Mathematical Chemistry (Schultz, 2011). The compounds identified were evaluated using this model, with 89% of them predicted to be non-binding. The other 11% (17 microcontaminants) were defined as weak binding (7 compounds), moderate binding (3 compounds), strong binding (6 compounds), and very strong binding (1 compound). Benzocaine was defined as weak binding (NH_2 group), while all other compounds were defined as possible binding (OH group). According to the model used, the compounds that presented estrogen receptor binding could be characterized as endocrine disruptors (Schultz, 2011).

After evaluation of mutagenicity (Ames test) using the CONSENSUS 1.0.3 model, the results with values ≥0.75 were evaluated by QSAR Toolbox prediction, based on two methods: *in vivo* mutagenicity (micronucleus) alerts by ISS and *in vitro* mutagenicity (Ames test) alerts by ISS. Out of the 150 microcontaminants evaluated, 8-hydroxyquinoline, carbendazim, isoniazid, L-lysine, naphthalene acetamide, and phenmedipham were considered as mutagenic, with score of 1 from the experimental values. Diuron presented a mutagenicity score of 0.75, considered a reliable indicator of mutagenicity. QSAR toolbox prediction using the *in vivo* mutagenicity (micronucleus) model alerts by ISS found 5 alerts of mutagenicity (Table S7, Section SVII, *Supplementary Material*). The compounds 8-hydroxyquinoline, carbendazim, isoniazid, and L-lysine showed H-acceptor-path3-H-acceptor alerts. Isoniazid (antibiotic) also presented hydrazines alert. On the other hand, 43 compounds were classified as non-mutagenic from the experimental values.

Finally, carcinogenicity was evaluated using 4 different models: i) IRFMN/Antares 1.0.0; ii) CAESAR 2.1.9; iii) ISS 1.0.2; and iv) IRFMN/ISSCAN-CGX 1.0.0. All the models used a classification scale from 0 (non-carcinogenic) to 1 (carcinogenic), both with experimental values (Table S2, Section SIV, *Supplementary Material*). Values from 0.00 to 0.25 were considered indicative of non-carcinogenicity, with good reliability, while values from 0.75 to 1.00 indicated carcinogenic compounds, with good reliability. For this endpoint, 13 microcontaminants were classified as non-carcinogenic and 21 as carcinogenic. The 13 non-carcinogenic microcontaminants included different classes of compounds, notably pharmaceuticals and pesticides. Out of the carcinogenic compounds, 67% were pharmaceuticals, including hormones (23.8% of the carcinogenic compounds), analgesics (9.5%), and psychoactive compounds (9.5%). Considering the detection frequencies observed in the screening results, three compounds deserved attention: the pesticide atrazine, identified in all the samples analyzed, with carcinogenicity score of 1; the antibiotic isoniazid, identified in 23 samples, with score of 0.75; and 4-acetamidoantipyrine, a dipyrone metabolite, identified in 21 samples, with score of 0.75. As a non-carcinogenic compound, caffeine was identified in all the samples, with score of 0.0.

3.3. Risk assessment ranking of microcontaminants by LC-QTOF MS with multi-criteria decision-making techniques: ToxPi vs. TOPSIS

The values obtained from the *in silico* (Q)SAR prediction models for the different endpoints were normalized (Table S3, Section SV, *Supplementary Material*) and weights were calculated for each endpoint using SMART (Eq. (1)), as indicated by the ToxPi tool. The weights obtained are presented in Fig. S5 (Section SVIII, *Supplementary Material*).

All the normalized values of the criteria (endpoints) for each compound were determined, together with their weights, followed by application of the ToxPi and TOPSIS tools to obtain the corresponding scores. The two risk assessment rankings of the identified compounds are provided in Table 2. Fig. 2 shows the raw and standardized scores, sorted by ranking, for the two tools used.

The Shapiro-Wilk test applied to the data (1 degree of freedom, 5% significance level) resulted in a p-value of 0.0077, so the null hypothesis of a normal distribution of the population was rejected, as the Fig. 2 makes clear. The application of Levene's test confirmed that the data from the

Table 2
Ranking of the screened compounds using the ToxPi and TOPSIS tools. Gradual color scale according to order of priority, from red (high priority) to green (low priority).

identified compounds	Class	Ranking	
		ToxPI	TOPSIS
Isoniazid	Pharmaceutical	1	2
Nimesulide	Pharmaceutical	2	1
Cortisol	Pharmaceutical	3	6
Estradiol	Pharmaceutical	4	5
Methylprednisolone	Pharmaceutical	5	7
Fluconazole	Pharmaceutical	6	9
19-nortestosterone	Pharmaceutical	7	3
Naphthaleneacetamide	Pesticide	8	8
Carbendazim	Pesticide	9	39
Cortisone	Pharmaceutical	10	13
4-acetamidoantipyrine	Metabolite pharmaceutical	11	22
Saflufenacil (Heat)	Pesticide	12	4
Oxyfluorfen	Pesticide	13	10
Desvenlafaxine	Pharmaceutical	14	23
O-Desmethylvenlafaxine	Pharmaceutical	15	31
Ethinylestradiol	Pharmaceutical	16	19
L-lysine	Pharmaceutical	17	27
Antipyrine	Pharmaceutical	18	12
2-aminoflubendazole	Veterinary drug	19	14
Phenmedipham	Pesticide	20	16
Atrazine	Pesticide	21	47
Gabapentin	Pharmaceutical	22	51
8-hydroxyquinoline	Pesticide	23	48
Benzocaine	Pharmaceutical	24	25
Flutriafol	Pesticide	25	17
Carbamateethyl-*N*-(3-hydroxyphenyl)	Pesticide	26	24
Hydroxytestosterone	Pharmaceutical metabolite	27	66
Cholecalciferol	Pharmaceutical	28	53
Dimethylanilin (*N.N*-)	Pigment	29	64
Prothioconazole-desthio	Pesticide metabolite	30	44
Imazapic	Pesticide	31	28
Imidacloprid	Pesticide	32	15
HMMA	Illicit drug metabolite	33	38
4-methylamino-antipyrine	Pharmaceutical metabolite	34	26
Norethisterone	Pharmaceutical	35	42
Carbamazepine	Pharmaceutical	36	76
Flumioxazin	Pesticide	37	60
Fipronilsulfide	Pesticide metabolite	38	18
Flutamide	Pharmaceutical	39	54
Fipronil	Pesticide	40	21
Adrenalone	Pharmaceutical	41	59
Pyraclostrobin	Pesticide	42	41
Fipronilsulfone	Pesticide metabolite	43	63
Chlorantraniliprole	Pesticide	44	75
Fipronildesulfinyl	Pesticide metabolite	45	83
2-hydroxyquinoline	Pharmaceutical metabolite	46	20
Pyridoxine	Pharmaceutical	47	55
Sebuthylazin	Pesticide	48	65
Atenolol	Pharmaceutical	49	57

2-aminonicotinic acid	Veterinary drug	50	79
Deisopropylatrazine (DIA)	Pesticide metabolite	51	29
Lincomycin	Pharmaceutical	52	49
Cyanazine	Pesticide	53	73
Paracetamol	Pharmaceutical	54	69
Acebutolol	Pharmaceutical	55	82
Deethylatrazine (DEA)	Pesticide metabolite	56	58
Exemestane	Pharmaceutical	57	100
Hydroxybupropion (as (RS.RS)-cyclic hemiketal)	Pharmaceutical metabolite	58	74
Fluxapyroxad	Pesticide	59	46
Azoxystrobinacid	Pesticide metabolite	60	92
Metominostrobin	Pesticide	61	32
Levetiracetam	Pharmaceutical	62	105
Difenoconazole	Pesticide	63	45
Pyrethrin 1	Pesticide	64	89
Simazine	Pesticide	65	91
Fluazifop	Pesticide	66	95
Haloxyfop	Pesticide	67	96
Pyrethrins: Jasmolin II (Pyrethrin 2)	Pesticide	68	35
Diuron	Pesticide	69	11
Acetanilide	Pharmaceutical	70	56
Nikethamide	Illicit drug	71	85
Fenpropimorphcarboxylicacid	Pesticide metabolite	72	77
Ecgoninemethylester	Illicit drug metabolite	73	112
N-Methylpregabalin	Pharmaceutical	74	43
Benzovindiflupyr	Pesticide	75	30
Tebuconazole	Pesticide	76	78
Carbofuran	Pesticide	77	110
Benzisothiazol-3(2H)-one-1-2-	Pesticide	78	90
Terbuthylazine	Pesticide	79	68
Divalproate	Pharmaceutical	80	108
Diclosulam	Pesticide	81	93
Cyproconazole	Pesticide	82	97
Methoxyfenozide	Pesticide	83	71
Dihydroquinoline 2-2-4-trimethyl-1-2-	Antioxidant	84	84
Tibolone	Pharmaceutical	85	80
Octhilinone	Pesticide	86	115
Imazethapyr	Pesticide	87	72
Flubendiamide	Pesticide	88	88
Sertralina	Pharmaceutical	89	40
Azoxystrobin	Pesticide	90	67
Icaridin	Pesticide	91	99
Acequinocyl	Pesticide	92	116
Venlafaxine	Pharmaceutical	93	106
Picoxystrobin	Pesticide	94	50
Theobromine	Pharmaceutical	95	114
Methyl*p*-hydroxybenzoate	Antioxidant	96	86
Propiconazole	Pesticide	97	111
Norcocaine	Illicit drug metabolite	98	81
Chlorfenvinphos	Pesticide	99	34
Myclobutanil	Pesticide	100	109

Dinoterb	Pesticide	101	94
Megestrolacetate	Pharmaceutical	102	52
Propazine	Pesticide	103	113
Isouron	Pesticide	104	103
Oxyphencyclimine	Pharmaceutical	105	117
Spirodiclofen	Pesticide	106	70
Aminorex	Pharmaceutical metabolite	107	118
Bethanidine	Pharmaceutical	108	87
Uniconazole	Pesticide	109	133
Embutramide	Veterinary drug	110	102
p-Hydroxybenzoicacid	Antioxidant	111	36
Tramadol-n,n-didesmethyl	Pharmaceutical metabolite	112	61
Apophedrin	Pharmaceutical	113	104
Molinate	Pesticide	114	128
Furmecyclox	Pesticide	115	120
Fexofenadine	Pharmaceutical	116	127
Amino acids: l-glutamicacid	Pharmaceutical	117	125
2-hydroxy-atrazine	Pesticide metabolite	118	107
Chlorimuronethyl	Pesticide	119	121
Trifloxystrobin	Pesticide	120	126
Nicotine	Pesticide	121	136
2-Phenethylamine	Pharmaceutical	122	123
DEET	Pesticide	123	62
Thioridazine	Pharmaceutical	124	98
Pseudoephedrine	Pharmaceutical	125	131
Losartan	Pharmaceutical	126	135
Propranolol	Pharmaceutical	127	122
Alpha-PVP	Illicit drug	128	138
CGA 321113 (Trifloxystrobinmetabolite)	Pesticide metabolite	129	119
Amprolium	Veterinary drug	130	124
Diphenylamine	Pesticide	131	130
Oxprenolol	Pharmaceutical	132	129
Eugenol	Pharmaceutical	133	132
Naftidrofuryl	Pharmaceutical	134	145
N.N-Diethyl-m-toluamide. DEET	Pesticide	135	33
Memantine	Pharmaceutical	136	101
Caffeine	Stimulant	137	140
Phenol	Pharmaceutical	138	37
Butoxycaine	Pharmaceutical	139	144
Triphenylphosphate (TPP)	Plasticizer	140	141
Linoleicacid	Pesticide	141	139
3-phenyl-2-propenal	Pharmaceutical	142	137
Benzododecinium	Pharmaceutical	143	146
Dodecylbenzenesulphonicacid	Saponifier	144	142
Bis(2-ethylhexyl) adipate	Plasticizer	145	134
Amino acids: l-tryptophan	Pharmaceutical	146	143
Diazepam	Pharmaceutical	147	147
2,4-D	Pesticide	148	148
Ibuprofen	Pharmaceutical	149	149
Ethoxyquin	Antioxidant	150	150

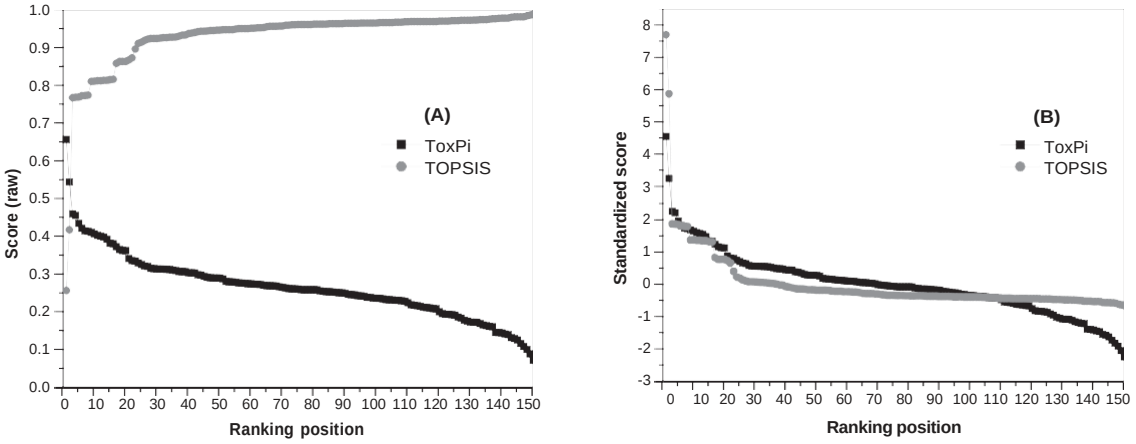


Fig. 2. (A) Ranking positions *vs.* raw scores calculated using ToxPi and TOPSIS. (B) Ranking positions *vs.* standardized scores calculated using ToxPi and TOPSIS.

two scores did not present statistically significant equality of variances, so they were not homogeneous. These tests showed that nonparametric comparison tests should be used in order to assess statistically significant differences between the two scores (and, therefore, the two rankings).

