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**DESARROLLO DE METODOLOGÍA ANALÍTICA
Y ESTUDIO MEDIOAMBIENTAL DE
AGENTES ANTIMICROBIANOS Y
DROGAS DE ABUSO**

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Memoria para optar al grado de Doctora en Química

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Autorizan,

A la licenciada **Dña. Iria González Mariño** a la presentación del trabajo recogido en la memoria titulada **“DESARROLLO DE METODOLOGÍA ANALÍTICA Y ESTUDIO MEDIOAMBIENTAL DE AGENTES ANTIMICROBIANOS Y DROGAS DE ABUSO”**, que ha realizado bajo su dirección en el Departamento de Química Analítica, Nutrición y Bromatología de la Universidad de Santiago de Compostela, para optar al Grado de Doctora en Química.

Y para que así conste, firmamos la presente autorización en Santiago de Compostela, marzo de 2012.

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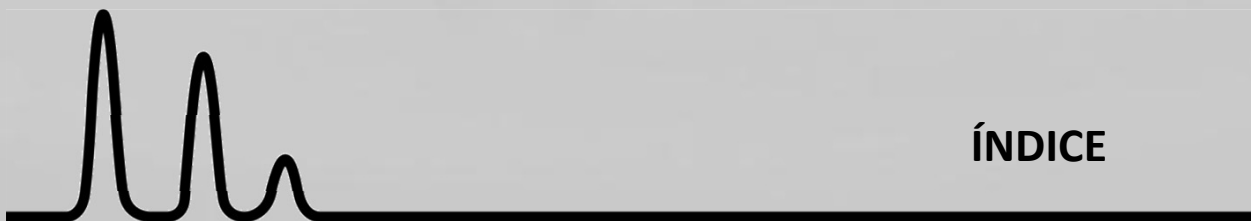
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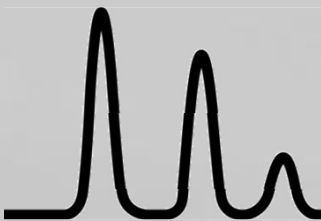
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SUMMARY

The following section is a summary of the PhD dissertation. It includes: (1) general aims, (2) a brief introduction about the pollutants and the analytical techniques considered, and (3) most relevant results and conclusions derived from each developed work. The content of the seven published articles is displayed in chapter III, covering the corresponding Experimental and Results and discussion subsections. General conclusions of the whole PhD dissertation are compiled in chapter IV.

1. AIM

The following PhD dissertation has performed the environmental study of two large families of emerging pollutants: antimicrobial agents (specifically parabens, their halogenated derivatives, triclosan, methyl triclosan and triclocarban) and drugs of abuse (including some of the major metabolites of the most frequently abused substances). Since both families of contaminants enter the environment, mainly, through urban wastewaters, the general aims of the present work were:

1. To **develop robust, sensitive and selective analytical methodologies** for the determination of the selected analytes in wastewaters (raw and treated) and in river waters affected by discharges of wastewater treatment plants (WWTPs). Compared to other existing methods, the analytical performance was attempted to be improved in terms of simplicity, economy, sensitivity and/or selectivity.
2. To **apply the developed methodologies to real samples** in order to validate them and to obtain data relating the environmental occurrence and fate of the aforementioned pollutants. In the case of drugs of abuse, measured concentrations in raw wastewater were used to evaluate the prevalence of their consumption within a specific population, following the guidelines of the *Sewage Epidemiology* approach. For parabens, occurrence data from 3 WWTPs allowed comparing the behaviour of the parent compounds with their halogenated derivatives during wastewater treatment processes (subsequently corroborated by a lab-scale biodegradability assay).
3. Finally, given that hydrophobicity of triclosan and its methylated derivative (methyl triclosan) leads to their adsorption on particulate matter, another of the considered objectives was **the development and application of a fast and simple procedure** for the simultaneous determination of both compounds in sludge and sediments.

2. INTRODUCTION

2.A. ANTIMICROBIAL AGENTS

Parabens (esters of the 4-hydroxybenzoic acid), triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol; TCS) and triclocarban (*N*-(4-chlorophenyl)-*N'*-(3,4-dichlorophenyl)-urea; TCC) are employed as bactericides and preservatives in a large variety of personal care products (PCPs) such as creams, shampoos, deodorants, toothpastes and soaps. TCS is also included in sportive clothes, footwear, carpets and kitchenware and parabens in pharmaceuticals, processed food and beverages.

The extensive inclusion of these chemicals in every-day consumption products has increased their concentration in urban sewage (up to the $\mu\text{g L}^{-1}$ level) [1-4]. Despite being considerably removed during conventional sewage treatments (particularly in the case of parabens), a variable percentage is released with effluents into surface waters, where they can still be detected in the $\text{ng-}\mu\text{g L}^{-1}$ range [5-10].

Once in the aquatic medium, antimicrobials are known to behave as weak endocrine disruptors. Parabens show oestrogenic potential [11] and, besides this, they can easily react with free chlorine when mixed with tap water [12], yielding mono and dihalogenated derivatives that display higher acute toxicity responses in the *Daphnia magna* test [13]. However, their environmental occurrence had been barely evaluated before this PhD dissertation, and their biodegradability (as well as the biodegradability of the parent compounds) remained unknown. Regarding TCS and TCC, laboratory studies have proved their transformation, under certain conditions, into more toxic and persistent species such as chlorinated phenols, polychlorinated diphenyl ethers or polychlorinated dibenzodioxins (for TCS [14-16]) and chlorinated anilines (for TCC [17]). TCS can also be methylated by several microorganisms to 5-chloro-2-(2,4-dichlorophenoxy)-anisole (methyl triclosan, MTCS [18]), a more lipophilic and, in some situations, more bio-accumulative compound that has already been detected in different environmental compartments: sludge, soils, sediments, etc. [19,20]. Evaluating its levels in the first matrix is a crucial issue in order to (1) understand the behaviour of TCS during wastewater treatments, (2) determine the existence of TCS methylation in the aquatic medium (previously to its intake by biota), and (3) evaluate the risk of introducing the methylated derivative in the terrestrial environment through the reutilization of biosolids as fertilisers.

Analytical methods for the determination of these pollutants in environmental samples are based on a pre-concentration step followed by the subsequent separation and detection by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). The former technique is more accessible to analytical laboratories and it is barely affected by matrix effects, but, as a disadvantage, it implies the derivatisation of polar and/or thermosensitive compounds (like TCC). On the contrary, LC does not usually require this step and, coupled to MS/MS on triple quadrupole instruments, renders an unmatched sensitivity. In recent years, quadrupole-time of flight (QTOF) mass analysers have also emerged as valuable systems in the field of environmental analysis, showing improved quantitative capabilities as well as extraordinary qualitative possibilities that allow the identification of not-originally defined compounds (e.g. pollutants by-products).

Among sample preparation techniques, one of the most common strategies for water matrices is solid-phase extraction (SPE) [6,16]: it is simple, robust, provides adequate enrichment factors and extraction yields and it is well established in most analytical laboratories. However, it usually requires the concentration of very large sample volumes, which is time-, solvent- and labour-consuming. Alternatively, microextraction techniques have come up like a promising approach and, in fact, some of them have already been applied to the determination of antimicrobial agents in water: solid-phase microextraction [21], stir-bar sorptive extraction [22], hollow-fibre liquid phase microextraction [23] or membrane-assisted liquid-liquid extraction [24]. Compared to the aforementioned methodologies, usually based on equilibrium processes, microextraction by packed sorbents (MEPS) is a miniaturised version of SPE expected to show better extraction yields, since it is based on adsorption phenomena. In MEPS, a small amount of sorbent is packed between the body and the metallic needle of a chromatographic syringe, so that the extraction may be performed automatically using a conventional injection system.

Concerning environmental solid samples, supercritical fluid extraction [19], pressurized liquid extraction [25], microwave assisted extraction [26] or ultrasound assisted extraction [27] usually provide good extraction efficiencies but, on the negative side, they frequently imply further time- and solvent-consuming clean-up procedures. Alternatively, matrix solid-phase dispersion (MSPD) is a simple strategy that reduces sample handling and time analysis, limits sample and solvent consumption and does not require expensive and specific instrumentation: the sample is mechanically dispersed with a sorbent in a mortar and the resulting material is packed into a column to perform the elution of the analytes.

2.B. DRUGS OF ABUSE

Abuse of illicit drugs has become a problem of global concern. According to the “World Drug Report 2011” of the United Nations Office of Drugs and Crime (UNODC), between 149 and 272 million people consumed any illicit substance at least once in the past year, and between 15 and 39 million were considered addicted [28]. Because of excretion after consumption, and occasional direct disposals into sewage systems, illicit drugs and their metabolites are continuously discharged into wastewaters, where they can be found up to the $\mu\text{g L}^{-1}$ level [29-39]. Since their removal during sewage treatments is usually incomplete, they are released into surface waters [29,36,37,40-42] and they have even reached drinking water sources [40,43-45].

The information existing about the toxicological impact of drugs of abuse in the environment is very limited, particularly in the case of synthetic substances. Most of the data available in the literature are on acute toxicity in humans or mammalian model organisms, whereas little is known regarding their adverse effects in aquatic species, main receptors of their environmental concentrations. Although a recent study has estimated a very low environmental risk for some of the most frequently abused drugs (morphine, amphetamine, cocaine and $\Delta 9$ -tetrahydrocannabinol, THC) [46], additional research is needed in order to identify potential synergistic or antagonistic effects, as well as long-term effects, for a complete eco-toxicological assessment.

Apart from the environmental impact, determining the levels of illicit substances in raw wastewater can also be used to monitor their consumption in a specific location. This approach, named as *Sewage Epidemiology*, was applied for the first time in 2005 by Zuccato et al. [47] and, since then, several research groups have followed it to estimate drug abuse in different countries [37,38,40,44,48-52]. In contrast to classical strategies of screening drugs consumption, analysis of water samples is cheaper, anonymous (avoiding potential conflicts over privacy) and provides real-time data, which would enable detecting changes in drugs usage if a long-term monitoring programme was carried out.

The great majority of the methods available in the literature for the determination of drugs of abuse in waters are based on an SPE step followed by the subsequent determination by LC-MS/MS on triple quadrupole systems [31,51,53-57]. As previously stated, SPE is a simple and robust technique that provides adequate enrichment factors and extraction yields and allows the simultaneous extraction of compounds with different physical and chemical properties (by selecting an adequate sorbent). However, its selectivity may be rather limited and, consequently, complex extracts, which usually lead to analytes ionisation suppression in electrospray interfaces (ESI), are obtained. Although deuterated analogues are available to

compensate for these matrix effects, they certainly result in a significant loss of sensitivity; therefore, developing selective SPE procedures is mandatory to minimise matrix components extraction and decrease limits of detection when the subsequent determination is performed by LC-MS.

Alternatively, GC-MS is rarely affected by matrix effects, it is accessible to most laboratories and has a long tradition for the determination of drugs of abuse in clinical and forensic sciences; however, it had not been applied with environmental purposes before this PhD dissertation.

Similarly, the quantitative possibilities of the hybrid quadrupole-time of flight mass spectrometers had never been evaluated for illicit drugs in the field of environmental analysis. These systems overcome some of the problems of the QqQ analysers and, furthermore, when working in MS mode as a single TOF, they offer the possibility to screen for a theoretically unlimited number of compounds after the LC-MS run (post-target analysis) and without the need of pure standards [58,59]. This may become very useful in the field of drugs of abuse to detect the consumption of new substances, appearing in the market continuously.

3. RESEARCH ACTIVITIES AND RESULTS

3.A. ANTIMICROBIAL AGENTS

The following PhD dissertation has considered the determination of parabens, TCS, TCC and MTCS in waters (using SPE followed by LC-MS/MS or microextraction by packed sorbents coupled at-line to GC-MS) and the determination of TCS and its O-methylated derivative in sludge and sediments (by matrix solid-phase dispersion combined with GC-MS). The main results of the developed works are stated below, according to the title of the derived published article.

Simultaneous determination of parabens, triclosan and triclocarban in water by liquid chromatography-electrospray ionisation-tandem mass spectrometry

The first developed research presents a simple and robust method for the simultaneous determination of TCS, TCC and seven parabens in sewage and river waters (including, for the first time, the distinction between branched and linear isomers of propyl and butyl paraben).