Comparison of the standardized ToxPi and TOPSIS scores using the Friedman test (5% significance level) resulted in a p-value of 0.05004, so the H_0 hypothesis could not be rejected and there was no significant difference between the scores. Therefore, the results obtained for risk assessment ranking of the identified compounds were consistent, enabling both methods to be recommended for use for this purpose. In addition, the results supported the suitability of using TOPSIS, which has never been used previously for this purpose. The similarity was most notable for the higher positions of the rankings, indicating the compounds with greatest environmental risk. Comparison of the rankings using 10-position intervals showed that the standard deviations of means of the positions assigned by TOPSIS, in relation to the ranking by ToxPi (considered as the reference in this comparison) were less than 5.0 for the intervals 1–10, 11–20, and 141–150 (Table S8, Section SVIII, *Supplementary Material*). This indicated that the dispersion of the positions from the mean remained inside the 10-position interval, supporting the information shown in Table 1 and Fig. 2.

In order to measure the robustness of the two MCDM tools, sensitivity tests were performed between the ranking positions, considering the weight sets obtained from SMART. Fig. 3A and C show sets of rankings compared to the original ranking (with weights obtained from relative importance values of 1, 2, and 3 between endpoints). Fig. 3B and D show the results of the Friedman tests, with the p-values of the two sets of rankings having statistically significant differences ($p < 0.05$). Post-hoc analyses were performed to identify the pairs of rankings that presented significant differences. In the case of TOPSIS, small variations of +1 or −0.5 in the SMART input values did not lead to significant differences, but differences were evident for higher variations (+2 values). The ToxPi sensitivity tests results showed significant differences between all the rankings, reflecting higher sensitivity of the ToxPi ranking tool. Therefore, the TOPSIS tool was more robust than ToxPi, indicating its greater suitability for the purpose of the present work. The TOPSIS results demonstrated that small variations in the input data did not lead to major changes in the scores obtained and, consequently, in the rankings assigned to the compounds. This was an important finding, because the changes made in the weights maintained the relative importance of the endpoints, with small modifications not altering the performance of TOPSIS. It should be noted that due to the subjectivity of the SMART input values used to calculate the weights, the high sensitivity observed for ToxPi could lead to highly variable results, affecting the ranking and, consequently, decision-making concerning the environmental risk of the identified compounds. In addition, the similarity of the results was more evident in the 20 highest positions of the TOPSIS rankings, as can be seen in Fig. 3A.

The microcontaminants presenting the greatest environmental risk, according to both MCDM, were the drugs isoniazid and nimesulide. Isoniazid, identified in 23 samples, is the most prescribed antitubercular drug and has absorption lower than 60% in humans (Bhandari et al., 2021). Nimesulide, detected in 4 samples, is a non-steroidal, antipyretic, and analgesic anti-inflammatory. Considering the first 20 ranked microcontaminants that presented greater risks to humans and environment, it can be seen that for both methods, the majority of the analytes belonged to the class of drugs (hormones, corticosteroid, antifungal, antidepressant, and analgesic/antidepressant metabolites). However, if only the results of the screening were considered for further quantitative analysis, without their association with (Q)SAR and MCDM methods, it could be mistakenly inferred that the most frequent microcontaminants in this study (pesticides) would be the priority group for future chemical analyses. On the other side of the ranking, there were 10 compounds that presented lower risks for humans and the environment. These compounds belonged to different classes and subclasses, presenting higher frequencies of detection, compared to the toxic compounds, with dodecylbenzenesulfonic acid, benzododecinium, linoleic acid, and ibuprofen present in 26, 24, 23, and 20 samples, respectively.

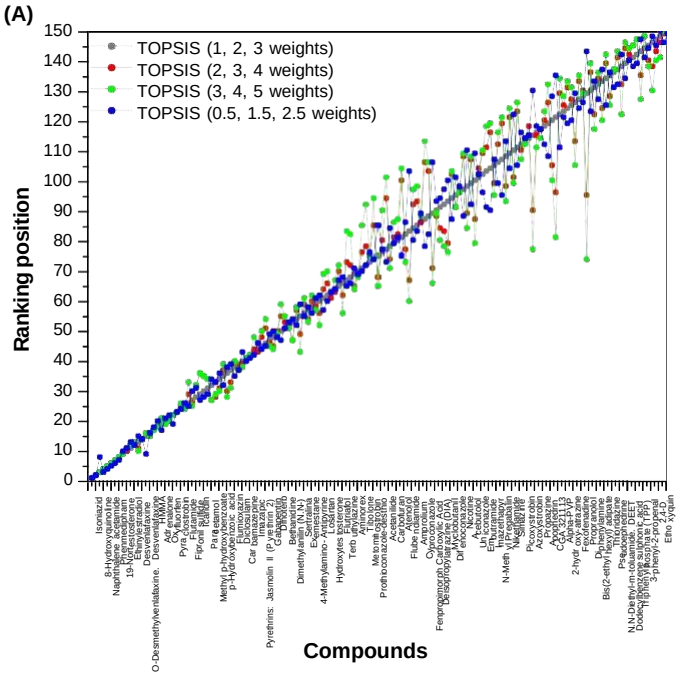
The results obtained in the present study reinforced the importance of carrying out extensive screening studies including various classes of microcontaminants, as well as the need for simultaneous assessment of environmental risk using (Q)SAR tools. The use of MCDM methods proved to be essential for evaluation of the endpoints in a weighted way, independent of the frequency of detection of the screened microcontaminants, enabling appropriate decisions to be made concerning environmental management.

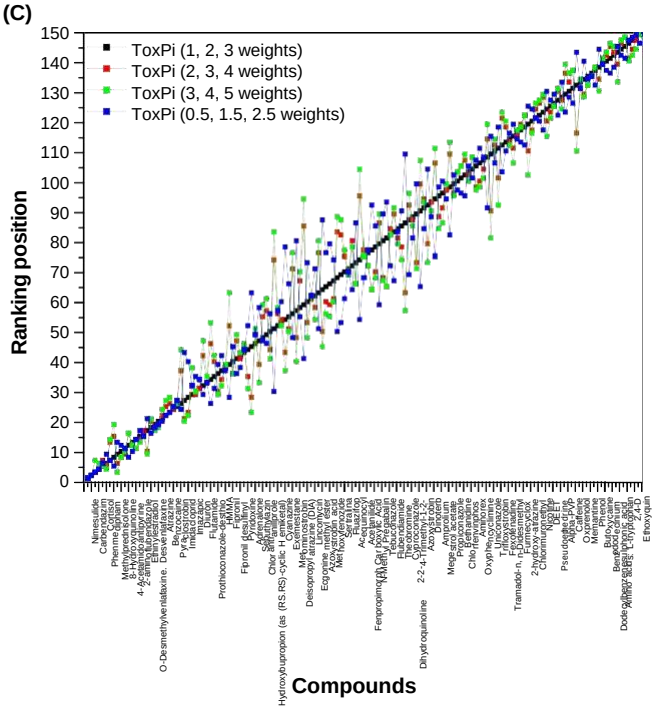
4. Conclusions

Qualitative analysis may be considered an important tool for the identification of contaminants, in a step prior to quantification. The major advantage of the approach used in screening analysis is the possibility of searching many compounds, using information available in purpose-built or commercial databases. In this study, screening indicated the presence of 150 suspected and confirmed microcontaminants in surface waters, especially pesticides and pharmaceuticals.

In silico predictions based on the use of (Q)SAR tools are easier to perform than experimental (*in vivo* or *in vitro*) bioassays of screened compounds. In this study, (Q)SAR results for the identified microcontaminants, employing eight different endpoints, were used as input data for successful ranking of the screened analytes according to their toxicity towards humans and the environment.

The application of MCDM techniques, implemented using the ToxPi and TOPSIS tools, enabled the ordering of the identified compounds in rankings according to their environmental risk. This





(B) Information		Value
Friedman chi-square (0.05 significance level)		38.328
Degrees of freedom		3
p-value		2.4×10^{-8}
Critical difference		58.993
Rankings comparison	**Observed difference**	**Significant difference?**
TOPSIS (1, 2, 3) – TOPSIS (0.5, 1.5, 2.5)	33	No
TOPSIS (1, 2, 3) – TOPSIS (2, 3, 4)	44	No
TOPSIS (1, 2, 3) – TOPSIS (3, 4, 5)	81	Yes
TOPSIS (0.5, 1.5, 2.5) – TOPSIS (2, 3, 4)	11	No
TOPSIS (0.5, 1.5, 2.5) – TOPSIS (3, 4, 5)	114	Yes
TOPSIS (2, 3, 4) – TOPSIS (3, 4, 5)	125	Yes

(D) Information		Value
Friedman chi-square (0.05 significance level)		115.52
Degrees of freedom		3
p-value		7.1×10^{-25}
Critical difference		58.993
Rankings comparison	**Observed difference**	**Significant difference?**
ToxPi (1, 2, 3) – ToxPi (0.5, 1.5, 2.5)	130	Yes
ToxPi (1, 2, 3) – ToxPi (2, 3, 4)	252	Yes
ToxPi (1, 2, 3) – ToxPi (3, 4, 5)	130	Yes
ToxPi (0.5, 1.5, 2.5) – ToxPi (2, 3, 4)	122	Yes
ToxPi (0.5, 1.5, 2.5) – ToxPi (3, 4, 5)	260	Yes
ToxPi (2, 3, 4) – ToxPi (3, 4, 5)	382	Yes

Fig. 3. (A) Sensitivity tests for TOPSIS rankings; (B) Friedman rank sum test for TOPSIS sensitivity test rankings; (C) Sensitivity tests for ToxPi rankings; (D) Friedman rank sum test for ToxPi sensitivity test rankings.

sorting would allow management decisions to be made concerning priority microcontaminants, taking into account the simultaneous effects of several physicochemical, biological, and toxicological parameters that are environmentally relevant, but have different relative importance. The weighted aggregation of these parameters enables ranking of the identified microcontaminants, regardless of their detection frequency. The appropriate application of MCDM methods to screening results assists in selecting the priority compounds for subsequent quantitative analysis during environmental monitoring.

Comparison of the rankings obtained by the two tools showed that there were no statistically significant differences between them, confirming the satisfactory consistency of the results. Sensitivity tests showed greater robustness of the TOPSIS technique, compared to the ToxPi software, in the face of small variations in the input data. Finally, application of the TOPSIS method is a pioneering approach with excellent potential to assist in chemical analysis decision-making in the environmental sciences field.

CRediT authorship contribution statement

Raquel Wielens Becker: Investigation; Formal analysis; Writing.
Letícia Alves Jachstet: Investigation; Formal analysis.
Alexsandro Dallegrave: Investigation; Formal analysis.
Alejandro Ruiz-Padillo: Writing; Investigation; Formal analysis.
Renato Zanella: Formal analysis; Supervision.
Carla Sirtori: Writing; Conceptualization; Supervision; Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.149002.