Analytes were pre-concentrated by SPE on Oasis HLB (60 mg) cartridges from 500 mL (river) or 200 mL (wastewater) samples at their natural pH. Subsequent elution with 4 mL of

methanol provided extraction recoveries higher than 85% except for raw wastewater, where they fell down to 65% for TCC (the most lipophilic of the considered compounds and the most likely to be adsorbed to particulate matter). Separation and detection were performed by LC-ESI-MS/MS, optimising and validating the whole method using two different triple quadrupole LC-MS systems: a low-mid (Varian 1200L) and a mid-high (API-4000) market range instrument. With the latter one, between 3- and 14-fold lower limits of quantification (LOQs) were obtained for parabens and TCC: 0.008-0.44 ng L⁻¹ versus 0.02-1.4 ng L⁻¹ in surface water and 0.02-1.11 ng L⁻¹ versus 0.05-3.5 ng L⁻¹ in wastewater. This effect was even more pronounced for TCS (0.23 and 0.57 ng L⁻¹ versus 20 and 50 ng L⁻¹ in surface and wastewater, respectively) probably due to a poor transmission efficiency for low masses in the low-mid market range system (TCS product ion: *m/z* 35). A comparison of matrix effects on both instruments showed a very different behaviour, particularly in the case of parabens. For these chemicals, signal suppression was observed in the 1200L system, while signal enhancement was noticed in the API-4000. As a result, different calibration approaches were chosen for them and this fact pointed out to the need of matrix effects re-evaluation whenever a method is transferred to a different LC-MS system.

Finally, the developed procedure was applied to the analysis of real samples, showing the ubiquity of methyl paraben (MeP) and *n*-propyl paraben (*n*-PrP, both at the 1-6 µg L⁻¹ level in raw wastewater) and the co-occurrence of both isomers of butyl paraben (*i*-BuP and *n*-BuP) at similar concentrations (ca. 100-200 ng L⁻¹ in raw wastewater).

Evaluation of the occurrence and biodegradation of parabens and halogenated by-products in wastewater by accurate-mass liquid chromatography-quadrupole-time-of-flight-mass spectrometry (LC-QTOF-MS)

The limited existing knowledge about the environmental occurrence of halogenated parabens led to adapt the above-described method to the determination of the same seven parabens together with the monochlorinated, the dichlorinated and the dibrominated derivatives of MeP in wastewater. In this case, separation and detection were performed by LC-ESI-MS using a liquid chromatograph coupled to a QTOF mass spectrometer. The performance of this system proved to be comparable to QqQ instruments in terms of quantitative capabilities, with good linearity ($R^2 > 0.99$ in the 5-500 ng mL⁻¹ range), repeatability (relative standard deviations, RSD < 5.6%) and limits of detection (LODs, in the 0.3-4.0 ng L⁻¹ range after SPE). Moreover, its accurate-mass determinations, in the full scan and MS/MS acquisition modes, allowed evaluating the presence of compounds not preselected originally as analytes and without the need of pure standards. In this way, it was

possible to extend the determination (qualitatively) to other halogenated species whose standards were not available in the laboratory.

Several wastewater samples were collected at three different WWTPs during April and May 2010, concentrated by SPE and analysed by LC-QTOF-MS as reported above. From these analysis, it is worth outlining that MeP and *n*-PrP were the most abundant parabens in raw wastewater ($0.3\text{-}10\ \mu\text{g L}^{-1}$), in accordance with the data displayed in the bibliography and reflecting their wider use in cosmetic formulations. Regarding the halogenated species, monochloro- and dichloro-methyl paraben (ClMeP and Cl₂MeP) were also determined in all the raw wastewater samples at levels between 0.01 and $0.1\ \mu\text{g L}^{-1}$. Halogenated derivatives of *n*-PrP could not be quantified due to the abovementioned lack of standards but, nevertheless, the monochlorinated species (ClPrP) was identified in several samples from its accurate precursor and product ion mass/charge ratios (m/z). An estimate of removal efficiencies (calculated from the average concentrations measured in influents and effluents) reported values higher than 94% for all the considered analytes, with the lowest percentages corresponding to the halogenated species. This trend was confirmed by an activated sludge biodegradation batch test, where non-halogenated parabens exhibited half-lives lower than 4 days, whereas halogenated derivatives of MeP turned out to be more stable (up to 10 days of half-life in the case of the dihalogenated species). A further stability test performed with real raw wastewater showed a similar tendency: parabens were rapidly degraded, while the dihalogenated species displayed half-lives longer than a week.

Fully automated determination of parabens, triclosan and methyl triclosan in wastewater by microextraction by packed sorbents and gas chromatography-mass spectrometry

Alternatively to the standard SPE, which requires the concentration of large sample volumes and is solvent- and time-consuming, the third of the developed methods was based on microextraction by packed sorbents for the extraction of six parabens, TCS and MTCS from wastewater. Its at-line coupling to GC-MS allowed the sample preparation strategy to be connected to the separation and detection step in a fully automated procedure that minimised sample and solvent consumption.

Under optimised conditions, analytes were extracted to a C₁₈ MEPS-sorbent (1 mg) from 2 mL samples adjusted at pH 3. Subsequently, they were eluted directly into the Programmable Temperature Vaporizer (PTV) injector of the gas chromatograph with two consecutive portions of 25 μL of ethyl acetate. After signal normalisation with isotopic labelled species as internal surrogates, no differences were noticed between the extraction efficiency for sewage and ultrapure water and, therefore, calibration was performed using standard

solutions in ultrapure water submitted to the same sample enrichment process. The proposed method reported lineal calibration curves from 0.1 to 10 ng mL⁻¹, relative standard deviations (%RSD) between 2.0 and 7.1% and limits of detection (LODs) varying from 0.001 to 0.015 ng mL⁻¹ in ultrapure water and from 0.02 to 0.59 ng mL⁻¹ in the most complex matrix (raw wastewater). Its application to real sewage samples demonstrated the abundance of MeP and *n*-PrP, in agreement with previously reported results.

From this particular case, it is worth outlining that further efforts in MEPS technique should be focused on the commercialisation of syringes packed with polymeric materials (alternative to silica based sorbents) in order to improve: (1) the absolute extraction efficiency for the most polar compounds (e.g. MeP); and (2) the selectivity of the enrichment step. The experience with SPE sorbents in conventional formats for PCPs determination points out to hydrophilic-lipophilic balance and mixed-mode sorbents.

Matrix solid-phase dispersion followed by gas chromatography-mass spectrometry for the determination of triclosan and methyl triclosan in sludge and sediments

Finally, the tendency of TCS and MTCS to get adsorbed to sludge, during wastewater treatment processes, and to sediments, when they reach surface waters, led to develop an expeditious method for their determination in both environmental matrices.

Alternatively to other classical solid sample preparation techniques, extraction and clean-up steps were integrated in the same process using matrix solid-phase dispersion (MSPD). Under final conditions, samples (0.5 g) were dispersed with diatomaceous earth (1 g) and transferred to a polypropylene syringe containing 2 g of silica impregnated with sulphuric acid (15%, w/w). The chemical stability of TCS and its methylated derivative allowed the use of such oxidative clean-up conditions without undergoing significant decomposition. In this way, the MSPD syringe was first rinsed with 5 mL of *n*-hexane (discarded) and both analytes were subsequently eluted with 10 mL of dichloromethane (which showed high extraction efficiency and good compatibility with oxidative conditions). After solvent exchange to ethyl acetate, TCS was converted into the *tert*-butyldimethylsilyl derivative and the extract was analysed by GC-MS. Obtained recoveries, for sludge and sediment samples spiked at different concentration levels, ranged from 86% to 113% and limits of quantification (LOQs) of the global method were 6 and 7 ng g⁻¹ for MTCS and TCS, respectively.

Analyses of sludge from several urban WWTPs demonstrated the ubiquitous distribution of MTCS in this matrix, and thus the occurrence of TCS methylation reactions during wastewater treatment processes. Maximum measured concentrations were 191 ng g⁻¹ (MTCS) and 2640 ng g⁻¹ (TCS); in general, levels of the methylated by-product represented

between 1 and 33% of the levels of TCS, a percentage that should be considered to improve the accuracy of TCS mass balances in WWTPs and also to assess the potential contamination of agriculture fields with this highly bio-accumulative halogenated diphenyl ether. On the other hand, MTCS was not detected in any analysed sediment, while TCS was found in 50% of these samples at a concentration one order of magnitude lower than in sludge (up to 200 ng g⁻¹).

3.B. DRUGS OF ABUSE

The following PhD dissertation has considered the analysis of waters for the determination of drugs of abuse and metabolites belonging to seven different chemical families: opioids, cannabinoids, alkaloids, amphetamine-like compounds, piperazine derivatives, hallucinogens and dissociative anesthetics. In all cases, SPE was selected as the sample preparation technique, performing the separation and detection by GC-MS/MS or LC-MS (/MS). This section displays the most relevant results of the developed works in this field.

Determination of drugs of abuse in water by solid-phase extraction, derivatisation and gas chromatography-ion trap-tandem mass spectrometry

Alternatively to the existing LC-MS methods, the first work was based on GC-MS/MS for the determination of fourteen illicit drugs and metabolites (belonging to four different chemical families) in surface and sewage waters.

Samples (500 mL for river water, 200 mL for treated wastewater and 100 mL for raw wastewater) were adjusted at pH 8.5 and concentrated using the Oasis HLB (200 mg) sorbent. Once completely dried, it was demonstrated that cartridges could be stored at -20 °C for at least 3 months without significant drugs degradation and/or inter-conversion reactions being observed; this procedure turned out to be a good alternative to classical storage of samples (frozen or acidified) not only as it saved space, but also because it avoided drugs degradation and/or adsorption to particulate matter. Previously to the determination step, analytes were sequentially eluted with 2 mL of ethyl acetate (amphetamine-like compounds) followed by 8 mL of acetone (remaining compounds) and derivatised by silylation with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, 60 min at 80 °C). The proposed method provided recoveries (63-137%) and LODs (0.8-15 ng L⁻¹) similar those reported by SPE-LC-MS/MS procedures, but at a lower cost and without the inconvenient of matrix effects. On the negative side, their main drawbacks were that it was not suitable for the analysis of the main metabolite of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP, a quaternary amine) and, to a minor extent, that it was slower than LC-MS based methods, as it required 60 min for the derivatisation step.

Analysis of a limited number of grab sewage and surface water samples showed the presence of several illicit drugs and metabolites in the aquatic environment. Thus, cocaine, benzoylecgonine (the main metabolite of cocaine) codeine, morphine and carboxy-THC (THCCOOH, the main metabolite of THC) could be quantified in most of the samples confirming their consideration as common emerging pollutants. Highest levels and frequency of detection corresponded to benzoylecgonine, highlighting the widespread consumption of cocaine in developed countries.

Comparison of molecularly imprinted, mixed-mode and hydrophilic balance sorbents performance in the solid-phase extraction of amphetamine drugs from wastewater samples for liquid chromatography-tandem mass spectrometry determination

Taking into account that matrix effects imply a serious limitation in LC-ESI-MS analysis, the following efforts were focused on the development of selective SPE procedures for extracting drugs of abuse from wastewaters.

In this sense, the second work was a result of the commercialization of SPE cartridges containing molecularly imprinted polymers (MIPs) for the retention of amphetamine-like compounds. The SPE protocol proposed by the manufacturer was applied to the extraction of five amphetamine derivatives (amphetamine, methamphetamine, MDMA, MDA and MDEA) from raw wastewater, and the analytical performance of the whole method (SPE-LC-MS/MS) was compared to the obtained using another two different commercial sorbents: a common hydrophilic-lipophilic balance sorbent (Oasis HLB) and a mixed-mode cation exchange material (Oasis MCX). Oasis HLB showed the worst performance, as three analytes (MDA, MDMA and MDEA) could not be determined because of interfering signals in the LC-MS/MS chromatogram, and amphetamine recoveries could not be corrected by the use of the deuterated analogous internal standard. Oasis MCX permitted the determination of all the target analytes, but still strong signal suppression was observed: ca. 70% signal drop in wastewater samples, which, in this case, could be corrected by internal standards providing acceptable trueness (overall recoveries: 101-137%), precision (RSD: 2.0-12%) and limits of detection (LOD: 1.5-5.2 ng L⁻¹). Alternatively, MIPs rendered cleaner extracts with lower matrix effects (ca. 30% signal drop), and thus lower LODs (0.5-2.7 ng L⁻¹) and even better trueness (92-114% overall recovery) and precision (RSD: 1.5-4.9%). However, the main drawbacks of this material were: (1) its lower capacity as compared to Oasis sorbents; and (2) the negative effects of sample flow-rate in the retention efficiency. Thus, sample concentration took 5 times longer (ca. 50 min) than in the case of MCX cartridges (ca. 10 min).