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SUPPLEMENTARY MATERIAL III

SUPPLEMENTARY MATERIAL

Multi-criteria decision-making techniques associated with (Q)SAR risk assessment for ranking surface water microcontaminants identified using LC-QTOF MS

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Section SI. Analytical Standards used to confirm the presence of analytes in the analysed samples

Table S1. Standards used to confirm screened microcontaminants.

Analyte	Supplier	Formula	Rt (min)
2,4-D	Sigma Aldrich	$C_8H_6Cl_2O_3$	11.5
Atrazine	Sigma Aldrich	$C_8H_{14}ClN_5$	11
Carbendazim	Sigma Aldrich	$C_9H_9N_3O_2$	4.96
Cyanazine	Sigma Aldrich	$C_9H_{13}ClN_6$	9.4
Deethylatrazine (DEA)	Dr. Ehrenstorfer	$C_6H_{10}ClN_5$	7.89
Deisopropylatrazine (DIA)	Sigma Aldrich	$C_5H_8ClN_5$	6.04
Diazepam	Sigma Aldrich	$C_{16}H_{13}ClN_2O$	12.35
Difenoconazole	Sigma Aldrich	$C_{19}H_{17}Cl_2N_3O_3$	14.5
Fipronil	Commercial product*	$C_{12}H_4Cl_2F_6N_4OS$	13.7
Fipronil-sulfone	Sigma Aldrich	$C_{12}H_4Cl_2F_6N_4O_2S$	14.17
Flutamide	Sigma Aldrich	$C_{11}H_{11}F_3N_2O_3$	12.57
Flutriafol	Commercial product*	$C_{16}H_{13}F_2N_3O$	11.24
Fluxapyroxad	Commercial product*	$C_{18}H_{12}F_5N_3O$	12.76
Imidacloprid	Commercial product*	$C_9H_{10}ClN_5O_2$	6.75
Losartan	Huaian Synniken	$C_{22}H_{23}ClN_6O$	11.61
Nimesulide	CosmeTrade	$C_{13}H_{12}N_2O_5S$	11.63
Paracetamol	Anqiu Lu'An Pharma	$C_8H_9NO_2$	3.62
Propazine	Sigma Aldrich	$C_9H_{16}N_5Cl$	12.11
Propranolol	CosmeTrade	$C_{16}H_{21}NO_2$	9.42
Saflufenacil	Commercial product*	$C_{17}H_{17}ClF_4N_4O_5S$	11.88
Simazine	Sigma Aldrich	$C_7H_{12}ClN_5$	9.65
Tebuconazole	Sigma Aldrich	$C_{16}H_{22}ClN_3O$	13.93

*Five active ingredients were confirmed by using commercial products using the strategy described previously by Cardoso et al. (2020).

Section SII. Sampling points

Three different sampling points at Conceição riverwere selected in present work. Hydrographic basin of the Conceição river is a tributary of the Ijuí river and is located in the northwest of Rio Grande do Sul State, (28°15' to 28°50'S and 53°30' to 54°10'W) with approximately 811.00 km^2 area, in five cities: Augusto Pestana, Boa Vista do Cadeado, Coronel Barros, Cruz Alta and Ijuí. The hydrographic basin of the Conceição river is 5th order and comprises 227 water courses, being 177 1st order, 38 2nd order, 9 3rd order, 2 4th order water courses and the main river, 5th order totalizing a length of 650.20 km and an average flow throughout the year of 185 $m^3.s^{-1}$ (Bernardi et al. 2015). A map of Hydrographic basin of the Conceição river is showed below (Figure S1). Sampling points are located in the in the Northwest region of Brazilian state of Rio Grande do Sul. Coordinates are: River spring (28°28'43.0"S 53°50'50.2"W); Point 1 (28°33'23.7"S 53°47'45.3"W); Point 2 (28°31'23.9"S 53°52'53.1"W) and Point 3 (28°27'47.6"S 53°57'17.2"W). Besides around urban area (Cruz Alta, Boa vista do Cadeado and Augusto Pestana cities), the major occupation of the land around Conceição River is agriculture and livestock activities, so it is possible to suggest that the three principals sources of Conceição River contamination may be agriculture, livestock and urban activities.

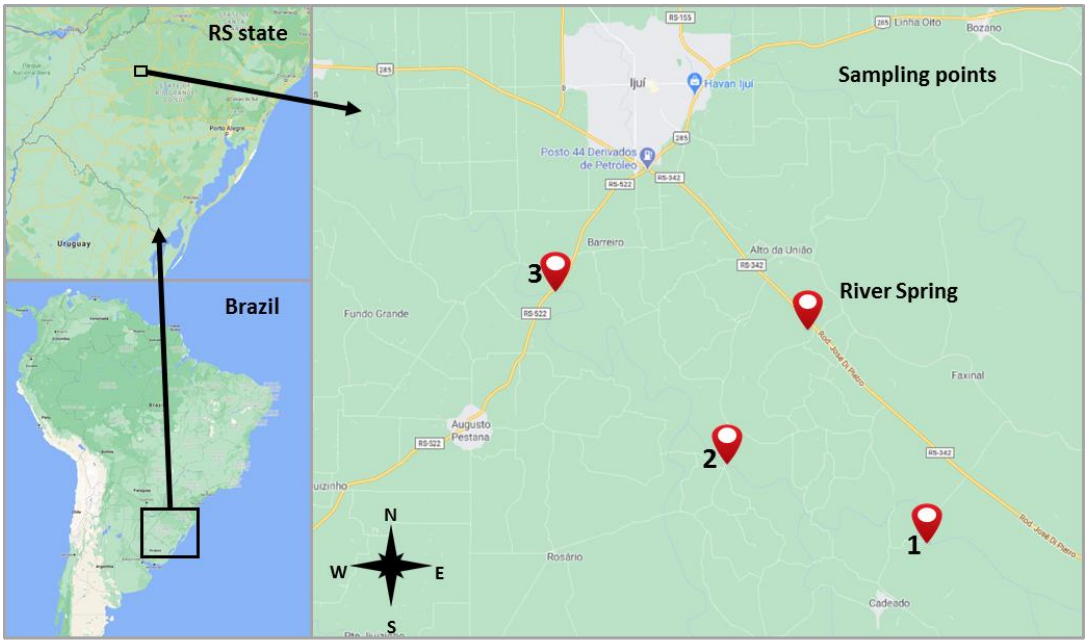


Figure S1. Sampling points location. Source: adapted from Google maps.

Section SIII. Sample preparation by SPE and LC-QTOF MS analyses

Samples were filtered through a 47 mm PVDF filtering membrane, with 0.45 µm porosity (Allcrom). After filtration, samples were extracted and preconcentrated using Oasis HLB SPE cartridges (500 mg, 6 mL). The cartridges were conditioned with 3 mL of methanol, followed by 3 mL of ultrapure water. 200 mL of sample was percolated through the cartridge and afterwards the cartridge was dried for 20 minutes under air flow. For the elution, 2 aliquots of 4 mL of methanol were used. The eluate was evaporated to dryness under nitrogen flow and reconstituted in 500 µL of Water:Methanol (50:50). The extract was vortexed for 1 minute and filtered through a 13 mm PVDF syringe filter with 0.22 µm posority (Allcrom) into a chromatographic vial.

The screening analysis to identify suspect compounds was performed using a Shimadzu Nexera X2 UHPLC system connected to an Impact II QTOF mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization source (ESI). MS and MS/MS information were recorded over the *m/z* range from 50 to 1200, calibrated with sodium formate. The QTOF MS system operated in broadband collision-induced dissociation (bbCID) acquisition mode, which provided MS and MS/MS spectra at the same time, operating with two different collision energies: low collision energy of 4 eV (MS) and high collision energy variation from 25 eV to 50 eV (MS/MS), using a scan rate of 2 Hz.

In this study, three different methods were used:

a) positive ionization mode method using a chromatographic separation column Hypersyl GOLD (100 mm x 2.1 mm x 1.9 µm). QTOF conditions were: capillary voltage, 4000 V; end plate offset, 500 V; nebulizer pressure, 4 bar (N_2); dry gas, 9 L min^{-1} (N_2) and dry temperature, 200 °C. Chromatographic separation occurred with the column thermostated at 35 °C and injection volume of 15 µL. Mobile phase was (A) MeOH

acidified with 0.1% (v/v) formic acid and (B) H_2O acidified with 0.1% (v/v) formic acid, at a flow rate of 0.3 mL min^{-1}. The gradient elution program was: 0 min, 95% B; 1 min, 95% B; 16 min, 5% B; 18 min, 5% B; 20 min, 95% B; 22 min, 95% B.

b) the second method to identified compounds in negative ionization mode method using a Hypersyl GOLD (100 mm x 2.1 mm x 1.9 µm) column. QTOF conditions were: capillary voltage, 2500 V; end plate offset, 500 V; nebulizer pressure, 3 bar (N_2); dry gas, 9 L min^{-1} (N_2) and dry temperature, 200 °C. Chromatographic separation was exactly as in method a).

c) positive ionization mode method using a chromatographic separation column Hypersyl GOLD HILIC (100 mm x 2.1 mm x 1.9 µm). QTOF conditions were the same as in method a). Chromatographic separation occurred with the column thermostated at 35 °C and injection volume of 15 µL. Mobile phase was (A) Acetonitrile and (B) Ammonium formate 60 mmol (pH 3.7), at a flow rate of 0.25 mL min^{-1}. The elution was in isocratic mode: 60% A and 40% B. After analysis, all data were processed using TASQ (2.2 version) software, from Bruker Daltonics.

Section IV. *In silico* quantitative structure-activity relationship ((Q)SAR) predictions

Table S2. Carcinogenicity classification by Vega models.

Score		Reability	
0	🟢	✓	Non-carcinogenic, experimental value
0.1	🟢	***	Non-carcinogenic
0.2	🟢	**	Non-carcinogenic
0.3	🟢	*	Non-carcinogenic
0.4	🟡	***	Possibly non-carcinogenic
0.5	🟡	**	Possibly non-carcinogenic
0.6	🟡	*	Possibly non-carcinogenic
0.7	🔴	*	Carcinogenic
0.8	🔴	**	Carcinogenic
0.9	🔴	***	Carcinogenic
1	🔴	✓	Carcinogenic, experimental value

Section SV. MCDM tools parameters

Table S3. Data treatment before ToxPi ranking.

Endpoint	Treatment	Logic	Normalization
Carcinogenicity Score	Linear(x)	Maximizing	X /Xmax
Mutagenicity Score	Linear(x)	Maximizing	X /Xmax
Estrogen Receptor Binding	Linear(x)	Maximizing	X /Xmax
Biodegradability	Linear(x)	Minimizing	Xmax/X
Half-Life	Sqrt(x)	Maximizing	X /Xmax
Mobility	Log10(x)	Minimizing	Xmax/X
PBT	Linear(x)	Maximizing	X /Xmax
STP Total Removal	Linear(x)	Minimizing	Xmax/X

Section SVI. Screening analyses

Table S4. Classification of identified compounds

Nº	Compound	Formula	CAS	Class	Subclass	Ionization Mode	Confirmed (C) or Suspected (S)	RT (min)
A1	19-Nortestosterone	C18H26O2	434-22-0	Pharmaceutical	Hormone	[M + H]+	S	13.17
A2	2,4-D	C8H6Cl2O3	94-75-7	Pesticide	Herbicide	[M - H]-	C	11.5
A3	2-aminoflubendazole	C14H10FN3O	82050-13-3	Veterinary drug	Anti-helmintic	[M + H]+	S	7.8
A4	2-aminonicotinic acid	C6H6N2O2	5345-47-1	Veterinarydrug	Anti-inflammatory	[M + H]+	S	0.89
A5	2-hydroxy-atrazine	C8H15N5O	2163-68-0	Pesticide metabolite	Herbicide	[M + H]+	S	6.36
A6	2-hydroxyquinoline	C9H7NO	59-31-4	Pharmaceutical metabolite	Quinoline metabolite	[M + H]+	S	6.58
A7	2-phenethylamine	C8H11N	64-04-0	Pharmaceutical	Psychoactive	[M + H]+	S	8.27
A8	3-phenyl-2-propenal	C9H8O	104-55-2	Pharmaceutical	Antibiotic, antitumoral	[M + H]+	S	14.7
A9	4-acetamidoantipyrine	C13H15N3O2	83-15-8	Pharmaceutical Metabolite	Analgesic, antipyretic	[M + H]+	S	5.66
A10	4-methylamino-antipyrine	C12H15N3O	519-98-2	Pharmaceutical Metabolite	Analgesic, antipyretic	[M + H]+	S	0.88
A11	8-hydroxyquinoline	C9H7NO	148-24-3	Pesticide	Fungicide	[M + H]+	S	6.57
A12	Acebutolol	C18H28N2O4	37517-30-9	Pharmaceutical	Antiarrhythmic and antihypertensive	[M + H]+	S	0.87
A13	Acequinocyl	C24H32O4	57960-19-7	Pesticide	Acaricide	[M + H]+	S	9.03
A14	Acetanilide	C8H9NO	103-84-4	Pharmaceutical	Analgesic	[M + H]+	S	5.92
A15	Adrenalone	C9H11NO3	99-45-6	Pharmaceutical	Vasoconstrictor	[M + H]+	S	0.89
A16	Alpha-PVP	$C_{15}H_{21}NO$	14530-33-7	Illicit drug	Illicit drug	[M + H]+	S	13.01
A17	L-glutamic acid	C5H9NO4	56-86-0	Pharmaceutical	Natural psychoactive	[M + H]+	S	0.88
A18	L-tryptophan	C11H12N2O2	73-22-3	Pharmaceutical	Psychoactive	[M + H]+	S	4.58
A19	Aminorex	C9H10N2O	2207-50-3	Pharmaceutical metabolite	Psychoactive	[M + H]+	S	0.89
A20	Amprolium	C14H20Cl2N4	137-88-2	Veterinary drug	Anti-coccidial	[M + H]+	S	0.87
A21	Anabasine	$C_{10}H_{14}N_2$	494-52-0	Pesticide	Insecticide	[M + H]+	S	0.88
A22	Antipyrine	C11H12N2O	60-80-0	Pharmaceutical	Analgesic, antipyretic	[M + H]+	S	10.3
A23	Apophedrin	C8H11NO	7568-93-6	Pharmaceutical	Nasal congestion, control of urinary incontinence, priapism and obesity	[M + H]+	S	0.89
A24	Atenolol	C14H22N2O3	29122-68-7	Pharmaceutical	Beta blocker	[M + H]+	S	0.88
A25	Atrazine	C8H14ClN5	1912-24-9	Pesticide	Herbicide	[M + H]+	C	11
A26	Azoxystrobin	C22H17N3O5	131860-33-8	Pesticide	Fungicide	[M + H]+	S	12.16
A27	Azoxystrobin acid	C21H15N3O5	1185255-09-7	Pesticide Metabolite	Fungicide	[M + H]+	S	11.38
A28	BAC 12	C21H38N+	8001-54-5	Pesticide	Biocide	[M + H]+	S	14
A29	Benzisothiazolone	C7H5NOS	2634-33-5	Pesticide	Biocide	[M + H]+	S	9
A30	Benzocaine	C9H11NO2	94-09-7	Pharmaceutical	Anesthetic	[M + H]+	S	8.69
A31	Benzododecinium	C21H38N+	10328-35-5	Pharmaceutical	Antiseptic	[M + H]+	S	14
A32	Benzovindiflupyr	C18H15Cl2F2N3O	1072957-71-1	Pesticide	Fungicide	[M + H]+	S	14.04
A33	Bethanidine	C10H15N3	55-73-2	Pharmaceutical	Antihypertensive	[M + H]+	S	4.56
A34	Bis(2-ethylhexyl) adipate	C22H42O4	103-23-1	Plasticizer	Plasticizer	[M + H]+	S	17.6
A35	Butoxycaine	C17H27NO3	3772-43-8	Pharmaceutical	Anesthetic	[M + H]+	S	13.81
A36	Caffeine	C8H10N4O2	58-08-2	Stimulant	Stimulant	[M + H]+	S	5.72
A37	Carbamate ethyl-*N*-(3-hydroxyphenyl)-	C9H11NO3	7159-96-8	Pesticide	Herbicide	[M + H]+	S	0.89
A38	Carbamazepine	C15H12N2O	298-46-4	Pharmaceutical	Psychoactive	[M + H]+	S	10.54
A39	Carbendazim	C9H9N3O2	10605-21-7	Pesticide	Fungicide	[M + H]+	C	4.96
A40	Carbofuran	C12H15NO3	1563-66-2	Pesticide	Insecticide, acaricide	[M + H]+	S	9.13
A41	CGA 321113 (Trifloxystrobin metabolite)	C19H17F3N2O4	252913-85-2	Pesticide metabolite	Fungicide	[M + H]+	S	14.04
A42	Chlorantraniliprole	C18H14BrCl2N5O2	500008-45-7	Pesticide	Insecticide	[M + H]+	S	11.69
A43	Chlorfenvinphos	C12H14Cl3O4P	470-90-6	Pesticide	Insecticide, acaricide	[M + H]+	S	14.04
A44	Chlorimuronethyl	C15H15ClN4O6S	90982-32-4	Pesticide	Herbicide	[M + H]+	S	12.61
A45	Cholecalciferol	C27H44O	67-97-0	Pharmaceutical	Vitamin	[M + H]+	S	17.51
A46	Cortisol/Hydrocortisone	C21H30O5	50-23-7	Pharmaceutical	Hormone	[M + H]+	S	13.12
A47	Cortisone	C21H28O5	53-06-5	Pharmaceutical	Hormone	[M + H]+	S	9.59
A48	Cyanazine	C9H13ClN6	21725-46-2	Pesticide	Herbicide	[M + H]+	C	9.4
A49	Cyproconazole	C15H18ClN3O	94361-06-5	Pesticide	Fungicide	[M + H]+	S	12.75
A50	DEET (Diethyltoluamide)	C12H17NO	134-62-3	Pesticide	Insect repellent	[M + H]+	S	11.26
A51	Deethylatrazine (DEA)	C6H10ClN5	6190-65-4	Pesticide metabolite	Herbicide	[M + H]+	C	7.89
A52	Deisopropylatrazine (DIA)	C5H8ClN5	1007-28-9	Pesticide metabolite	Herbicide	[M + H]+	C	6.04

Nº	Compound	Formula	CAS	Class	Subclass	Ionization Mode	Confirmed (C) or Suspected (S)	RT (min)
A53	Desvenlafaxine	C16H25NO2	93413-62-8	Pharmaceutical	Psychoactive	[M + H]+	S	6.95
A54	Diazepam	C16H13ClN2O	439-14-5	Pharmaceutical	Psychoactive	[M + H]+	C	12.35
A55	Diclosulam	C13H10Cl2FN5O3S	145701-21-9	Pesticide	Herbicide	[M + H]+	S	10.68
A56	Difenoconazole	C19H17Cl2N3O3	119446-68-3	Pesticide	Fungicide	[M + H]+	C	14.5
A57	Dihydroquinoline-2-2-4-trimethyl-1-2-	C12H15N	147-47-7	Antioxidant	Antioxidant	[M + H]+	S	11.25
A58	Dimethylanilin	C8H11N	121-69-7	Pigment	Pigment	[M + H]+	S	0.89
A59	Dinoterb	C10H12N2O5	1420-07-1	Pesticide	Herbicide	[M - H]-	S	14.08
A60	Diphenylamine	C12H11N	122-39-4	Pesticide	Fungicide	[M + H]+	S	12.89
A61	Diuron	C9H10Cl2N2O	330-54-1	Pesticide	Herbicide	[M + H]+	S	11.44
A62	Divalproate	C8H16O2	99-66-1	Pharmaceutical	Psychoactive	[M + H]+	S	12.52
A63	Dodecylbenzenesulphonic acid	C18H30O3S	27176-87-0	Saponifier	Saponifier	[M - H]-	S	16.4
A64	Ecgonine methyl ester	C10H17NO3	7143 09 - 1	Illicit drug metabolite	Illicit drug metabolite	[M + H]+	S	0.88
A65	Embutramide	C17H27NO3	15687-14-6	Veterinary drug	Preparation for euthanasia	[M + H]+	S	13.81
A66	Estradiol	C18H24O2	50-28-2	Pharmaceutical	Hormone	[M + H]+	S	13.46
A67	Ethinylestradiol	C20H24O2	57-63-6	Pharmaceutical	Hormone	[M + H]+	S	13.46
A68	Ethoxyquin	C14H19NO	91-53-2	Antioxidant	Antioxidant	[M + H]+	S	12.15
A69	Eugenol	C10H12O2	97-53-0	Pharmaceutical	Antiseptic	[M + H]+	S	16.93
A70	Exemestane	C20H24O2	107868-30-4	Pharmaceutical	Breast cancer treatment	[M + H]+	S	13.46
A71	Fenpropimorph carboxylic acid	C20H31NO3	121098-45-1	Pesticide metabolite	Fungicide	[M + H]+	S	8.83
A72	Fexofenadine	C32H39NO4	83799-24-0	Pharmaceutical	Antihistamine	[M + H]+	S	10.95
A73	Fipronil	C12H4Cl2F6N4OS	120068-37-3	Pesticide	Insecticide	[M - H]-	S	13.7
A74	Fipronil desulfinyl	C12H4Cl2F6N4	205650-65-3	Pesticide metabolite	Insecticide	[M - H]-	S	13.55
A75	Fipronil sulfide	C12H4Cl2F6N4S	120067-83-6	Pesticide metabolite	Insecticide	[M - H]-	S	13.9
A76	Fipronilsulfone	C12H4Cl2F6N4O2S	120068-36-2	Pesticide metabolite	Insecticide	[M - H]-	C	14.17
A77	Fluazifop	C15H12F3NO4	83066-88-0	Pesticide	Herbicide	[M + H]+	S	12.08
A78	Flubendiamide	C23H22F7IN2O4S	272451-65-7	Pesticide	Insecticide	[M - H]-	S	13.77
A79	Fluconazole	C13H12F2N6O	86386-73-4	Pharmaceutical	Antifungal	[M + H]+	S	7.59
A80	Flumioxazin	C19H15FN2O4	103361-09-7	Pesticide	Herbicide	[M + H]+	S	15.15
A81	Flutamide	C11H11F3N2O3	13311-84-7	Pharmaceutical	Cancer treatment	[M - H]-	C	12.57
A82	Flutriafol	C16H13F2N3O	76674-21-0	Pesticide	Fungicide	[M + H]+	S	11.25
A83	Fluxapyroxad	C18H12F5N3O	907204-31-3	Pesticide	Fungicide	[M + H]+	S	12.76
A84	Furmecyclox	C14H21NO3	60568-05-0	Pesticide	Fungicide	[M + H]+	S	10.23
A85	Gabapentin	C9H17NO2	60142-96-3	Pharmaceutical	Psychoactive	[M + H]+	S	6.78
A86	Haloxyfop	C15H11ClF3NO4	69806-34-4	Pesticide	Herbicide	[M + H]+	S	13.99
A87	HMMA	C11H17NO2	117652-28-5	Illicit drug metabolite	Illicit drug metabolite	[M + H]+	S	9.59
A88	Hydroxybupropion	C13H18ClNO2	92264-81-8	Pharmaceutical metabolite	Psychoactive	[M + H]+	S	7.88
A89	Hydroxytestosterone	C19H28O3	2141-17-5	Pharmaceutical metabolite	Hormone	[M + H]+	S	13.47
A90	Ibuprofen	C13H18O2	15687-27-1	Pharmaceutical	Anti-inflammatory	[M + H]+	S	9.83
A91	Icaridin	C12H23NO3	119515-38-7	Pesticide	Insect repellent	[M + H]+	S	11.94
A92	Imazapic	C14H17N3O3	104098-48-8	Pesticide	Herbicide	[M + H]+	S	0.88
A93	Imazethapyr	C15H19N3O3	81335-77-5	Pesticide	Herbicide	[M + H]+	S	9.27
A94	Imidacloprid	C9H10ClN5O2	138261-41-3	Pesticide	Insecticide	[M + H]+	S	6.76
A95	Isoniazid	C6H7N3O	54-85-3	Pharmaceutical	Antibiotic	[M + H]+	S	0.89
A96	Isouron	C10H17N3O2	55861-78-4	Pesticide	Herbicide	[M + H]+	S	0.88
A97	Levetiracetam	C8H14N2O2	102767-28-2	Pharmaceutical	Psychoactive	[M + H]+	S	0.87
A98	Lincomycin	C18H34N2O6S	154-21-2	Pharmaceutical	Antibiotic	[M + H]+	S	9.04
A99	Linoleic acid	C18H32O2	60-33-3	Pesticide	Formicide	[M + H]+	S	15.21
A100	L-Lysine	C6H14N2O2	56-87-1	Pharmaceutical	Amino acid	[M + H]+	S	0.88
A101	Losartan	C22H23ClN6O	114798-26-4	Pharmaceutical	Anti hypertensive	[M + H]+	C	11.61
A102	Megestrol acetate	595-33-5	C24H32O4	Pharmaceutical	Hormone	[M + H]+	S	9.05
A103	Memantine	C12H21N	19982-08-2	Pharmaceutical	Alzheimer's treatment	[M + H]+	S	12.19
A104	Methoxyfenozide	C22H28N2O3	161050-58-4	Pesticide	Insecticide	[M + H]+	S	10.32
A105	Methyl *p*-hydroxybenzoate	C8H8O3	99-76-3	Antioxidant	Antioxidant	[M + H]+	S	6.42
A106	Methylprednisolone	C22H30O5	83-43-2	Pharmaceutical	Antiallergic and anty-inflamatory	[M + H]+	S	13.46
A107	Metominostrobin 2	C16H16N2O3	133408-50-1	Pesticide	Fungicide	[M + H]+	S	11.64
A108	Molinate	C9H17NOS	2212-67-1	Pesticide	Herbicide	[M + H]+	S	10.86
A109	Myclobutanil	C15H17ClN4	88671-89-0	Pesticide	Fungicide	[M + H]+	S	12.94
A110	Naftidrofuryl	C24H33NO3	31329-57-4	Pharmaceutical	Vasodilator	[M + H]+	S	0.88
A111	Naphthalene acetamide	C12H11NO	86-86-2	Pesticide	Growth regulator	[M + H]+	S	10
A112	Nicotine	C10H14N2	54-11-5	Pesticide	Insecticide	[M + H]+	S	0.88