Finally, the application of the method based on MIP cartridges to real samples showed the ubiquity of MDA and MDMA in both raw and treated wastewater at the 4-20 ng L⁻¹ level.

Screening and selective quantification of illicit drugs in wastewater by mixed-mode solid-phase extraction and quadrupole-time-of-flight liquid chromatography-mass spectrometry

In the last developed method, the number of analytes was increased to 24, including drugs and metabolites belonging to seven different families. Oasis MCX was selected as sorbent to optimise an SPE procedure whose main difference to other existing methods was carrying out a fractionation of basic analytes from neutral and acidic species during cartridge elution. Under final working conditions, samples (200 mL for raw wastewater, 500 mL for treated wastewater) were acidified at pH 4.5 and extracted on Oasis MCX (150 mg) cartridges. Cannabinoids (together with neutral and acidic matrix components) were first eluted with 2 mL of methanol, whereas remaining (basic) compounds were recovered subsequently by passing 4 mL of basified methanol (5% ammonium hydroxide). Both fractions were analysed separately by LC-MS/MS (MS for opioids) in a QTOF instrument, showing that matrix effects during ESI ionisation were significantly reduced for the analytes included in the second fraction, as compared to the effects reported for hydrophilic-lipophilic balance reversed-phase sorbents or the same mixed-mode polymer but without performing sequential elution. Recoveries above 63% and 82% were attained for all the species in raw and treated sewage, respectively, and limits of quantification ranged from 2 to 50 ng L⁻¹. These data highlighted the suitability of QTOF mass spectrometers to perform the quantification of drugs of abuse in waters (in this field, they had only been used with qualitative purposes). Although instrumental LOQs were, in some cases, higher than other values reported with QqQ systems, they were still low enough to allow the determination of several substances in real samples

In this sense, analysis of raw wastewater confirmed the ubiquity of cocaine, benzoylecgonine and THCCOOH in this matrix, up to 300, 700 and 200 ng L⁻¹, respectively. Concentrations from 24 h-composite samples were translated into mean loads and correlated to drug consumption following the guidelines of the *Sewage Epidemiology* approach: for amphetamine and cocaine, estimated abuse was within the ranges reported in Europe (2.6 and 4.6 doses day⁻¹ 1000 inh⁻¹, respectively) whereas, for cannabis, it was the highest calculated value to date (68 doses day⁻¹ 1000 inh⁻¹).

Finally, the post-target capabilities of the QTOF system were used for the identification of non-target pollutants. First, high resolution MS chromatograms were automatically searched against an in-house built database; a reduced list of candidate drugs was generated and the corresponding extracted ion chromatograms were obtained. In a further LC run,

tandem mass spectrometry (MS/MS) spectra of unknown peaks were acquired using different collision energies and compared with those existing in public libraries, or interpreted, to assign the unknown peak to one of the previously selected candidates. This methodology permitted the identification of ephedrine and ecgonine methyl ester (two substances already reported in wastewater) in influent samples.

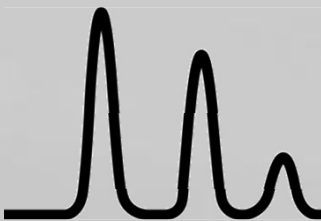
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I. JUSTIFICACIÓN Y OBJETIVOS

Desde mediados de los años 90, una creciente atención por parte de los estudios ambientales se ha centrado en los denominados contaminantes emergentes. La red NORMAN (*Network of reference laboratories for monitoring of emerging environmental pollutants*, [1]) define como contaminante emergente a una sustancia actualmente no regulada y no incluida en ningún programa de monitorización de rutina, pero que es candidata potencial para una posterior regulación en función de las conclusiones que se desprendan sobre su posible impacto ambiental. En su mayoría, se trata de compuestos cuya utilización está aprobada en multitud de productos de uso doméstico y que, por ello, no necesitan ser persistentes para causar efectos nocivos, ya que su elevada tasa de degradación se ve compensada por su continua introducción en el medio ambiente [2]. Entre estos contaminantes se incluye una amplia variedad de sustancias como son los fármacos, surfactantes, edulcorantes, fragancias, filtros solares, etc.

En la presente tesis doctoral se ha abordado el estudio de dos grandes familias de contaminantes emergentes:

- A. Agentes antimicrobianos.** En concreto, se han considerado el triclosán y el triclocarbán, ampliamente utilizados en productos de cuidado personal (PCPs), y siete ésteres del ácido 4-hidroxibenzoico (parabenes) presentes también en PCPs y, adicionalmente, en alimentos procesados y productos farmacéuticos. Los dos primeros son conocidos disruptores endocrinos y pueden transformarse, una vez liberados al ambiente, en compuestos considerablemente más tóxicos como las dioxinas o las anilinas cloradas [3,4]. Para los parabenes, el mayor riesgo ambiental descrito radica en su potencial estrogénico [5]. En el presente trabajo se han considerado, además, el metil triclosán (producto de la biometilación del triclosán y ambientalmente más persistente que éste [6]) y los derivados mono y diclorados/bromados de los parabenes (susceptibles de formarse como consecuencia de la halogenación del parabén correspondiente [7]).

- B. Drogas de abuso.** En este caso, se han estudiado compuestos pertenecientes a siete subgrupos diferentes (opioides, cannabinoides, alcaloides, derivados anfetamínicos, derivados de la piperacina, alucinógenos y anestésicos disociativos) y algunos de los principales metabolitos de las sustancias más consumidas de acuerdo con los últimos informes de la Oficina de las Naciones Unidas para las Drogas y el Crimen (*United Nations Office of Drugs and Crime*, UNODC) [8,9] y el Observatorio Europeo de las Drogas y Toxicomanías (*European Monitoring Centre for Drugs and Drug Addiction*, EMCDDA) [10,11]. El impacto eco-toxicológico de las drogas de abuso ha sido hasta la

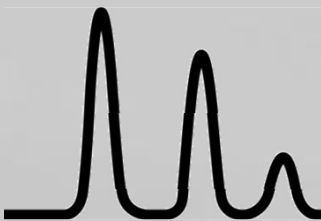
fecha muy poco evaluado y, aunque los datos disponibles sugieren que se degradan con rapidez, se necesitan estudios adicionales para poder afirmar si existe o no un riesgo real.

Ambas familias de contaminantes se introducen en el medio ambiente, fundamentalmente, a través de las aguas residuales urbanas (como resultado, en último lugar, de tratamientos de depuración incompletos o inexistentes). Por este motivo, los principales objetivos de la presente tesis doctoral han sido:

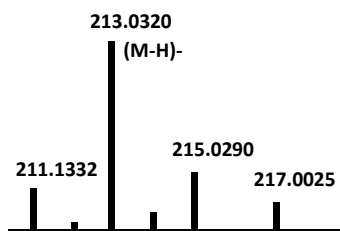
1. El **desarrollo de metodologías analíticas** robustas, sensibles y selectivas para la determinación de los analitos seleccionados en aguas residuales (tratadas y sin tratar) y en aguas fluviales sometidas a descargas de estaciones depuradoras. En la medida de lo posible, se han pretendido mejorar las prestaciones con respecto a los métodos existentes en la bibliografía, en términos de simplicidad, economía, sensibilidad y/o selectividad.
2. La **aplicación de estas metodologías a muestras reales** para validarlas y obtener datos de distribución de estos contaminantes en el medio ambiente. En el caso de las drogas de abuso, las concentraciones medidas en agua residual se han usado para evaluar la prevalencia de su abuso dentro de una población determinada, de acuerdo con las directrices de una nueva metodología denominada *Epidemiología de Aguas Residuales* [12]. Para los parabenos, los datos de distribución en tres estaciones depuradoras han permitido comparar el comportamiento de los compuestos nativos con el de sus derivados halogenados frente a los tratamientos de depuración. Posteriormente, este comportamiento ha sido corroborado mediante un ensayo de biodegradabilidad aeróbica a escala de laboratorio.
3. Finalmente, y dado que el carácter hidrofóbico del triclosán y del metil triclosán favorece su adsorción sobre el material particulado, otro de los objetivos que se plantearon fue el **desarrollo y aplicación de un procedimiento rápido y sencillo** para la determinación simultánea de estos dos compuestos en lodos y sedimentos.

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II. INTRODUCCIÓN



A. AGENTES ANTIMICROBIANOS

1. ASPECTOS GENERALES

1.1. DEFINICIÓN Y APLICACIONES

1.1.1. Parabenes

Los parabenes son ésteres del ácido 4-hidroxibenzoico ampliamente utilizados como agentes antimicrobianos en numerosos productos de consumo diario: alimentos procesados (conservas, salsas, refrescos, bollería industrial), productos de cuidado personal (cremas, geles, champús, cosméticos) y compuestos farmacéuticos (jarabes, píldoras, colirios). Su adición a alimentos está regulada en la Unión Europea por la Directiva 95/2/CE del 20 de febrero de 1995 [1], que permite una concentración máxima global del 0.1% (p/p) expresada como concentración de ácido 4-hidroxibenzoico. En cosméticos, este límite está fijado en el 0.8% (p/p) (Directiva 76/768/CEE del 27 de julio de 1976) [2].

El extenso campo de aplicación de estos agentes se debe a su efectividad, pero también a su bajo coste, elevada estabilidad química y baja toxicidad. Son activos contra hongos, levaduras, bacterias Gram+ y una pequeña proporción de bacterias Gram-, provocando disrupción en los procesos de transporte de la membrana, inhibición de la síntesis de ADN y ARN o inactivación de enzimas claves en su desarrollo [3]. Su actividad bactericida y fungicida se incrementa con la longitud de la cadena hidrocarbonada del grupo éster, pero la paralela disminución de su solubilidad en agua, donde habitan la mayoría de los microorganismos, hace que los de mayor tamaño sean, precisamente, los que menos se utilizan. Por el contrario, los más comunes son el metil parabén o 4-hidroxibenzoato de metilo y el propil parabén o 4-hidroxibenzoato de propilo, cuya combinación, además, produce un efecto aditivo [4].

En presencia de halógenos reaccionan dando lugar a la formación de los correspondientes hidroxibenzoato de alquilo halogenados, sin aplicación comercial conocida. En la presente tesis doctoral se ha considerado el estudio cuantitativo de siete parabenes y de tres de los derivados halogenados del metil parabén: el metil parabén monoclorado, el diclorado y el dibromado. Adicionalmente y de forma cualitativa, se ha evaluado la presencia en agua residual de todos los derivados mono y diclorados/bromados de los restantes parabenes.

1.1.2. Triclosán

El triclosán (5-cloro-2-(2,4-dicloro-fenoxi)-fenol) se comercializa con diferentes nombres (Irgacare MP, Irgasan DP300, Aquasept, Sapoderm, Ster-Zac) y se utiliza desde hace

más de 40 años como agente antimicrobiano en el tratamiento de superficies (e.g. Silestone) y en jabones, cremas, desodorantes y, especialmente, productos de higiene dental. La Directiva 76/768/CEE [2] permite su adición a productos de cuidado personal a concentraciones inferiores al 0.3% (p/p). Recientemente, la Unión Europea ha prohibido su adición a materiales textiles y envoltorios plásticos de alimentos, si bien en Estados Unidos (EEUU) se sigue utilizando en fibras y polímeros con estos fines a niveles comprendidos entre el 0.075 y el 0.5% (p/p) [5].

El triclosán ofrece protección contra bacterias Gram+, Gram-, levaduras y otros tipos de hongos. De acuerdo con Levy et al. [6], el mecanismo de actuación de este compuesto se basa en que inhibe a la proteína transportadora enoil-acil-reductasa, bloqueando la biosíntesis de ácidos grasos necesarios para la formación de las paredes celulares y la reproducción.