Nº	Compound	Formula	CAS	Class	Subclass	Ionization Mode	Confirmed (C) or Suspected (S)	RT (min)
A113	Nikethamide	C10H14N2O	59-26-7	Illicit drug	Anti doping respiratory,illicit drug	[M + H]+	S	0.88
A114	Nimesulide	C13H12N2O5S	51803-78-2	Pharmaceutical	Anti-inflammatory	[M - H]-	C	11.63
A115	*N*-Methyl pregabalin	C9H19NO2	1155843-61-0	Pharmaceutical	Psychoactive	[M + H]+	S	10.47
A116	Norcocaine	C16H19NO4	18717-72-1	Illicit drug metabolite (cocaine)	Illicit drug	[M + H]+	S	6.6
A117	Norethisterone	C20H26O2	68-22-4	Pharmaceutical	Hormone	[M + H]+	S	14.07
A118	Octhilinone	C11H19NOS	25339-53-1	Pesticide	Fungicide	[M + H]+	S	13.36
A119	Oxprenolol	C15H23NO3	6452-71-7	Pharmaceutical	Antihypertensive	[M + H]+	S	0.87
A120	Oxyfluorfen	C15H11ClF3NO4	42874-03-3	Pesticide	Herbicide	[M + H]+	S	13.99
A121	Oxyphencyclimine	C20H28N2O3	125-53-1	Pharmaceutical	Anticholinergic	[M + H]+	S	0.88
A122	Paracetamol	C8H9NO2	103-90-2	Pharmaceutical	Analgesic	[M + H]+	C	3.62
A123	Phenmedipham	C16H16N2O4	13684-63-4	Pesticide	Herbicide	[M + H]+	S	8.36
A124	Phenol	C6H6O	108-95-2	Pharmaceutical	Antiseptic	[M + H]+	S	8.75
A125	*p*-Hydroxybenzoic acid	C7H6O3	99-96-7	Antioxidant	Antimicrobial preservative	[M + H]+	S	6.21
A126	Picoxystrobin	C18H16F3NO4	117428-22-5	Pesticide	Fungicide	[M + H]+	S	13.64
A127	Propazine	C9H16N5Cl	139-40-2	Pesticide	Herbicide	[M + H]+	C	12.11
A128	Propiconazole	C15H17Cl2N3O2	60207-90-1	Pesticide	Fungicide	[M + H]+	S	14.01
A129	Propranolol	C16H21NO2	525-66-6	Pharmaceutical	Antihypertensive	[M + H]+	C	9.42
A130	Prothioconazole-desthio	C14H15Cl2N3O	120983-64-4	Pesticide metabolite	Fungicide	[M + H]+	S	13.38
A131	Pseudoephedrine	C10H15NO	90-82-4	Pharmaceutical	Nasal decongestant	[M + H]+	S	11.12
A132	Pyraclostrobin	C19H18ClN3O4	175013-18-0	Pesticide	Fungicide	[M + H]+	S	14.1
A133	Pyrethrin 1	C21H28O3	121-21-1	Pesticide	Insecticide	[M + H]+	S	14.23
A134	Pyrethrin 2	C22H28O5	121-29-9	Pesticide	Insecticide	[M + H]+	S	13.45
A135	Pyridoxine	C8H11NO3	65-23-6	Pharmaceutical	Vitamin	[M + H]+	S	0.89
A136	Saflufenacil	C17H17ClF4N4O5S	372137-35-4	Pesticide	Herbicide	[M + H]+	S	11.88
A137	Sebuthylazin	C9H16ClN5	7286-69-3	Pesticide	Herbicide	[M + H]+	S	12.34
A138	Sertralina	C17H17Cl2N	79617-96-2	Pharmaceutical	Psychoactive	[M + H]+	S	11.95
A139	Simazine	C7H12ClN5	122-34-9	Pesticide	Herbicide	[M + H]+	C	9.65
A140	Spirodiclofen	C21H24Cl2O4	148477-71-8	Pesticide	Acaricide, insecticide	[M + H]+	S	16.89
A141	Tebuconazole	C16H22ClN3O	80443-41-0	Pesticide	Fungicide	[M + H]+	C	13.93
A142	Terbuthylazine	C9H16ClN5	5915-41-3	Pesticide	Herbicide	[M + H]+	S	12.35
A143	Theobromine	C7H8N4O2	83-67-0	Pharmaceutical	Vasodilator,diuretic, and heart stimulant	[M + H]+	S	4.59
A144	Thioridazine	C21H26N2S2	50-52-2	Pharmaceutical	Psychoactive	[M + H]+	S	11.16
A145	Tibolone	C21H28O2	5630-53-5	Pharmaceutical	Hormone	[M + H]+	S	14.69
A146	Tramadol-*n,n*-Didesmethyl	C15H23NO2	73806-55-0	Pharmaceutical metabolite	Analgesic	[M + H]+	S	15.24
A147	Trifloxystrobin	C20H19F3N2O4	141517-21-7	Pesticide	Fungicide	[M + H]+	S	14.66
A148	Triphenylphosphate	C18H15O4P	115-86-6	Plasticizer	Plasticizer	[M + H]+	S	14.06
A149	Uniconazole	C15H18ClN3O	83657-22-1	Pesticide	Growth control	[M + H]+	S	12.75
A150	Venlafaxine	C17H27NO2	93413-69-5	Pharmaceutical	Psychoactive	[M + H]+	S	11.63

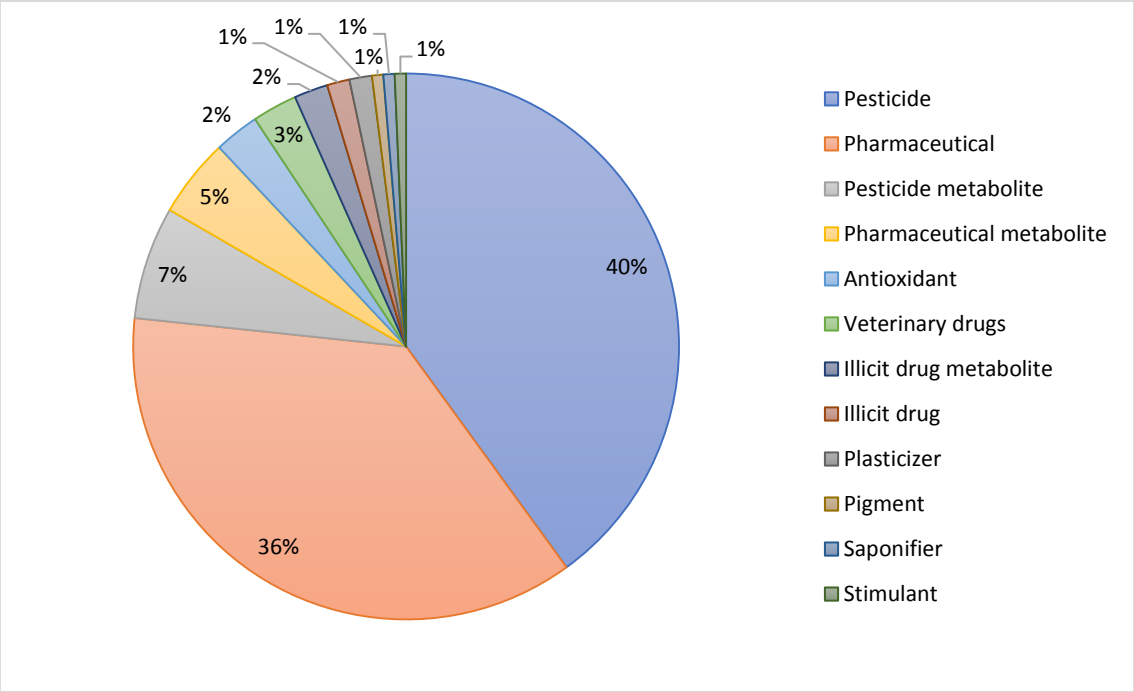


Figure S2. Classification of identified microcontaminants.

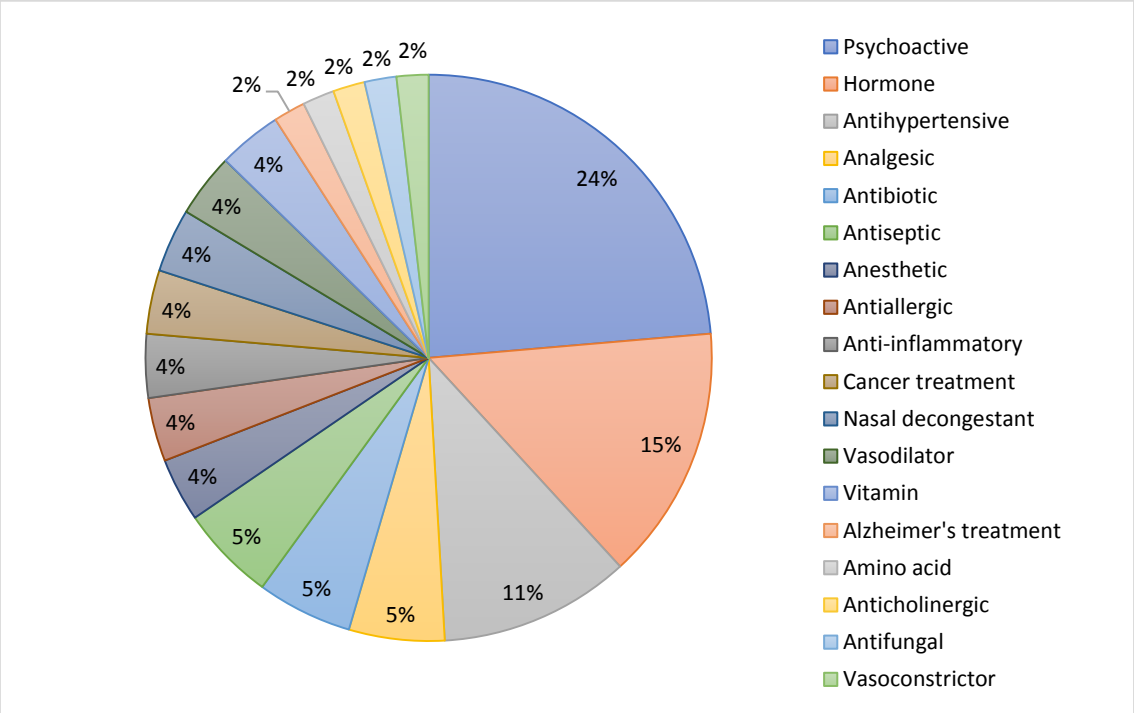


Figure S3. Subclassification of identified pharmaceuticals.