En condiciones aeróbicas puede ser O-metilado por la acción de diversos microorganismos originando la formación de 5-cloro-2-(2,4-diclorofenoxi)-anisol, más comúnmente conocido como metil triclosán [7].

1.1.3. Triclocarbán

El triclocarbán (*N*-(4-clorofenil)-*N'*-(3,4-diclorofenil)-urea) se ha utilizado desde 1957 como agente antimicrobiano en numerosos productos de cuidado personal, especialmente jabones, a niveles de hasta el 1.5% (p/p). Desde 1976, la Directiva 76/768/CEE [2] permite su adición a cosméticos hasta una concentración máxima del 0.2% (p/p).

Resulta efectivo contra bacterias Gram+ y Gram-, actuando como disruptor de los procesos de transporte que tienen lugar en la membrana celular [8].

Su producción en EEUU ronda las 500 toneladas anuales, lo que ha llevado a la Agencia para la Protección del Medio Ambiente (*Environmental Protection Agency*, EPA) a catalogarlo como producto químico de gran producción (*High production volume chemical*) [9], a diferencia de su consideración como producto químico de baja producción (*Low production volume chemical*) por parte de la Comisión Europea [10].

1.2. ESTRUCTURA Y PROPIEDADES FÍSICO-QUÍMICAS

En la **Tabla II.1** se recogen las estructuras de los agentes antimicrobianos y derivados considerados en la presente tesis doctoral, sus fórmulas empíricas, pesos moleculares monoisotópicos y propiedades físico-químicas más relevantes desde el punto de vista analítico: pK_a , logaritmo de la constante de partición octanol-agua ($\log K_{ow}$) y presión de vapor

(P_v). Todos los datos son experimentales (recogidos en la base de datos PhysProp, Syracuse Research Corporation [11]) excepto los marcados con (ª), que corresponden a valores calculados mediante *software* proporcionados por la base de datos SciFinder Scholar [12].

Los parabenes son relativamente solubles en agua e hidrolíticamente estables en un amplio intervalo de pH. Presentan una acidez entre baja e intermedia y, al pH al que se encuentran la mayoría de las aguas (5.5-8), están en forma neutra. Su solubilidad en este medio aumenta al aumentar el pH (pasan a forma aniónica) y desciende con el incremento de la longitud de la cadena hidrocarbonada del grupo éster. Sus derivados halogenados son más ácidos y más lipofílicos que los correspondientes compuestos nativos.

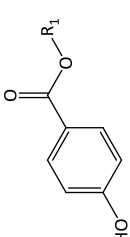
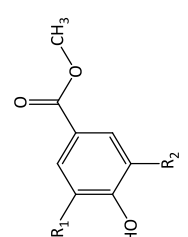
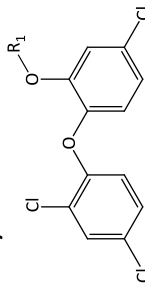
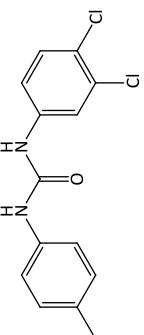
El triclosán es resistente a la hidrólisis, pero no a la degradación fotoquímica o térmica [13,14]. Es menos soluble en agua que los parabenes y más lipofílico, por lo que presenta una mayor tendencia a adherirse a materiales sólidos. Su distribución en medios acuosos viene determinada por el pH: es ligeramente ácido y, en medios básicos, se encuentra en forma aniónica (más soluble). Su derivado O-metilado es más estable frente a la degradación fotoquímica [14] y presenta un carácter lipofílico más acusado.

El triclocarbán es el menos ácido y, exceptuando al metil triclosán, el más apolar de todos los compuestos estudiados. Es muy poco soluble en agua, poco volátil y térmicamente inestable.

1.3. DISTRIBUCIÓN EN EL MEDIO AMBIENTE

La sistemática inclusión de antimicrobianos en productos de cuidado personal ha disparado su presencia en las aguas residuales urbanas, convertidas en la principal vía de entrada de estos compuestos en el medio ambiente [15-18]. A pesar de que un porcentaje variable es eliminado durante los tratamientos de depuración, parte es emitido con los efluentes a las aguas superficiales [19-24], alcanzando incluso las aguas potables [19,25-29]. Además, el carácter hidrofóbico de algunos compuestos (el triclocarbán, el triclosán y su derivado O-metilado) favorece su deposición sobre los sedimentos en aguas contaminadas [30-32] y su adsorción a los lodos en las estaciones depuradoras [32-35], pudiendo distribuirse posteriormente en los suelos como resultado de la utilización de biosólidos como fertilizantes [36,37]. Por el contrario, su baja volatilidad limita su tendencia a pasar a la fase gaseosa y, hasta la fecha, sólo un estudio ha detectado algunos parabenes en aire [38]. Finalmente, y como consecuencia de su omnipresencia medioambiental, estos antimicrobianos pueden ser asimilados por los seres vivos dando lugar a fenómenos de bioacumulación y biomagnificación derivados de su carácter lipofílico [39,40].

Tabla II.1. Estructura y propiedades físico-químicas de los agentes antimicrobianos considerados. Datos experimentales recogidos en la base de datos PhysProp, Syracuse Research Corporation [11], o calculados mediante *software* (a) proporcionados por la base de datos SciFinder Scholar [12].

| Clasificación y estructura | Compuesto | Abreviatura | R ₁ | R ₂ | Fórmula Empírica | Pm monoisotópico | pK _a | log K _{ow} | P _v (mm Hg) |
|---|---------------------------|---------------------|--|----------------|---|------------------|--------------------|---------------------|------------------------|
| Parabenes  | Metil parabén | MeP | CH ₃ | - | C ₈ H ₈ O ₃ | 152.05 | 8.30 ^a | 1.96 | 2.37 E-4 |
| | Etil parabén | EtP | CH ₂ -CH ₃ | - | C ₉ H ₁₀ O ₃ | 166.06 | 8.34 | 2.47 | 9.29 E-5 |
| | <i>i</i> -Propil parabén | <i>i</i> -PrP | CH-(CH ₃) ₂ | - | C ₁₀ H ₁₂ O ₃ | 180.08 | 8.40 ^a | 2.91 | 1.16 E-3 |
| | <i>n</i> -Propil parabén | <i>n</i> -PrP | CH ₂ -CH ₂ -CH ₃ | - | C ₁₀ H ₁₂ O ₃ | 180.08 | 8.23 ^a | 3.04 | 5.55 E-4 |
| | <i>i</i> -Butil parabén | <i>i</i> -BuP | CH ₂ -CH-(CH ₃) ₂ | - | C ₁₁ H ₁₄ O ₃ | 194.09 | 8.17 ^a | 3.40 | 3.81 E-4 |
| | <i>n</i> -Butil parabén | <i>n</i> -BuP | CH ₂ -CH ₂ -CH ₂ -CH ₃ | - | C ₁₁ H ₁₄ O ₃ | 194.09 | 8.47 | 3.57 | 1.86 E-4 |
| | Bencil parabén | BzP | CH ₂ -Ph | - | C ₁₄ H ₁₂ O ₃ | 228.08 | 8.18 ^a | 3.56 | 3.37 E-6 |
| Parabenes halogenados  | Metil parabén monoclorado | ClMeP | Cl | H | C ₈ H ₇ ClO ₃ | 186.01 | 6.89 ^a | 2.64 | 8.32 E-4 |
| | Metil parabén diclorado | Cl ₂ MeP | Cl | Cl | C ₈ H ₆ Cl ₂ O ₃ | 219.97 | 5.43 ^a | 3.48 ^a | 5.44 E-4 ^a |
| | Metil parabén dibromado | Br ₂ MeP | Br | Br | C ₈ H ₆ Br ₂ O ₃ | 307.87 | 5.30 ^a | 2.83 ^a | 7.75 E-4 ^a |
| Triclosán y metil triclosán  | Triclosán | TCS | H | - | C ₁₂ H ₇ Cl ₃ O ₂ | 287.95 | 7.80 ^a | 4.76 | 6.45 E-7 |
| | Metil triclosán | MTCS | CH ₃ | - | C ₁₃ H ₉ Cl ₃ O ₂ | 301.97 | - | 5.27 ^a | 5.18 E-5 ^a |
| Triclocarbán  | Triclocarbán | TCC | - | - | C ₁₃ H ₉ Cl ₃ N ₂ O | 313.98 | 12.77 ^a | 4.90 | 3.61 E-9 |

1.3.1. Aguas

1.3.1.1. Aguas residuales

En la **Tabla II.2** se recogen los niveles detectados para estos compuestos en agua residual sin tratar (influyente) y tratada (efluente) y en agua superficial (río) procedente de diversos países.

Entre los parabenos, el metil y el *n*-propil parabén fueron las especies detectadas con mayor frecuencia y a mayor concentración en los influentes: hasta 26.2 $\mu\text{g L}^{-1}$ [42] y 2.8 $\mu\text{g L}^{-1}$ [41], respectivamente, en dos estudios llevados a cabo en España. En general, los valores fueron también elevados en el resto de casos evaluados, poniendo de manifiesto la mayor ubiquidad de estos compuestos con respecto a otros parabenos en las formulaciones de productos de cuidado personal. Siguiendo con esta tendencia, el etil parabén y el *n*-butil parabén se cuantificaron a niveles de hasta 2 $\mu\text{g L}^{-1}$ [42] y 0.9 $\mu\text{g L}^{-1}$ [15], respectivamente, mientras que el bencil parabén no superó los 5 ng L^{-1} [42]. El único trabajo que consideró el estudio del *i*-propil parabén en agua residual sin tratar notificó concentraciones mucho más bajas que las de su isómero *n*- (2.2-5.4 ng L^{-1} frente a 1200-1700 ng L^{-1}), en claro contraste con los valores detectados para los dos isómeros del butil parabén, del mismo orden de magnitud (40-106 ng L^{-1} y 88-172 ng L^{-1}) [42]. Estos datos coinciden con los obtenidos en un trabajo incluido en la presente memoria [43], y reflejan que, mientras el *n*-propil parabén es mucho más utilizado que su isómero *iso*-, en el caso del butil parabén ambas especies son incluidas por igual en productos de cuidado personal.

Como resultado también de su amplio uso, el triclosán y el triclocarbán fueron cuantificados a niveles muy elevados en agua residual sin tratar: hasta 16.6 $\mu\text{g L}^{-1}$ [34] y 6.7 $\mu\text{g L}^{-1}$ [20], respectivamente, en EEUU. Aunque el primero fue determinado a concentraciones muy altas en influentes de otros países [25,44-46], los valores notificados para el triclocarbán en el único estudio no estadounidense considerado [17] fueron sensiblemente inferiores (entre no detectado y 362 ng L^{-1}), reflejo de su menor utilización allende del país norteamericano. El producto de *O*-metilación del triclosán se mantuvo por debajo de los límites de detección/cuantificación en dos de los cuatro estudios que lo determinaron [34,47], alcanzando los 354 ng L^{-1} en uno realizado en España. En este último, la concentración máxima determinada en el efluente fue de 51 ng L^{-1} [25].

Los niveles en agua residual tratada recogidos en la **Tabla II.2** constituyen un reflejo de la literatura existente en torno a la eficacia de los procesos de depuración sobre los agentes antimicrobianos considerados. En general, los parabenos son extensamente eliminados de las aguas tras su paso por las estaciones depuradoras, tal y como se aprecia en los valores de concentración medidos en los efluentes (con un máximo de 423 ng L^{-1} para el metil parabén [15]). Por el contrario, el porcentaje de eliminación del triclosán es muy variable y depende en

parte de los tratamientos aplicados. Así, en plantas con procesos convencionales de lodo activado se sitúa en torno al 90%, del cual entre el 40 y el 60% se atribuye a la biodegradación (más efectiva en condiciones aeróbicas que anóxicas, prácticamente inexistente en medios anaeróbicos [7,34]) y el resto a la adsorción sobre los lodos [44,48,49]. Finalmente, la información disponible acerca de la eliminación del triclocarbán en las estaciones depuradoras es muy escasa y, en base a los estudios existentes, se puede concluir que es muy variable y que está fuertemente influenciada por su adsorción sobre los lodos.

1.3.1.2. Aguas superficiales

Coincidiendo con su mayor presencia en aguas residuales, el metil parabén, el *n*-propil parabén, el triclosán y el triclocarbán fueron los compuestos determinados a mayor concentración en aguas superficiales (hasta 1, 3, 5 y 6.7 $\mu\text{g L}^{-1}$, respectivamente, **Tabla II.2.**) [20,22,23].

En el caso de los parabenes, los niveles más elevados se determinaron en muestras de ríos localizados en un área de China densamente poblada y que reciben las descargas de numerosas estaciones depuradoras de aguas residuales urbanas [22]. En el resto de los estudios, su concentración sólo superó los 200 ng L^{-1} en dos trabajos llevados a cabo en Reino Unido (MeP) [50] y Japón (MeP y *n*-PrP) [24]. El etil parabén, el *i*-propil parabén, los dos isómeros del butil parabén y el bencil parabén fueron cuantificados a niveles inferiores.

El valor de 5.2 $\mu\text{g L}^{-1}$ es el más alto notificado hasta la fecha para el triclosán en un agua superficial y corresponde a aguas fluviales indias afectadas por la actividad industrial [23]; los autores atribuyeron las elevadas concentraciones a las descargas procedentes de industrias textiles de la zona, ya que en India este agente antimicrobiano todavía se utiliza como aditivo habitual en fibras y polímeros para la fabricación de ropa y calzado deportivos. En general, las concentraciones determinadas en los demás estudios fueron sensiblemente inferiores, con la excepción de dos trabajos realizados en China (hasta 1 $\mu\text{g L}^{-1}$) [22] y EEUU (hasta 1.6 $\mu\text{g L}^{-1}$) [20].

En aguas superficiales, el triclocarban sólo ha sido determinado en EEUU, mostrando valores que varían entre inferiores al límite de detección y 6.7 $\mu\text{g L}^{-1}$ [20].

1.3.1.3. Aguas potables

Ocasionalmente, se ha evaluado la presencia de algunos de los agentes antimicrobianos considerados en la presente memoria en aguas potables [19,25-29]. De los compuestos incluidos en los citados estudios (metil parabén, triclocarbán, metil triclosán y triclosán) sólo éste último fue cuantificado a un nivel de 734 ng L^{-1} en una muestra tomada durante el verano en una planta potabilizadora de California; en el resto de muestras tomadas a lo largo del año (15 en total) no superó el límite de detección [28].

Tabla II.2. Rango de concentraciones o concentración media (ng L⁻¹) de los agentes antimicrobianos considerados en aguas residuales y fluviales. N: número de estaciones depuradoras (agua residual) o puntos de muestreo (agua fluvial) incluidos en el estudio; nd: inferior al límite de detección; nc: inferior al límite de cuantificación.

| Concentración (ng L ⁻¹) | N | Influyente | Efluente | N | Río | País | Ref. |
|-------------------------------------|-------------------------|------------|----------|--------|---------|-------------|--------|
| Parabenes | | | | | | | |
| Metil parabén | 2 | 1658-5613 | nc | - | - | España | [17] |
| | 1 | nc | nc | 3 | nd | España | [41] |
| | 1 | 6810-26200 | nd | 1 | nd-54 | España | [42] |
| | 7 | 65-9980 | 4.6-423 | 3 | 3.1-17 | Suíza | [15] |
| | 1 | 2642 | nc | 3 | nc-10 | Reino Unido | [51] |
| | - | - | - | 10 | nc-400 | Reino Unido | [50] |
| | - | - | - | 29 | nd-23 | India | [23] |
| | - | - | - | 9 | nc-1062 | China | [22] |
| | - | - | - | 12 | 25-676 | Japón | [24] |
| 8 | 100-1470 | 20-30 | - | - | Canadá | [16] | |
| Etil parabén | 2 | 196-625 | nd-48 | - | - | España | [17] |
| | 1 | nc | nd | 3 | nd | España | [41] |
| | 1 | 480-1943 | nd-57 | 1 | 29-30 | España | [42] |
| | 7 | 2.2-719 | nc-17 | 3 | nc-1.6 | Suíza | [15] |
| | 1 | 1036 | 50 | 3 | 6-13 | Reino Unido | [51] |
| | - | - | - | 10 | nc-15 | Reino Unido | [50] |
| | - | - | - | 29 | 2.5-147 | India | [23] |
| | - | - | - | 12 | nd-64 | Japón | [24] |
| | 8 | 20-270 | nd | - | - | Canadá | [16] |
| <i>i</i> -Propil parabén | 1 | 2.2-5.4 | nd-0.9 | 1 | nd-0.8 | España | [42] |
| | - | - | - | 12 | nd-46 | Japón | [24] |
| <i>n</i> -Propil parabén | 2 | 77-1945 | nd-39 | - | - | España | [17] |
| | 1 | 2784 | nc | 3 | nd | España | [41] |
| | 1 | 1227-1737 | nd | 1 | nd-105 | España | [42] |
| | 7 | 43-1540 | nc-28 | 3 | nc-5.8 | Suíza | [15] |
| | 1 | 1393 | 63 | 3 | 6-7 | Reino Unido | [51] |
| | - | - | - | 10 | nc-24 | Reino Unido | [50] |
| | - | - | - | 29 | nd-57 | India | [23] |
| | - | - | - | 9 | 5-3142 | China | [22] |
| | - | - | - | 12 | nd-207 | Japón | [24] |
| | 8 | 200-2430 | nd-40 | - | - | Canadá | [16] |
| | 2 | 760-2000 | nc-3.7 | - | - | EEUU | [52] |
| | <i>i</i> -Butil parabén | 1 | 40-106 | nd-2.7 | 1 | nd-4.8 | España |
| - | | - | - | 12 | nd-13 | Japón | [24] |
| 2 | | 83-390 | nc-3.6 | - | - | EEUU | [52] |

A.1. ASPECTOS GENERALES

Tabla II.2 (continuación). Rango de concentraciones o concentración media (ng L^{-1}) de los agentes antimicrobianos considerados en aguas residuales y fluviales.

| Concentración (ng L^{-1}) | N | Influyente | Efluente | N | Río | País | Ref. |
|--------------------------------------|---|------------|----------|----|---------|-------------|------|
| <i>n</i> -Butil parabén | 1 | 318 | nc | 3 | nd | España | [41] |
| | 1 | 88-172 | nd-2.4 | 1 | nd-6.4 | España | [42] |
| | 7 | 9.7-864 | nc-12 | 3 | nc-2.8 | Suíza | [15] |
| | 1 | 52 | nc | 3 | nc | Reino Unido | [51] |
| | - | - | - | 10 | nc-52 | Reino Unido | [50] |
| | - | - | - | 29 | nd-6.6 | India | [23] |
| | - | - | - | 9 | nd | China | [22] |
| | - | - | - | 12 | nd-163 | Japón | [24] |
| | 8 | 20-260 | nd-10 | - | - | Canadá | [16] |
| Bencil parabén | 2 | nd | nc | - | - | España | [17] |
| | 1 | nd-4.7 | nd-2.1 | 1 | nd-2.4 | España | [42] |
| | 7 | nc-4.1 | 0.2-16 | 3 | nc-4.4 | Suíza | [15] |
| | 1 | - | - | 12 | nd-2.3 | Japón | [24] |
| Triclosán, metil triclosán | | | | | | | |
| Triclosán | 2 | nd-87 | nd | - | - | España | [17] |
| | 1 | 343 | nd | 3 | nd | España | [41] |
| | 1 | 423-1142 | 141-178 | 1 | 58-138 | España | [42] |
| | 1 | 728 | 74-104 | 1 | 26-105 | España | [47] |
| | 8 | 231-12500 | 20-1283 | 16 | nd-285 | España | [25] |
| | 1 | 70 | 33 | 3 | 10-15 | Reino Unido | [51] |
| | - | - | - | 10 | nc-24 | Reino Unido | [50] |
| | 2 | 4800-7300 | 300-620 | 1 | nd-10 | Alemania | [44] |
| | - | - | - | 29 | 10-5160 | India | [23] |
| | - | - | - | 9 | 35-1023 | China | [22] |
| | 2 | 2700-11900 | 260-270 | - | - | Japón | [45] |
| | 1 | 497-6920 | - | - | - | Japón | [46] |
| | 8 | 870-1830 | 50-360 | - | - | Canadá | [16] |
| | 5 | 3800-16600 | 240-2700 | - | - | EEUU | [34] |
| | 1 | 6100 | 35 | 19 | nd-1600 | EEUU | [20] |
| Metil triclosán | 1 | nd | nc | 1 | nd | España | [47] |
| | 8 | nd-354 | nd-51 | 16 | nd-12 | España | [25] |
| | 2 | 1-2 | 7-14 | 1 | nd-5 | Alemania | [44] |
| | 5 | nc | nc | - | - | EEUU | [34] |
| Triclocarbán | 2 | nd-362 | nd | - | - | España | [17] |
| | 1 | 6700 | 110 | 19 | nd-6750 | EEUU | [20] |
| | - | - | - | 18 | nd-250 | EEUU | [53] |
| | 2 | 6650-6750 | - | 26 | nd-5600 | EEUU | [19] |

1.3.2. Lodos, biosólidos, suelos y sedimentos

En la **Tabla II.3** se recogen los niveles determinados para el triclosán, su derivado O-metilado, el triclocarbán y algunos parabenes en matrices sólidas ambientales: lodos procedentes de estaciones depuradoras, biosólidos, suelos (mayoritariamente agrícolas) y sedimentos fluviales y marinos.

El triclosán ha sido uno de los agentes antimicrobianos más estudiados en muestras de esta naturaleza. Se estima que entre un 30 y un 50% del compuesto presente en las aguas residuales se adhiere a los lodos durante los tratamientos de depuración, de ahí las concentraciones tan elevadas detectadas en esta matriz (hasta $55 \mu\text{g g}^{-1}$ de residuo seco en un estudio estadounidense [49]). En suelos agrícolas y sedimentos fluviales sus niveles descienden considerablemente, aunque superaron los 100 ng g^{-1} en algunos casos [30,36,40].

A pesar de su mayor hidrofobicidad, la distribución del derivado O-metilado del triclosán en matrices sólidas ambientales ha sido evaluada con una frecuencia significativamente menor. Las concentraciones determinadas para este compuesto en lodos son en general muy inferiores a las del compuesto de partida, alcanzando $1 \mu\text{g g}^{-1}$ en un estudio en el que los niveles de triclosán oscilaron entre 0.53 y $15.6 \mu\text{g g}^{-1}$ [34]). En los tres únicos estudios considerados que evaluaron su presencia en suelos agrícolas, sedimentos fluviales y sedimentos marinos, sus niveles oscilaron entre 0.3 y 3.8 ng g^{-1} [37], entre no detectado y 450 ng g^{-1} [54] y entre no detectado y 11 ng g^{-1} [31], respectivamente.

Al igual que el triclosán, el triclocarbán ha sido cuantificado a concentraciones muy elevadas en lodos y biosólidos de EEUU (hasta 51 y $36 \mu\text{g g}^{-1}$ [55,56]) a diferencia de las muestras procedentes de otros países [33,35,57]. En sedimentos, sus niveles descienden sensiblemente [33].

Debido a su mayor polaridad y menor persistencia, los parabenes han sido detectados con menor frecuencia que el triclosán y el triclocarbán en muestras sólidas ambientales. Núñez et al. [58] determinaron las concentraciones de varios parabenes en suelos de diversa naturaleza y encontraron que estaban comprendidos entre 0.18 y 6.35 ng g^{-1} , siendo el metil y el *n*-propil parabén los más abundantes en la mayoría de las muestras. Este último compuesto fue detectado también por Ferreira et al. [59] en un suelo de jardín a 1.5 ng g^{-1} (datos no incluidos en la **Tabla II.3**). En lodos, el único estudio considerado mostró valores de entre 46 y 202 ng g^{-1} para el metil parabén, entre 6 y 10 ng g^{-1} para el *n*-propil parabén y entre no detectado y 5 ng g^{-1} para el bencil parabén [35].