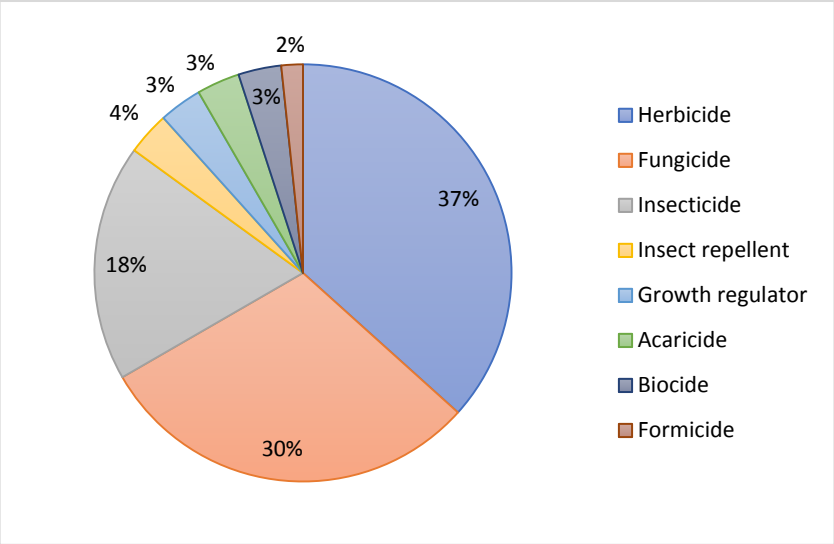


Figure S4. Subclassification of identified pesticides.

Table S5. Identified microcontaminants by LC-QTOF MS analysis in surface waters evaluated.

Nº	Compound	Screening Results																												
		Sampling Point 1									Sampling Point 2									Sampling Point 3									Blank
		Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						
		1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
A1	19-Nortestosterone		x																	x									
A2	2,4-D	x	x	x							x	x	x							x	x	x		x		x			
A3	2-aminoflubendazole													x									x	x				x	
A4	2-aminonicotinic acid	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A5	2-hydroxy-atrazine	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A6	2-hydroxyquinoline						x						x								x								
A7	2-phenethylamine					x																							
A8	3-phenyl-2-propenal	x		x						x																			
A9	4-acetamidoantipyrine			x	x	x	x	x	x		x			x	x	x	x	x	x	x	x	x	x	x	x	x		x	
A10	4-methylamino-antipyrine		x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x			x	x		x			x	
A11	8-hydroxyquinoline						x								x	x		x			x		x		x				
A12	Acebutolol	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x		x	x	
A13	Acequinocyl		x																					x				x	
A14	Acetanilide																							x					
A15	Adrenalone														x						x								
A16	Alpha-PVP	x	x	x	x	x	x	x			x	x	x	x		x		x		x	x	x	x	x	x	x			
A17	L-glutamic acid																				x								
A18	L-tryptophan		x	x	x	x	x				x	x	x	x	x						x	x	x		x			x	
A19	Aminorex													x															
A20	Amprolium																					x							
A21	Anabasine		x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x			x	x	
A22	Antipyrine / Phenazone					x																							
A23	Apophedrin			x	x		x						x	x									x	x					
A24	Atenolol	x	x	x	x	x	x			x	x		x	x	x	x				x	x	x	x	x	x	x			
A25	Atrazine	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A26	Azoxystrobin	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A27	Azoxystrobin acid	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	
A28	BAC 12		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x		x	x	x	x	x	x	x	x	x	
A29	Benzisothiazolone	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	
A30	Benzocaine																				x								x
A31	Benzododecinium		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x		x	x	x	x	x	x	x	x	x	
A32	Benzovindiflupyr				x		x	x																					
A33	Bethanidine		x	x				x	x	x	x					x	x		x	x				x	x	x	x	x	
A34	Bis(2-ethylhexyl) adipate																				x		x						
A35	Butoxycaine																			x									
A36	Caffeine	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A37	Carbamate ethyl-*N*-(3-hydroxyphenyl)-												x		x														
A38	Carbamazepine	x		x		x		x	x																				
A39	Carbendazim	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A40	Carbofuran	x		x							x									x									
A41	CGA 321113 (Trifloxystrobin metabolite)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A42	Chlorantraniliprole		x	x	x	x	x	x	x	x		x	x	x	x	x		x	x			x		x	x	x	x	x	
A43	Chlorfenvinphos						x																						
A44	Chlorimuronethyl	x	x	x							x	x	x							x	x	x							
A45	Cholecalciferol																			x									
A46	Cortisol/Hydrocortisone			x	x	x													x			x			x			x	
A47	Cortisone			x			x																						

Nº	Compound	Screening Results (continue...)																										
		Sampling Point 1									Sampling Point 2									Sampling Point 3									Blank
		Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						
		1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
A48	Cyanazine				x	x				x									x									x	
A49	Cyproconazole		x			x	x	x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x	x	x	x	
A50	DEET (Diethyltoluamide)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A51	Deethylatrazine (DEA)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A52	Deisopropylatrazine (DIA)	x	x	x			x				x	x	x							x	x	x							
A53	Desvenlafaxine						x																						
A54	Diazepam				x						x									x									
A55	Diclosulam		x	x								x	x								x	x							
A56	Difenoconazole								x						x			x	x					x			x	
A57	Dihydroquinoline-2-2-4-trimethyl-1-2-					x				x	x							x	x	x		x	x	x					
A58	Dimethylanilin			x	x		x											x						x				x	
A59	Dinoterb																			x									x
A60	Diphenylamine		x	x	x						x		x							x		x	x	x					x
A61	Diuron	x																											
A62	Divalproate	x			x	x					x	x					x			x	x				x				
A63	Dodecylbenzenesulphonic acid	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x
A64	Ecgonine methyl ester																				x								
A65	Embutramide																			x									
A66	Estradiol	x																											
A67	Ethinylestradiol															x													
A68	Ethoxyquin												x								x								
A69	Eugenol						x																						
A70	Exemestane										x																		
A71	Fenpropimorph carboxylic acid								x	x							x	x	x							x	x		
A72	Fexofenadine								x																				
A73	Fipronil	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A74	Fipronil desulfinyl		x	x	x	x	x	x	x	x		x	x	x	x	x		x	x		x	x	x	x	x	x	x		
A75	Fipronil sulfide				x	x	x		x						x	x			x					x			x		
A76	Fipronilsulfone	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A77	Fluazifop						x																						
A78	Flubendiamide	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
A79	Fluconazole								x																				
A80	Flumioxazin													x							x		x						
A81	Flutamide	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
A82	Flutriafol				x			x	x	x	x		x	x				x	x			x	x	x	x			x	
A83	Fluxapyroxad		x	x	x	x	x	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A84	Furmecyclox																						x						
A85	Gabapentin	x																											
A86	Haloxyfop																								x				
A87	HMMA							x											x							x			
A88	Hydroxybupropion							x	x																	x			
A89	Hydroxytestosterone															x									x				
A90	Ibuprofen	x		x	x	x	x	x	x	x		x	x	x	x	x	x	x			x	x	x	x	x				
A91	Icaridin	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
A92	Imazapic		x																x										
A93	Imazethapyr	x	x	x	x						x	x	x	x						x	x	x							
A94	Imidacloprid	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	
A95	Isoniazid		x	x		x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	
A96	Isouron		x		x	x		x	x	x							x	x	x			x	x	x	x	x	x	x	

Nº	Compound	Screening Results (continue...)																												
		Sampling Point 1									Sampling Point 2									Sampling Point 3									Blank
		Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						
		1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
A97	Levetiracetam	x	x	x	x	x		x	x	x		x	x	x		x	x	x	x	x		x	x		x	x	x	x		
A98	Lincomycin													x		x														
A99	Linoleic acid	x		x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x		x	x	x		x	x		
A100	L-Lysine					x														x										
A101	Losartan			x			x												x											
A102	Megestrol acetate										x				x					x					x					
A103	Memantine		x	x		x			x	x		x	x		x		x		x	x	x		x	x	x	x	x	x		x
A104	Methoxyfenozide			x			x			x					x										x	x				
A105	Methyl *p*-hydroxybenzoate									x																x				
A106	Methylprednisolone	x				x	x	x	x	x	x					x	x	x	x					x			x	x		
A107	Metominostrobin 2						x																							
A108	Molinate			x	x		x	x	x	x	x			x	x	x	x	x	x		x	x		x	x	x	x			
A109	Myclobutanil		x																											
A110	Naftidrofuryl					x																x						x		
A111	Naphthalene acetamide														x															
A112	Nicotine	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
A113	Nikethamide					x	x										x	x					x							
A114	Nimesulide	x				x					x									x										
A115	*N*-Methyl pregabalin	x																												
A116	Norcocaine		x									x		x						x										
A117	Norethisterone	x										x		x														x		
A118	Octhilinone					x																				x				
A119	Oxprenolol																									x				
A120	Oxyfluorfen																								x					
A121	Oxyphencyclimine				x		x													x		x	x	x						
A122	Paracetamol			x	x	x					x			x	x	x					x		x				x			
A123	Phenmedipham																									x				
A124	Phenol																			x										
A125	*p*-Hydroxybenzoic acid					x	x						x	x	x				x			x							x	
A126	Picoxystrobin			x	x	x	x	x	x	x				x	x	x	x	x	x	x			x	x	x	x	x			
A127	Propazine		x	x								x									x									
A128	Propiconazole	x	x	x	x	x	x	x			x	x	x	x		x				x	x	x	x	x	x	x				
A129	Propranolol	x	x	x	x	x	x	x	x	x	x	x		x		x	x	x	x		x	x		x		x		x		
A130	Prothioconazole-desthio				x	x	x	x	x	x				x	x	x	x	x	x					x	x	x	x	x		
A131	Pseudoephedrine																		x									x		
A132	Pyraclostrobin																							x						
A133	Pyrethrin 1																						x							
A134	Pyrethrin 2						x	x							x															
A135	Pyridoxine					x				x			x																	
A136	Saflufenacil	x		x									x							x		x								
A137	Sebuthylazin	x		x					x		x				x				x		x							x		
A138	Sertralina																					x								
A139	Simazine	x	x	x							x	x	x							x	x	x								
A140	Spirodiclofen					x																								
A141	Tebuconazole		x			x	x			x					x		x								x	x				
A142	Terbuthylazine	x	x	x					x		x	x			x				x		x						x			
A143	Theobromine																					x								
A144	Thioridazine								x																					
A145	Tibolone																x													

Nº	Compound	Screening Results (continue...)																												
		Sampling Point 1									Sampling Point 2									Sampling Point 3										Blank
		Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2						Sampling Campaign 1			Sampling Campaign 2							
		1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
A146	Tramadol-*n*,*n*-Didesmethyl													x															
A147	Trifloxystrobin				x		x	x						x	x	x	x							x	x				
A148	Triphenylphosphate	x	x	x		x	x	x	x	x		x	x	x		x		x	x	x	x		x	x	x		x	x	
A149	Uniconazole		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	
A150	Venlafaxine																									x			
Total		47	55	64	54	62	64	46	54	52	52	47	54	56	51	52	45	49	54	56	56	57	54	58	53	49	47	48	7
Average of compounds identified bysample point		53																											

Section SVII. *In silico* predictions for identified microcontaminants – (Q)SAR data.

Table S6. (Q)SAR results for identified microcontaminants.