A.1. ASPECTOS GENERALES

Tabla II.3. Rango de concentraciones o concentración media (ng g^{-1}) de los agentes antimicrobianos considerados en lodos, biosólidos, suelos y sedimentos. N: número de muestras incluidas en el estudio; nd: inferior al límite de detección; nc: inferior al límite de cuantificación.

| Concentración (ng g^{-1}) | N | Tipo muestra | Concentración | País | Ref. |
|--------------------------------------|-----------------|-------------------|---------------|-----------|--------|
| Parabenes | | | | | |
| Metil parabén | 1 | Lodo | 46-202 | España | [35] |
| <i>n</i> -Propil parabén | 1 | Lodo | 6-10 | España | [35] |
| Bencil parabén | 1 | Lodo | nd-5 | España | [35] |
| Triclosán, metil triclosán | | | | | |
| Triclosán | 1 | Lodo | 1300-1490 | España | [35] |
| | 19 | Lodo | 54-2987 | España | [37] |
| | 3 | Lodo | 420-5400 | España | [32] |
| | 3 | Lodo | 865-5940 | Escocia | [33] |
| | 20 | Lodo | 400-8800 | Alemania | [48] |
| | 2 | Lodo digerido | 10000-24600 | Irlanda | [36] |
| | 2 | Lodo activado | 620-1450 | Canadá | [57] |
| | 1 | Lodo | 20000-55000 | EEUU | [49] |
| | 5 | Lodo | 530-15600 | EEUU | [34] |
| | 4 | Biosólidos | 680-11550 | Canadá | [57] |
| | 94 | Biosólidos | 12640 | EEUU | [56] |
| | 1 | Biosólidos | 12100-18800 | EEUU | [60] |
| | 5 | Suelo agrícola | 0.8-4.7 | España | [37] |
| | 3 | Suelo agrícola | nd-178 | Irlanda | [36] |
| | 26 | Suelo agrícola | nd-66.6 | EEUU | [60] |
| | 3 | Suelo agrícola | nd-160 | EEUU | [40] |
| | 3 | Sedimento fluvial | nd-36 | España | [32] |
| | 6 | Sedimento marino | 0.27-131 | España | [30] |
| | 30 | Sedimento marino | 5-27 | Australia | [31] |
| | Metil triclosán | 19 | Lodo | 4-311 | España |
| 5 | | Lodo | nc-1030 | EEUU | [34] |
| 5 | | Suelo agrícola | 0.3-3.8 | España | [37] |
| 9 | | Sedimento fluvial | nd-450 | Alemania | [54] |
| 30 | | Sedimento marino | nd-11 | Australia | [31] |
| Triclocarbán | | | | | |
| Triclocarbán | 1 | Lodo | 5-7 | España | [35] |
| | 3 | Lodo | 516-2829 | Escocia | [33] |
| | 2 | Lodo activado | 2170-4820 | Canadá | [57] |
| | 1 | Lodo digerido | 51000 | EEUU | [55] |
| | 4 | Biosólidos | 3050-5970 | Canadá | [57] |
| | 94 | Biosólidos | 36060 | EEUU | [56] |
| | 6 | Sedimento fluvial | nd-139 | Escocia | [33] |

1.3.3. Atmósferas interiores

Como en el caso de otros compuestos presentes en productos de uso doméstico, los agentes antimicrobianos considerados en la presente memoria han sido determinados en ambientes interiores de diversos países. Rudel et al. [38] aislaron varios disruptores endocrinos (entre ellos el metil, el etil y el *n*-butil parabén) en aire y polvo de 120 hogares estadounidenses y encontraron que el 90% de las muestras presentaban niveles cuantificables de metil parabén (valores medios de 2.9 ng m^{-3} y 900 ng g^{-1} , respectivamente), mientras que el etil y el *n*-butil parabén se mantenían por debajo de los límites de cuantificación en la mayoría de los casos considerados. En dos estudios diferentes, Canosa et al. [61,62] determinaron las concentraciones de metil, etil, *n*-propil, *n*-butil parabén y triclosán en dos series diferentes de 10 muestras de polvo procedentes de edificios españoles y obtuvieron unos valores medios de entre 76 ng g^{-1} (*n*-BuP) y 934 ng g^{-1} (TCS). En ambientes interiores canadienses, los niveles de parabenos más elevados correspondieron al metil parabén (media de $1 \text{ } \mu\text{g g}^{-1}$) y al *n*-propil parabén (460 ng g^{-1}); la concentración media del triclosán fue de 380 ng g^{-1} y el bencil parabén y el metil triclosán no superaron el límite de detección en ningún caso [63].

1.4. IMPLICACIONES PARA LA SALUD Y CONSIDERACIONES ECO-TOXICOLÓGICAS

Como ingredientes incluidos en la formulación de numerosos productos de consumo diario, la toxicidad de los agentes antimicrobianos considerados en la presente memoria ha sido evaluada, clásicamente, en mamíferos y humanos. De forma más reciente, su detección en diferentes compartimentos ambientales ha promovido la evaluación de sus potenciales efectos tóxicos sobre los ecosistemas.

1.4.1. Parabenes

Los parabenos absorbidos a través de la piel no se hidrolizan de forma completa (al contrario de lo que ocurre cuando son absorbidos en el tracto gastrointestinal), eliminándose con la orina en forma nativa [64] o, en el peor de los casos, acumulándose en los tejidos. Darbre et al. [65] aislaron estos compuestos en tumores de mama humanos y plantearon su posible relación con el desarrollo de cáncer de mama, presumiblemente favorecido por el uso de desodorantes y antitranspirantes. Esta hipótesis, no demostrada, se apoya en el descubrimiento de su actividad agonista estrogénica en 1998 por Routledge et al. [66], posteriormente corroborada por numerosos ensayos *in vitro* e *in vivo* [67] y que ha demostrado incrementarse con la longitud, la ramificación y la inclusión de anillos aromáticos en la cadena hidrocarbonada del grupo éster [66].

En el medio acuático, los efectos estrogénicos de estos compuestos se manifiestan porque inducen la síntesis de vitelogenina en peces macho [68]. Estudios con protozoos, peces e invertebrados han demostrado que, al igual que ocurre con las bacterias, su toxicidad aguda se incrementa al incrementar la longitud del sustituyente hidrocarbonado, siendo el bencil parabén el que origina la respuesta tóxica más elevada [24,68-70]. Entre los pocos trabajos que evalúan la toxicidad crónica de estos compuestos en organismos acuáticos, Dobbins et al. [69] llevaron a cabo ensayos con el crustáceo *Daphnia magna* y el pez *Pimephales promelas* y encontraron que también el butil y el bencil parabén eran los parabenes más tóxicos. Basándose en los efectos a corto y a largo plazo y teniendo en cuenta los niveles ambientales, estimaron unos cocientes de riesgo ($RQ = MEC/PNEC$; MEC = concentración ambiental medida; PNEC = concentración prevista sin efectos) de 0.00023 para el bencil parabén, 0.00011 para el *i*-butil parabén y 0.000090 para el metil parabén. Yamamoto et al. [24] realizaron estudios similares sobre algas (*Pseudokirchneriella subcapitata*), crustáceos (*Daphnia magna*) y peces (*Oryzias latipes*) y estimaron un valor máximo de RQ de 0.01 para el propil parabén, seguido del *n*-butil parabén (0.0086) y el metil parabén (0.0042). Estos datos sugieren un riesgo ecológico despreciable por parte de esta familia de antimicrobianos, si bien ambos autores señalan la importancia de evaluar su toxicidad a lo largo del ciclo de vida completo de los organismos, su efecto disruptor endocrino y su potencial efecto aditivo, o incluso sinérgico, cuando coexisten compuestos diferentes [24].

1.4.2. Triclosán

Una vez que penetra en el cuerpo (por contacto con la piel o las mucosas) el triclosán es rápidamente metabolizado a sus conjugados glucurónidos y sulfatos y excretado a través de la orina. La extensión y velocidad de su eliminación, junto con los bajos niveles a los que habitualmente se emplea, limitan su exposición al ser humano, que no es susceptible de verse afectado por trastornos genotóxicos, teratogénicos, mutagénicos o carcinogénicos directamente derivados del uso de productos conteniendo triclosán. No obstante, la similitud estructural de este compuesto con las hormonas tiroideas y con ciertos disruptores androgénicos y estrogénicos conocidos (como los bifenilos y los difenil éteres halogenados) ha despertado la preocupación sobre sus posibles efectos endocrinos. Ensayos con animales han demostrado que interactúa con los receptores de las hormonas tiroideas disminuyendo los niveles de tiroxina en el plasma [71]. Adicionalmente, actúa como disruptor de hormonas sexuales presentando actividad antiestrogénica o antiandrogénica según el ensayo considerado [72].

El mayor efecto tóxico descrito para este compuesto se produce en el caso de los organismos acuáticos [68]. Las algas son especialmente sensibles a la exposición a triclosán,

hasta el punto que de la concentración más alta sin efecto adverso observado (NOEC) para algunas especies (inferior a $1 \mu\text{g L}^{-1}$) está en ocasiones por debajo de las concentraciones registradas en aguas superficiales, lo que sugiere un importante riesgo ecológico. Los invertebrados y algunas especies de peces son también muy vulnerables a este agente antimicrobiano. Estudios con la trucha arcoíris (*Oncorhynchus mykiss*), el pez cebra (*Danio rerio*) y el medaka japonés (*Oryzias latipes*) demostraron que sus efectos tóxicos son mayores durante las primeras etapas del desarrollo; la NOEC para individuos adultos de esta última especie se estimó en $1.7 \mu\text{g L}^{-1}$, si bien para el resto de especies consideradas los valores de NOEC variaron entre 34.1 y $200 \mu\text{g L}^{-1}$. En exposiciones crónicas a concentraciones de triclosán ambientalmente relevantes, las algas y los invertebrados demostraron ser nuevamente los organismos más vulnerables, mientras que los peces y las plantas acuáticas no se vieron afectados. A niveles superiores a $250 \mu\text{g L}^{-1}$, el antimicrobiano resultó genotóxico para algas y bivalvos, y por encima de $300 \mu\text{g L}^{-1}$ demostró efectos adversos en el desarrollo y reproducción de varios tipos de peces.

Finalmente, el impacto del triclosán en los ecosistemas terrestres ha sido hasta la fecha escasamente considerado. Reiss et al. [73] llevaron a cabo una revisión de los 31 estudios publicados y concluyeron que, a las concentraciones a las que habitualmente se detecta en el suelo, no existe riesgo para ningún organismo terrestre, incluidos microorganismos, gusanos, plantas, pájaros y mamíferos.

1.4.3. Triclocarbán

Los estudios *in vitro* realizados hasta la fecha no muestran evidencia de que el triclocarbán presente actividad genotóxica, mutagénica o teratogénica en humanos [9].

En el medio acuático, sus efectos adversos han sido evaluados en algas, peces e invertebrados, siendo estos últimos los organismos más sensibles a su exposición: estudios a 7 días sobre el crustáceo *Ceriodaphnia dubia* permitieron estimar un valor de PNEC para esta especie de $0.146 \mu\text{g L}^{-1}$, inferior a la concentración de triclocarbán habitualmente detectada en el medio acuático y que llevó a establecer un Cociente de Riesgo para este compuesto de entre 0.009 y 0.34 [9].

El único trabajo publicado que ha evaluado sus efectos en ecosistemas terrestres concluye que las comunidades microbianas del suelo apenas se ven afectadas por la presencia de triclocarbán, mientras que para los gusanos de la especie *Eisenia fetida* la concentración letal (LC_{50}) alcanza los 40 mg Kg^{-1} [74].

1.5. PRODUCTOS DE TRANSFORMACIÓN

Una vez en el medio ambiente, los parabenos, el triclosán y el triclocarbán pueden experimentar diversas reacciones como consecuencia de procesos naturales o antropogénicos. En algunos casos, los productos de transformación resultantes presentan una toxicidad mayor que la del compuesto de partida, implicando un mayor riesgo medioambiental y una mayor necesidad de control sobre el mismo.

1.5.1. Productos de transformación de los parabenos

Los compuestos que contienen grupos hidroxilo fenólicos, como los parabenos y el triclosán, exhiben cinéticas de cloración favorables en presencia de concentraciones de cloro libre del orden de los pocos mg L^{-1} ; estos niveles son similares a las concentraciones residuales de cloro en agua de grifo e inferiores a las utilizadas para la depuración de aguas residuales o para la potabilización de aguas superficiales, de modo que la transformación puede tener lugar en diferentes localizaciones: en los hogares, en las estaciones depuradoras, en las plantas potabilizadoras, etc. En el caso de los parabenos, los productos resultantes de esta reacción son el 3-cloro-4-hidroxibenzoato de alquilo y el 3,5-dicloro-4-hidroxibenzoato de alquilo [75]. Análogamente, el bromuro presente en las aguas naturales de zonas costeras (a niveles de entre 50 y 400 ng mL^{-1} [76]) puede oxidarse a bromo por la acción del cloro dando lugar a la formación de los correspondientes hidroxibenzoatos de alquilo bromados.

Canosa et al. [75] demostraron la presencia en agua residual sin tratar de los derivados diclorados de los dos parabenos de mayor aplicación comercial, el metil parabén y el *n*-propil parabén. Posteriormente, Terasaki y Makino [77] detectaron dicloro-metil, dicloro-*i*-propil y monocloro-bencil parabén en aguas de piscina a concentraciones de hasta 28 ng L^{-1} . En un intento de evaluar sus efectos tóxicos, estos mismos autores determinaron que el potencial estrogénico de los parabenos disminuía al convertirlos en compuestos clorados [78]. En otro estudio, compararon la toxicidad aguda de los derivados mono- y diclorados con la de sus compuestos de partida mediante bioensayos con *Daphnia magna* y *Vibrio fischeri*, concluyendo que, si bien para este último organismo no se podía establecer una relación entre cloración y toxicidad, el test con *Daphnia magna* revelaba respuestas tóxicas mayores al aumentar el grado de halogenación de las especies analizadas [79].

En la presente memoria se ha considerado la determinación cuantitativa de 3 parabenos halogenados: el metil parabén monoclorado, el diclorado y el dibromado. Sus estructuras y propiedades físico-químicas se recogen en la **Tabla II.1**. Comparados con el parabén análogo, son más ácidos y más lipofílicos, mostrando una mayor tendencia a la bioacumulación que podría explicar en parte el mayor efecto tóxico descrito para estos

derivados en el bioensayo con *Daphnia magna*. Adicionalmente, y tal como se ha demostrado en la presente tesis doctoral, son más resistentes a la biodegradación y, por tanto, son eliminados menos eficazmente de las aguas residuales durante los tratamientos de depuración.

1.5.2. Productos de transformación del triclosán

Las dioxinas son, probablemente, los productos de transformación del triclosán más preocupantes. Se pueden generar por fotólisis [13], por ejemplo si el antimicrobiano está presente en aguas residuales expuestas a la radiación solar [80], o por combustión, como ocurre al incinerar los lodos procedentes de estaciones depuradoras [81].

Adicionalmente, y al igual que los parabenos, este compuesto se clora con facilidad en presencia de cloro libre. Como consecuencia de esta reacción, se forman clorofenoles e hidroxidifenil éteres tetra y pentaclorados que, de hecho, ya han sido detectados en agua residual [82].

Finalmente, y aunque los mecanismos biológicos implicados todavía no se conocen, diversos organismos pueden O-metilar el triclosán originando la formación de 5-cloro-2-(2,4-diclorofenoxi)-anisol, más comúnmente conocido como metil triclosán. Chen et al. [7] observaron que esta transformación la experimentaba alrededor del 1% del triclosán contenido en las aguas en condiciones aeróbicas, un porcentaje menor en condiciones anóxicas y que no tenía lugar en medios anaeróbicos. La estructura y propiedades físico-químicas más relevantes este compuesto se recogen en la **Tabla II.1**. En comparación con el antimicrobiano de partida, es más lipofílico y más estable frente a la degradación biológica y fotoquímica [14]. La mayor bioacumulación de uno u otro depende sin embargo del pH del medio en el que se encuentran y de las características fisiológicas del organismo evaluado. Varios estudios han demostrado su presencia en tejidos de seres vivos expuestos a descargas continuas de aguas conteniendo triclosán [39,83,84], aunque no está claro si la metilación es previa (tiene lugar en las aguas) o posterior a la incorporación de triclosán (dentro del organismo). En relación a sus efectos tóxicos, Farré et al. [85] llevaron a cabo bioensayos de inhibición de la luminiscencia de *Vibrio fischeri* en aguas residuales contaminadas con triclosán y metil triclosán y encontraron que ambos compuestos presentaban valores similares de concentración efectiva (EC_{50}): 0.28 y 0.21 $\mu\text{g mL}^{-1}$, respectivamente. Por el contrario, la toxicidad aguda en algas (*Daphnia magna* y *Scenedesmus subspicatus*) resultó sensiblemente inferior para el derivado metilado [68].

1.5.3. Productos de transformación del triclocarbán

La transformación ambiental del triclocarbán puede conducir a la liberación de metabolitos carcinogénicos como las anilinas mono y dicloradas [86]. La ruptura del enlace C-N (observada sólo a temperaturas y pHs elevados) origina la formación de aminas primarias aromáticas, que se asocian con el desarrollo de metahemoglobinemia en humanos [20].

2. PREPARACIÓN DE MUESTRAS DE AGUA

La preparación de muestra es una de las etapas clave dentro del proceso analítico. Su principal objetivo es aislar al analito de la matriz que lo contiene convirtiéndolo a un estado químico compatible con el sistema de determinación utilizado; en la medida de lo posible, se pretende también separarlo de especies potencialmente interferentes (mejorando la selectividad final del método) e incrementar su concentración (mejorando la sensibilidad).

En la presente tesis doctoral se ha abordado la determinación de parabenes, triclosán, metil triclosán y triclocarbán en agua y la determinación de triclosán y su derivado O-metilado en lodo y sedimento. Entre esta sección y la sección 3 se recoge una revisión bibliográfica de las metodologías de pretratamiento que han sido aplicadas para aislar los citados agentes antimicrobianos en muestras de esta naturaleza, ofreciendo una explicación más detallada de aquéllas que han sido utilizadas en la presente tesis: la extracción en fase sólida y una nueva versión miniaturizada (la microextracción con adsorbentes empaquetados) y la dispersión de la matriz en fase sólida.

2.1. EXTRACCIÓN LÍQUIDO-LÍQUIDO

La extracción líquido-líquido (*liquid-liquid extraction*, LLE) es la metodología clásica para aislar compuestos orgánicos en matrices acuosas. Está basada en la distribución o reparto de los analitos entre dos fases inmiscibles, generalmente un medio acuoso (la muestra) y un disolvente orgánico. La selectividad y eficacia del proceso dependen de la correcta elección de este disolvente, pero también de otros factores como el pH y la fuerza iónica [87]. Es una técnica lenta, laboriosa e implica un elevado consumo de disolventes orgánicos y numerosos problemas prácticos (como la formación de emulsiones) que dificultan su automatización. A pesar de ello, está incluida en numerosos métodos oficiales de análisis y sigue siendo ampliamente utilizada, aunque la tendencia es que vaya siendo sustituida por la extracción en fase sólida (SPE) u otras metodologías más novedosas.

Nishi et al. [88] optimizaron un procedimiento de LLE combinado con cromatografía de líquidos-espectrofotometría ultravioleta-visible (LC-UV-VIS) para determinar triclosán en aguas superficiales. Extrajeron 500 mL de muestra (previamente filtrada y acidificada a pH 2-3) con dos porciones de 50 mL de hexano; secaron el extracto con Na_2SO_4 , lo llevaron a sequedad y finalmente lo reconstituyeron con 0.5 mL de metanol, obteniendo una recuperación del 95% y un límite de cuantificación de 3 ng L^{-1} .

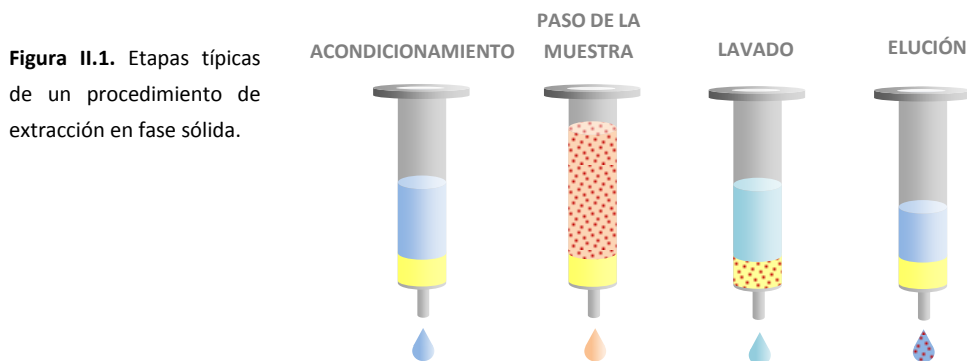
Bester [44] extrajo triclosán y metil triclosán en muestras de agua residual sin filtrar (1 L) mediante LLE con 10 mL de tolueno; para eliminar las trazas de agua, congeló la fase orgánica a $-20\text{ }^{\circ}\text{C}$ y, una vez seca, la concentró a 1 mL y la analizó mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS). Los porcentajes de recuperación fueron del 88% y el 102% para el compuesto nativo y el derivado O-metilado, respectivamente, y los límites de cuantificación se situaron en 3 y 0.3 ng L^{-1} , respectivamente.

2.2. EXTRACCIÓN EN FASE SÓLIDA

La extracción en fase sólida (*solid-phase extraction*, SPE) se desarrolló a mediados de los años 70 como alternativa a la extracción líquido-líquido convencional, convirtiéndose hoy en día en una de las técnicas de preparación de muestras líquidas más ampliamente utilizadas. Está basada en la adsorción de los analitos sobre una fase sólida y en su posterior elución con un disolvente adecuado; de esta forma se logra su concentración, su purificación e incluso su separación, si se realiza una elución fraccionada con distintos disolventes [89].

2.2.1. Etapas

En la **Figura II.1** se recogen las etapas típicas de un procedimiento de SPE [87]:



- *Acondicionamiento de la fase estacionaria.* Imprescindible cuando se trabaja con fases enlazadas sobre partículas de sílice, particularmente con fases apolares. Consiste en pasar uno o varios disolventes adecuados con el objetivo de solvatar los grupos funcionales y facilitar su posterior contacto con los componentes de la matriz. Entre los disolventes más empleados están el metanol (que interacciona tanto con los grupos silanoles polares como con los grupos funcionales ligados apolares), el acetonitrilo, el isopropanol, el tetrahidrofurano y la acetona. Con otras fases estacionarias, aunque no es necesario

realizar un acondicionamiento como tal, si es habitual pasar un pequeño volumen de disolvente para eliminar posibles impurezas.

- *Paso de la muestra.* A continuación se hace pasar la muestra a través del adsorbente, generalmente aplicando presión positiva o negativa para acelerar el proceso.
- *Lavado o elución de la matriz.* En ocasiones, se pasa un disolvente que permite eliminar algunas interferencias sin llegar a eluir los analitos, para lo cual se debe tener en cuenta no sólo la naturaleza del disolvente sino también la de la fase estacionaria.
- *Elución de los analitos.* Finalmente, los analitos son recuperados del adsorbente mediante una fase líquida que interacciona más fuertemente con ellos que las partículas de fase sólida. Si esta fase líquida es inmiscible con la muestra o con el disolvente de lavado (en el caso de llevar a cabo un lavado) es necesario secar previamente el adsorbente.

2.2.2. Dispositivos comerciales

En un principio, los adsorbentes empleados en SPE estaban empaquetados en pequeñas columnas de acero que se utilizaban como precolumnas para la preconcentración *on-line* de compuestos traza en cromatografía líquida. Posteriormente, estas columnas originaron el desarrollo de otras realizadas en materiales poliméricos, más baratas, desechables y diseñadas para extracciones *off-line*: los **cartuchos** [89]. En la actualidad, existen además otros dos formatos comerciales: los **cartuchos tipo jeringa** y los **discos o membranas**, comercializados por primera vez en 1989 por la compañía 3M [90].

En los dos primeros dispositivos, el adsorbente (entre 50 mg y 10 g) está empaquetado en un cuerpo de polietileno, polipropileno o vidrio que presenta una (en el caso de las jeringas) o dos terminales Luer (en el caso de los cartuchos). El terminal Luer es un estándar utilizado en el laboratorio clínico que ha sido aceptado a nivel analítico para otro tipo de aplicaciones y que facilita las conexiones entre materiales, permitiendo, por ejemplo, ensamblar varios cartuchos o varias jeringas en serie, acoplarles una jeringa de uso clínico o conectar el sistema de vacío. Los modos de operar con cualquiera de estos dos dispositivos son variables y dependen del volumen de la muestra y de la naturaleza de la matriz. La carga y la elución pueden realizarse por simple gravedad, aplicando presión o vacío, o incluso, en el caso de las jeringas, centrifugando mientras la fase líquida atraviesa el adsorbente [87,89].