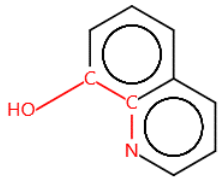
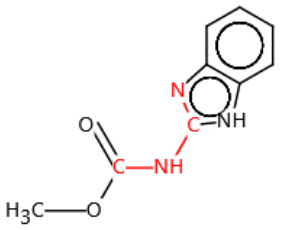
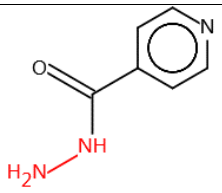
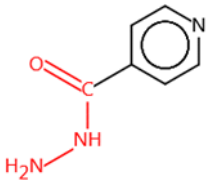
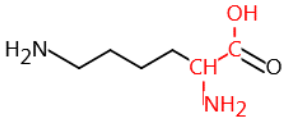
Nº	Compound	Mobility (log Koc)	Half-life (days)	Biodegradability (Bowin 5)	STP Total removal (%)	PBT	Estrogen Receptor Binding	Mutagenicity	Carcinogenicity
A1	19-Nortestosterone	3.15	37.5	0.375	3.49	0.395	15	0.05	0.8
A2	2,4-D	1.47	37.5	0.551	4.36	0.185	0	0	0
A3	2-aminoflubendazole	3.63	60.0	0.133	2.52	0.405	0	0.5	0.65
A4	2-aminonicotinic acid	1	37.5	0.546	1.87	0.314	0	0	0.5
A5	2-hydroxy-atrazine	2.98	15.0	0.324	1.85	0.296	0	0	0.4
A6	2-hydroxyquinoline	1.84	15.0	0.345	1.92	0.22	0	0.1	0.7
A7	2-phenethylamine	2.7	15.0	0.401	1.96	0.273	0	0	0.375
A8	3-phenyl-2-propenal	1.57	15.0	0.569	2.36	0.311	0	0	0.025
A9	4-acetamidoantipyrine	1.82	37.5	0.319	1.85	0.351	0	0.35	0.75
A10	4-methylamino-antipyrine	1.89	15.0	0.317	1.86	0.406	0	0.1	0.65
A11	8-hydroxyquinoline	3.31	15.0	0.366	2.16	0.265	5	1	0.25
A12	Acebutolol	1.4	37.5	0.438	2.05	0.402	0	0	0.5
A13	Acequinocyl	5.2	37.5	0.644	93.9	0.448	0	0.15	0.75
A14	Acetanilide	1.55	15.0	0.413	1.9	0.193	0	0	0.625
A15	Adrenalone	2.06	15.0	0.446	1.86	0.208	10	0	0.5
A16	Alpha-PVP	3.17	37.5	0.154	26.1	0.354	0	0	0.475
A17	L-glutamic acid	1.13	8.7	0.924	1.85	0.188	0	0	0.325
A18	L-tryptophan	2.51	15.0	0.496	1.85	0.273	0	0	0
A19	Aminorex	2.57	15.0	0.185	1.9	0.29	0	0.1	0.425
A20	Amprolium	1.12	15.0	0.116	1.85	0.411	0	OD*	OD*
A21	Anabasine	2.98	37.5	0.202	1.88	0.211	0	0	0.575
A22	Antipyrine	2.12	15.0	0.334	1.86	0.301	0	0	1
A23	Apophedrin	1.59	15.0	0.355	1.85	0.206	0	0	0.4
A24	Atenolol	1.83	37.5	0.394	1.85	0.316	0	0	0.6
A25	Atrazine	2.35	60.0	0.235	3.45	0.332	0	0	1
A26	Azoxystrobin	3.45	60.0	0.408	3.1	0.508	0	0	0.5
A27	Azoxystrobin acid	2.84	37.5	0.474	1.92	0.456	0	0	0.55
A28	BAC 12	5.43	15.0	0.392	5.14	0.413	0	OD*	OD*
A29	Benzisothiazolone	1.54	15.0	0.318	1.86	0.372	0	0.05	0.45
A30	Benzocaine	1.77	15.0	0.466	2.14	0.286	5	0.1	0.7
A31	Benzododecinium	5.29	15.0	0.447	75.4	0.413	0	0	0.425
A32	Benzovindiflupyr	4.33	60.0	-0.218	41	0.49	0	0.15	0.7
A33	Bethanidine	3.07	15.0	0.384	1.99	0.206	0	0.15	0.5
A34	Bis(2-ethylhexyl) adipate	4.56	8.7	0.821	94	0.243	0	0	0.5
A35	Butoxycaine	3.31	37.5	0.534	32.9	0.294	0	0	0.4
A36	Caffeine	1	15.0	0.368	1.85	0.308	0	0	0
A37	Carbamate Ethyl-*N*-(3-hydroxyphenyl)-	2.26	15.0	0.177	1.95	0.285	10	0.15	0.475
A38	Carbamazepine	3.12	37.5	0.173	2.96	0.432	0	0.15	0.725
A39	Carbendazim	2.58	37.5	0.0976	1.98	0.198	0	1	0.575
A40	Carbofuran	1.98	37.5	0.117	2.68	0.331	0	0	0.575
A41	CGA 321113 (Trifloxystrobin metabolite)	5.87	60.0	0.523	93.2	0.364	0	0	0.55
A42	Chlorantraniliprole	2.69	180.0	-0.113	4.1	0.355	0	0.05	0.6
A43	Chlorfenvinphos	3.1	60.0	-0.0262	22.2	0.47	0	0	0.625
A44	Chlorimuronethyl	1.86	60.0	0.281	9.22	0.202	0	0	0.5
A45	Cholecalciferol	1.38	37.5	0.183	94	0.385	15	0	0.475
A46	Cortisol/Hydrocortisone	2.05	60.0	0.226	2.01	0.33	15	0.05	0.7
A47	Cortisone	2.05	60.0	0.136	1.96	0.329	0	0.2	0.85
A48	Cyanazine	2.13	180.0	0.169	2.51	0.327	0	0.1	0.375
A49	Cyproconazole	3.08	60.0	-0.0259	4.92	0.375	0	0	0.675

Nº	Compound	Mobility (log Koc)	Half-life (days)	Biodegradability (Bowin 5)	STP Total removal (%)	PBT	Estrogen Receptor Binding	Mutagenicity	Carcinogenicity
A50	DEET (Diethyltoluamide)	2.05	37.5	0.423	2.45	0.158	0	0	0.375
A51	Deethylatrazine (DEA)	2.02	60.0	0.214	1.98	0.31	0	0	0.55
A52	Deisopropylatrazine (DIA)	1.84	60.0	0.287	1.9	0.295	0	0	0.55
A53	Desvenlafaxine	3.24	60.0	-0.0658	3.9	0.439	15	0	0.625
A54	Diazepam	3.88	37.5	0.153	4.42	0.475	0	0	0
A55	Diclosulam	2.46	180.0	0.0381	13.3	0.391	0	0	0.5
A56	Difenoconazole	3.77	180.0	-0.134	45.3	0.585	0	0	0.575
A57	Dihydroquinoline-2-2-4-trimethyl-1-2-	3.04	37.5	0.149	9.37	0.343	0	0	0.8
A58	Dimethylanilin	1.9	37.5	0.274	5.51	0.258	0	0	1
A59	Dinoterb	3.51	60.0	-0.166	16.6	0.376	0	0.15	0.6
A60	Diphenylamine	2.92	37.5	0.164	13.2	0.358	0	0	0.425
A61	Diuron	2.04	37.5	0.19	3.73	0.311	0	0.75	0.2
A62	Divalproate	1.43	15.0	0.76	4.24	0.232	0	0	0.75
A63	Dodecylbenzenesulphonic acid	4.1	15.0	0.502	69.5	0.354	0	0	0.4
A64	Ecgonine methyl ester	1	15.0	0.591	1.85	0.277	0	0	0.475
A65	Embutramide	2.63	37.5	0.493	5.29	0.261	0	0	0.625
A66	Estradiol	4.19	37.5	0.212	30.5	0.398	20	0	0.95
A67	Ethinylestradiol	4.65	60.0	-0.0902	17.5	0.403	15	0	0.825
A68	Ethoxyquin	3.1	37.5	0.226	24.5	0.245	0	0	0.075
A69	Eugenol	2.83	15.0	0.465	3.18	0.248	5	0	0.225
A70	Exemestane	3.74	60.0	0.093	5.29	0.33	0	0.05	0.8
A71	Fenpropimorph carboxylic acid	3.29	37.5	0.276	1.87	0.346	0	0	0.575
A72	Fexofenadine	4.94	60.0	-0.099	4.36	0.47	0	0	0.475
A73	Fipronil	3.77	180.0	0.0167	30.1	0.544	0	0.1	0.575
A74	Fipronil desulfinyl	4.48	180.0	0.0925	41	0.502	0	0.15	0.65
A75	Fipronil sulfide	4.73	180.0	0.0419	71.2	0.552	0	0.15	0.65
A76	Fipronilsulfone	3.82	180.0	-0.0086	51.8	0.498	0	0.1	0.675
A77	Fluazifop	2.91	60.0	0.649	6.64	0.414	0	0	0.725
A78	Flubendiamide	4.2	180.0	-0.673	39.9	0.389	0	0.05	0.6
A79	Fluconazole	3.59	180.0	0.115	1.86	0.462	0	0.15	0.825
A80	Flumioxazin	2.96	60.0	0.258	3.28	0.468	0	0.2	0.625
A81	Flutamide	3.06	60.0	0.139	10	0.439	0	0.25	0.725
A82	Flutriafol	3.6	180.0	0.0981	2.62	0.503	0	0	0.625
A83	Fluxapyroxad	4.54	180.0	0.17	12.4	0.547	0	0.05	0.575
A84	Furmecyclox	2.63	37.5	0.293	49.6	0.406	0	0	0.6
A85	Gabapentin	1.73	15.0	0.585	1.85	0.202	0	0	1
A86	Haloxyfop	3.13	180.0	0.555	10.6	0.34	0	0	0.65
A87	HMMA	2.87	37.5	0.432	2	0.236	10	0.05	0.5
A88	Hydroxybupropion	1.82	37.5	0.244	2.82	0.375	0	0.15	0.525
A89	Hydroxytestosterone	2.24	37.5	0.369	2.4	0.36	0	0.05	0.8
A90	Ibuprofen	2.63	15.0	0.499	28.7	0.222	0	0	0.225
A91	Icaridin	1.9	15.0	0.288	3.31	0.406	0	0.25	0.425
A92	Imazapic	2.25	60.0	0.429	3.01	0.435	0	0.15	0.65
A93	Imazethapyr	2.53	60.0	0.466	3.41	0.248	0	0	0.65
A94	Imidacloprid	2.99	60.0	0.159	1.86	0.457	0	0.15	0.55
A95	Isoniazid	1	37.5	0.0034	1.85	0.351	0	1	0.75
A96	Isouron	2.19	37.5	0.301	2.23	0.398	0	0	0.4
A97	Levetiracetam	1.07	37.5	0.388	1.85	0.193	0	0.15	0.425
A98	Lincomycin	1.84	37.5	0.47	1.85	0.31	0	0	0.575
A99	Linoleic acid	4.07	15.0	0.751	93.9	0.318	0	0	0.475
A100	L-Lysine	1	8.7	0.644	1.85	0.194	0	1	0.325
A101	Losartan	5.15	37.5	0.159	30.5	0.389	0	0.15	0.45
A102	Megestrol acetate	3.85	60.0	0.217	30.1	0.373	0	0.05	0.7
A103	Memantine	2.82	60.0	0.0671	9.63	0.287	0	0	0.35
A104	Methoxyfenozide	3.04	60.0	-0.208	18.4	0.337	0	0.25	0.625

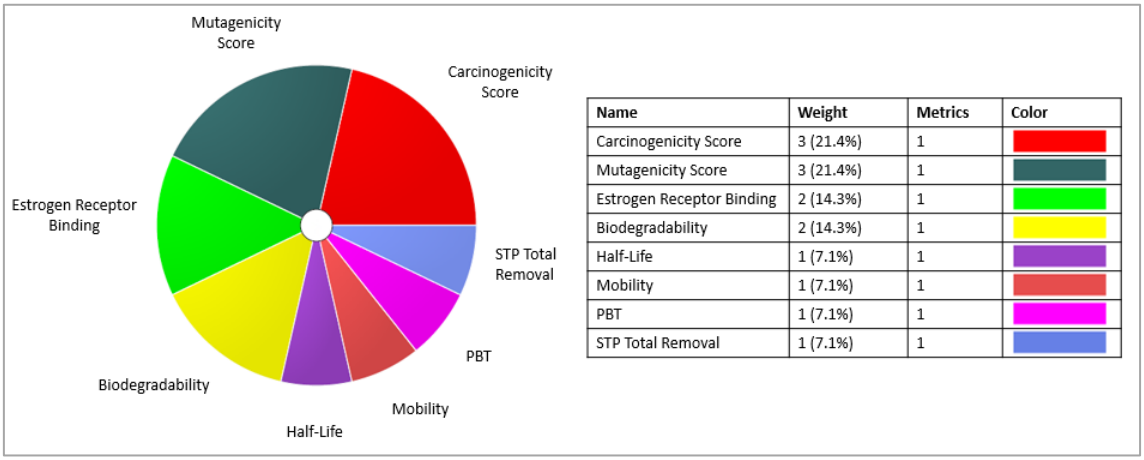
Nº	Compound	Mobility (log Koc)	Half-life (days)	Biodegradability (Bowin 5)	STP Total removal (%)	PBT	Estrogen Receptor Binding	Mutagenicity	Carcinogenicity
A105	Methyl *p*-hydroxybenzoate	1.94	15.0	0.626	2.21	0.209	5	0	0.45
A106	Methylprednisolone	2.26	60.0	0.2	2.11	0.275	15	0.05	0.7
A107	Metominostrobin 2	4.03	37.5	0.3	2.68	0.356	0	0.05	0.725
A108	Molinate	2.26	37.5	0.212	8.13	0.357	0	0	0.55
A109	Myclobutanil	3.78	37.5	0.113	5.21	0.444	0	0	0.625
A110	Naftidrofuryl	4.94	60.0	0.222	86.4	0.241	0	0.05	0.525
A111	Naphthalene acetamide	3.1	37.5	0.322	2.06	0.219	0	1	0.625
A112	Nicotine	2.72	37.5	0.0731	1.91	0.256	0	0	0.3
A113	Nikethamide	1.54	37.5	0.427	1.86	0.267	0	0	0.5
A114	Nimesulide	2.75	37.5	0.0021	3.41	0.492	0	0.5	0.775
A115	*N*-Methyl pregabalin	1.45	15.0	0.647	1.85	0.272	0	0	0.55
A116	Norcocaine	2.89	15.0	0.661	2.21	0.338	0	0	0.575
A117	Norethisterone	3.62	60.0	0.0728	5.44	0.503	0	0	0.85
A118	Octhilinone	2.93	15.0	0.425	2.96	0.39	0	0	0.675
A119	Oxprenolol	2.26	37.5	0.428	2.35	0.246	0	0	0.225
A120	Oxyfluorfen	4.6	180.0	0.149	67.4	0.65	0	0.475	0.7
A121	Oxyphencyclimine	3.66	37.5	0.311	13.5	0.338	0	0	0.75
A122	Paracetamol	1.65	15.0	0.426	1.86	0.214	5	0	0.525
A123	Phenmedipham	3.41	37.5	-0.262	15.3	0.434	0	1	0.55
A124	Phenol	2.27	15.0	0.446	1.98	0.234	5	0	0
A125	*p*-Hydroxybenzoic acid	1.33	15.0	0.721	2	0.193	5	0	0.25
A126	Picoxystrobin	3.89	180.0	0.536	17.5	0.368	0	0	0.575
A127	Propazine	2.54	60.0	0.163	5.14	0.374	0	0	0.55
A128	Propiconazole	3.19	60.0	-0.0643	19.1	0.496	0	0	0.625
A129	Propranolol	2.95	15.0	0.35	12.6	0.309	0	0	0.55
A130	Prothioconazole-desthio	3.04	180.0	-0.117	6.14	0.447	0	0.05	0.7
A131	Pseudoephedrine	1.86	15.0	0.301	1.9	0.212	0	0	0.275
A132	Pyraclostrobin	4.68	60.0	0.0952	29.6	0.591	0	0.3	0.7
A133	Pyrethrin 1	4.01	37.5	0.427	91.7	0.375	0	0.25	0.8
A134	Pyrethrin 2	3.47	37.5	0.589	45.3	0.356	0	0.15	0.825
A135	Pyridoxine	1	15.0	0.651	1.85	0.215	0	0	0.65
A136	Saflufenacil	2.14	180.0	0.034	1.97	0.419	0	0.15	0.6
A137	Sebuthylazin	2.63	60.0	0.239	3.45	0.369	0	0.1	0.675
A138	Sertralina	5.23	60.0	-0.211	85	0.651	0	0.15	0.575
A139	Simazine	2.17	60.0	0.308	2.45	0.364	0	0	0.55
A140	Spirodiclofen	4.63	180.0	0.268	91.1	0.405	0	0	0.55
A141	Tebuconazole	3.19	60.0	-0.0104	18.4	0.522	0	0	0.7
A142	Terbuthylazine	2.5	60.0	0.198	11	0.33	0	0.1	0.675
A143	Theobromine	1	15.0	0.351	1.85	0.303	0	0	0.375
A144	Thioridazine	5.09	60.0	-0.174	91.7	0.537	0	0	0.475
A145	Tibolone	3.66	60.0	0.0964	8.47	0.392	0	0	0.725
A146	Tramadol-*n*,*n*-didesmethyl	2.71	37.5	0.277	4.31	0.338	0	0	0.525
A147	Trifloxystrobin	6.48	60.0	0.456	56	0.383	0	0.15	0.55
A148	Triphenylphosphate	4.03	37.5	-0.0331	60.7	0.384	0	0	0.375
A149	Uniconazole	2.97	37.5	0.122	17.6	0.495	0	0	0.6
A150	Venlafaxine	2.94	60.0	-0.0053	7.8	0.428	0	0	0.625

* OD (out of domain) – The method used was not applicable to this molecule.

Table S7. *In vivo* mutagenicity (Micronucleus) alerts by ISS for identified microcontaminants.

Compound	Alert	Structure
8-Hydroxyquinoline	H-acceptor-path3-H-acceptor	
Carbendazim	H-acceptor-path3-H-acceptor	
Diuron	No alert found	
Isoniazid	Hydrazines	
	H-acceptor-path3-H-acceptor	
L-lisine	H-acceptor-path3-H-acceptor	
Naphthalene acetamide	No alert found	
Phenmedipham	No alert found	

Section SVIII. Ranking by multi-criteria decision-making techniques: ToxPi *vs*. TOPSIS



Name	Weight	Metrics	Color
Carcinogenicity Score	3 (21.4%)	1	
Mutagenicity Score	3 (21.4%)	1	
Estrogen Receptor Binding	2 (14.3%)	1	
Biodegradability	2 (14.3%)	1	
Half-Life	1 (7.1%)	1	
Mobility	1 (7.1%)	1	
PBT	1 (7.1%)	1	
STP Total Removal	1 (7.1%)	1	

Figure S5. SMART model: endpoints and weights.

Table S8. Statistical test results of dispersion using Standard Deviation from mean for TOPSIS ranking in relation to ToxPi linear ranking (standard deviation from mean = 0.95)

Ranking range TOPSIS	Standard Deviation - Mean
1-10	3.5
11-20	2.6
21-30	5.3
31-40	6.4
41-50	5.9
51-60	6.9
61-70	10.6
71-80	8.7
81-90	6.4
91-100	9.0
101-110	7.7
111-120	10.0
121-130	7.3
131-140	13.6
141-150	1.7

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GENERAL CONCLUSIONS

For centuries, humans have been using all the available natural resources, without any concern about environment protection and preservation. It is now necessary to develop new approaches to identify contamination, understand the effects of the presence of contaminants in nature, and to develop new approaches for the recuperation of contaminated environments. All the studies developed during the present doctoral work had the aims of testing, expanding, and deepening information regarding contamination of aquatic environments, supported by analytical screening analysis based on LC-QTOF MS. A search was made for new strategies to improve the detection and identification of contaminants, as well as prediction of the risks posed by these substances to humans and the environment, using different freely available software tools.

The first study used a purpose-built database to investigate the presence of metabolites and pharmaceuticals in RHW samples analyzed by LC-HRMS. The strategy resulted in the identification of 31 metabolites and 43 pharmaceuticals in the samples, with antibiotics being the most detected class. Application of the common fragmentation approach enabled the identification of four additional metabolites not present in the purpose-built database. These results demonstrated the importance of using wide-scope screening, including both pharmaceuticals and metabolites, for comprehensive evaluation of contamination caused by wastewaters. The risk assessment of pharmaceuticals and their metabolites based only on predicted environmental concentration (PEC), predicted no effect concentration (PNEC), and risk quotient (RQ) (PEC/PNEC) might underestimate the risks to the environment and humans. There were some cases where low or medium RQ values were obtained, but the compounds were predicted to be mutagenic and carcinogenic. The inclusion of *in silico* (Q)SAR predictions allowed assessment of the environmental fates and effects of such compounds, in terms of biodegradability, possible PBT, and potential hazard to the aquatic environment. The approach proposed in this study enables a more proactive prioritization of pharmaceutical and metabolite compounds in complex matrices such as RHW. This should greatly assist in the proposal of additional treatment systems in conventional wastewater treatment plants (WWTPs), in order to ensure efficient removal of all these compounds. Although they could be further improved, reliable and freely available (Q)SAR models can be

employed for the risk assessment of metabolites and biotic and abiotic TPs present in the aquatic environment.

The second study explored the results of the first work, considering the problem of incomplete removal of pharmaceuticals during conventional wastewater treatment. Investigation was made of the degradation of DZP by an AOP based on SPFP, with LC-QTOF MS used to identify TPs formed during the degradation process. It was possible to elucidate six transformation products generated during the degradation of DZP, four of which had not been reported previously in the literature. After identification of the TPs, *in silico* (Q)SAR predictions demonstrated that two of the TPs (TP1 and TP2) presented mutagenicity alerts, indicating that they could present a greater environmental risk, compared to the parent compound. This was a very important result that demonstrated the need for monitoring to ensure that a treatment process not only provides complete degradation of DZP, but also is effective in degrading all the TPs formed. The proposed new strategy based on a DLLME method for extraction and preconcentration of DZP and its TPs was shown to be fast, cheap, and simple, with its use allowing the elucidation of a TP that could not be detected without this step. Three different matrices were used to validate and test the method. For UPW and SW, the proposed DLLME method provided results very similar to those obtained using a traditional SPE method. For RHW, the DLLME method showed lower preconcentration efficiency, compared to SPE, but nonetheless achieved the same goal, since it succeeded in extracting/preconcentrating all the TPs also found by the SPE method. Considering the cost and time of the method, DLLME was much cheaper and faster than SPE, with a significant reduction of the quantity of solvents used in the process, so it would be an attractive technique for use in routine laboratory analyses.

After the screening and degradation studies, the challenge was to expand the study to the testing of surface waters, but not considering only pharmaceuticals, metabolites, and TPs. Screening was performed, evaluating thousands of contaminants from different chemical classes, which indicated the presence of 150 suspected and confirmed microcontaminants in surface water, especially pesticides and pharmaceuticals. *In silico* predictions using (Q)SAR, with eight different endpoints, were performed to identify compounds, with the data then being used for successful ranking of the screened analytes according to their toxicity towards humans and the environment. The use of MCDM techniques (ToxPi and TOPSIS) enabled the ordering of the identified compounds according to their environmental risk. Comparison of the rankings obtained by the two

tools showed that there were no statistically significant differences between them, confirming the satisfactory consistency of the results. Sensitivity tests showed greater robustness of the TOPSIS technique, compared to ToxPi, given small variations in the input data. This sorting would allow management decisions to be made concerning priority microcontaminants, taking into account the simultaneous effects of several physicochemical, biological, and toxicological parameters that are environmentally relevant. The appropriate application of MCDM methods for screening can assist in selecting the priority compounds for subsequent quantitative analysis during environmental monitoring. The combination of these three different strategies, LC-QTOF MS → *in silico* (Q)SAR predictions → MCDM ToxPi and TOPSIS ranking, constitutes a pioneering approach that has excellent potential to assist in chemical analysis decision-making in the environmental sciences field.

During the course of these studies, there was progressive evolution, expansion, and incorporation of new approaches for the evaluation of environmental contamination. The combination of the detailed studies showed that the selection of analytical strategies is much more complex than only considering qualitative or quantitative analyses. The multidisciplinary approach developed here was demonstrated to have the potential to guide environmental analysis in a highly efficient way.

The next step will be to quantify the most hazardous compounds, as well as to propose advanced treatment processes capable of degrading contaminants and mitigating environmental risks, with savings of both time and money.

FINAL DISPOSAL OF WASTES

All the wastes generated during this work were separated, stored in appropriate bottles, labeled, and taken to the UFRGS Chemical Waste Management and Treatment Center (CGTRQ), where specialized outsourced companies were contracted for final disposal of the materials.

LIST OF COLABORATION STUDIES DEVELOPED DURING THE PhD

A. Della-Flora, **R.W. Becker**, M.F. Ferrão, A.T. Toci, G.A. Cordeiro, M. Boroski, C. Sirtori, Fast, cheap and easy routine quantification method for atrazine and its transformation products in water matrixes using a DLLME-GC/MS method, Anal. Methods. 10 (2018) 5447–5452. https://doi.org/10.1039/C8AY02227E.

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