En los discos, la fase sólida está inmovilizada en una membrana de politetrafluoroetileno o de fibra de vidrio de 0.5 mm de espesor, de forma que el proceso de extracción se lleva a cabo utilizando un aparato de filtración estándar y forzando el paso de la muestra y los disolventes con ayuda de una fuente de vacío. El menor tamaño de partícula (8-12 μm frente a 40-80 μm en los otros dos sistemas) asegura una mayor eficacia de extracción y

permite operar a flujos más elevados, convirtiéndolos en especialmente útiles en la concentración de grandes volúmenes de muestra. Para volúmenes inferiores, se comercializan pequeños discos de menor tamaño introducidos en el interior de cartuchos o jeringas [87,89]. No obstante, los cartuchos y jeringas clásicos continúan siendo los formatos más empleados en la mayoría de aplicaciones, probablemente debido a su menor precio y a la mayor disponibilidad de fases estacionarias [91].

2.2.3. Adsorbentes

La elección del adsorbente depende en gran parte de la clase de analitos a extraer y de sus grupos funcionales, aunque también está condicionada por la naturaleza de la matriz y las interacciones que ésta pueda establecer con los analitos y con el propio adsorbente. Los más comunes son los óxidos inorgánicos, las sílices enlazadas, el carbón grafitizado y los materiales poliméricos, aunque también se utilizan materiales de acceso restringido, adsorbentes de afinidad y polímeros impresos molecularmente [91-93].

Los *óxidos inorgánicos* más importantes en SPE son la sílice, la alúmina, el Florisil (silicato de magnesio sintético) y la tierra de diatomeas. Retienen fuertemente a los compuestos con grupos funcionales susceptibles de establecer puentes de hidrógeno (carboxilos, hidroxilos), en menor extensión a las moléculas con significativo carácter dipolar (ésteres, cetonas) y débilmente a las que presentan grupos funcionales polarizables (anillos aromáticos, alquenos). La sílice y la alúmina pueden actuar además como intercambiadores iónicos en muestras acuosas tamponadas [91].

Las *sílices enlazadas* se obtienen por reacción de organosilanos con sílice activada mediante la formación de enlaces silil-éter. En función de la polaridad del grupo enlazado se clasifican en tres grupos [87,89]:

- *Fase normal*: la unión con los analitos se establece a través de interacciones polares como puentes de hidrógeno o interacciones dipolo-dipolo (igual que en el caso de los óxidos inorgánicos). Sirven para aislar analitos más polares que la matriz, y se utilizan principalmente para la purificación de extractos orgánicos o para la extracción de analitos desde líquidos apolares (no se aplican a muestras acuosas).
- *Fase reversa*: el mecanismo de interacción, fundamentalmente, son las fuerzas de Van der Waals; permiten aislar analitos orgánicos de matrices polares como el agua.
- *Intercambiadores iónicos*: contienen grupos iónicos enlazados, de tal forma que las interacciones son de naturaleza electrostática y se establecen entre las moléculas de analito cargadas y los grupos funcionales del adsorbente con carga opuesta. Los intercambiadores catiónicos presentan grupos ácidos (fuertes, como los sulfonatos, o débiles, como los

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carboxilatos), mientras que los aniónicos están funcionalizados con grupos básicos (fuertes, como las aminas cuaternarias, o débiles, como las aminas primarias, secundarias o terciarias). Para que la retención sea efectiva, la matriz debe estar a un pH tal que ambos, analito y adsorbente, estén cargados, y además no debe presentar concentraciones elevadas de otras especies iónicas. La elución puede realizarse por neutralización (los iones retenidos se convierten a su forma molecular y se eluyen con un disolvente orgánico) o por desplazamiento (utilizando un disolvente con una concentración relativamente elevada de un ion desplazante, que se une a los sitios activos con más fuerza que los analitos).

Las sílices enlazadas se encuentran entre las fases más comunes en SPE, pero son inadecuadas para algunas aplicaciones. Pueden contener grupos silanol libres que interaccionan de forma irreversible con algunas clases de compuestos, son inestables a pH extremos y los volúmenes de rotura de las sílices en fase reversa para moléculas pequeñas y altamente polares son, con frecuencia, insuficientes para su cuantificación a niveles traza [91]. En la **Tabla II.4** se recogen algunas de las sílices enlazadas y algunos de los intercambiadores iónicos con base de sílice más utilizados en SPE [89].

Tabla II.4. Sílices enlazadas de fase normal, fase reversa e intercambio iónico comunes en SPE [89].

| Adsorbente | Tipo de fase | Estructura |
|--------------------------------------|--------------------------|--|
| Cianopropilsilano (CN) | Normal | -Si-CH ₂ -CH ₂ -CH ₂ -CN |
| Diolsilano (2OH) | Normal | -Si-(CH ₂) ₄ -CHOH-CH ₂ OH |
| Aminopropilsilano (NH ₂) | Normal | -Si-CH ₂ -CH ₂ -CH ₂ NH ₂ |
| N-propiletilen-diaminosilano (PSA) | Normal | -Si-(CH ₂) ₃ -NH-(CH ₂) ₂ -NH ₂ |
| Octadecilsilano (C ₁₈) | Reversa | -Si-(CH ₂) ₁₇ -CH ₃ |
| Octilsilano (C ₈) | Reversa | -Si-(CH ₂) ₇ -CH ₃ |
| Etilsilano (C ₂) | Reversa | -Si-CH ₂ -CH ₃ |
| Fenilsilano (PH) | Reversa | -Si-Ph |
| Ciclohexilsilano (CH) | Reversa | -Si-C ₆ H ₁₁ |
| Bencenosulfonil-propilsilano (SCX) | Intercambiador catiónico | -Si-(CH ₂) ₃ -Ph-SO ₃ ⁻ |
| Sulfonilpropilsilano (PRS) | Intercambiador catiónico | -Si-(CH ₂) ₃ -SO ₃ ⁻ |
| Carboximetilsilano (CBA) | Intercambiador catiónico | -Si-CH ₂ -COO ⁻ |
| Dietilaminopropilsilano (DBA) | Intercambiador aniónico | -Si-(CH ₂) ₃ -NH ⁺ -(CH ₂ -CH ₂) ₂ |
| Trimetilaminopropilsilano (SAX) | Intercambiador aniónico | -Si-(CH ₂) ₃ -N ⁺ -(CH ₃) ₃ |

El **carbón grafitizado** es un adsorbente no específico y no poroso con un área superficial específica de entre 100 y 200 m² g⁻¹. Permite concentrar compuestos orgánicos altamente polares mediante interacciones hidrofóbicas e interacciones π-π, pero además, se piensa que contiene complejos carbono-oxígeno que proporcionan sitios con carga positiva

para el intercambio de aniones; en este sentido se puede considerar como un adsorbente mixto (fase reversa y cambiador aniónico). Una de sus desventajas es la excesiva retención de algunos analitos, efecto que puede remediarse (si la retención no es irreversible) realizando la elución en sentido opuesto al de la carga [91].

Los *adsorbentes poliméricos*, como su nombre indica, presentan estructura polimérica y, por lo general, retienen a los analitos a través de interacciones π - π y fuerzas de Van der Waals. Los más comunes son los copolímeros de poliestireno-divinilbenceno (PS-DVB) macroporosos, caracterizados por un área superficial específica de hasta $800 \text{ m}^2 \text{ g}^{-1}$. Superan algunas de las limitaciones de las sílices enlazadas, ya que son estables en un intervalo de pH más amplio y se unen más fuertemente a las moléculas polares (basta con que tengan estructura orgánica); sin embargo, su capacidad y selectividad en la extracción de este tipo de compuestos es limitada, y la aparición de nuevos materiales poliméricos ha suscitado un considerable descenso en su popularidad [94]. Por ejemplo, las resinas Amberlite XAD-2 y XAD-4 (utilizadas comúnmente para la extracción de contaminantes orgánicos desde matrices acuosas) han sido desplazadas de muchas aplicaciones debido a los problemas que plantean: bajos volúmenes de rotura y flujo de muestreo y necesidad de inclusión de etapas de limpieza antes del paso de la muestra.

Un modo de mejorar la capacidad de adsorción de un polímero es incrementar el área superficial específica y, con ello, el número de puntos de interacción entre el adsorbente y el analito. Los polímeros altamente entrecruzados (conocidos por la nomenclatura anglosajona *hypercrosslinked*) persiguen este objetivo. Se sintetizan utilizando las polimerizaciones usuales para la obtención de los adsorbentes poliméricos macroporosos pero incluyendo un elevado contenido de agente entrecruzante (DVB); como alternativa, el post-entrecruzamiento de las cadenas lineales de poliestireno utilizando reactivos bifuncionales crea puentes estructurales entre los diferentes anillos fenílicos, produciendo resinas altamente microporosas y con un área superficial de hasta $2000 \text{ m}^2 \text{ g}^{-1}$. Estas propiedades las hacen mucho más retentivas que los polímeros macroporosos convencionales, aunque también les confieren gran hidrofobicidad y, en consecuencia, baja capacidad para la extracción de compuestos polares [94].

Una solución al marcado carácter apolar de los polímeros *hypercrosslinked* (y aplicable también a los polímeros macroporosos) es copolimerizar un monómero entrecruzante (generalmente DVB) con otro polar. En esta línea, Applied Separations desarrolló las fases Amberlite XAD-7 y Amberlite XAD-8, de metacrilato-DVB (MA-DVB). Posteriormente apareció Oasis HLB (*Hydrophilic lipophilic balance*), de Waters Corporation, que es un copolímero macroporoso basado en poli(*N*-vinilpirrolidona-divinilbenceno) (PVP-DVB) con un área superficial específica de $800 \text{ m}^2 \text{ g}^{-1}$. Oasis HLB es probablemente el adsorbente polimérico más

utilizado debido a su capacidad para extraer compuestos con un amplio rango de polaridad y, de hecho, ha sido empleado en cuatro de los trabajos desarrollados en esta tesis doctoral. Presenta la ventaja adicional de ser íntegramente hidratable, con lo que la etapa previa de acondicionamiento no es necesaria y los analitos pueden recuperarse totalmente aunque la fase llegue a secarse durante o tras el procesamiento de la muestra. Además del metacrilato y la PVP, otras casas comercializan copolímeros hidrofílicos con base de poliamida (SampliQ OPT, Agilent Technologies) [94].

Otra alternativa para incrementar la polaridad de los polímeros de PS-DVB es modificar químicamente su estructura con grupos funcionales polares (acetilo, hidroximetilo, sulfónico, benzoilo). De esta forma, se reduce además la tensión interfacial entre la superficie polimérica y la fase acuosa, facilitando el contacto entre la matriz de la muestra y el adsorbente. Uno de los clásicos es Isolute ENV+ ($1100 \text{ m}^2 \text{ g}^{-1}$) comercializado por International Sorbent Technology (IST) como polímero de PS-DVB hidroxilado. Más adelante, Phenomenex comercializó Strata X ($800 \text{ m}^2 \text{ g}^{-1}$) que es PS-DVB modificado con grupos pirrolidona. Varian, en colaboración con Polymer Laboratories, ofrece Bond Elut Plexa, que, según información de los proveedores, es un adsorbente con un gradiente de polaridad a lo largo de su superficie, yendo desde una superficie exterior hidroxilada hacia un núcleo central hidrofóbico (descripción que concuerda con la de un polímero químicamente modificado con grupos hidroxilo [94]). No obstante y, hasta la fecha, ninguno de estos adsorbentes hidrofílicos ha tenido el impacto que ha causado Oasis HLB, probablemente debido a su aparición más reciente.

Finalmente, dentro las fases poliméricas no sólo se ha buscado incrementar su capacidad, sino también mejorar su selectividad. En este sentido, la aparición de los adsorbentes en modo mixto supuso un cambio significativo. Estos materiales combinan un esqueleto polimérico con grupos iónicos y, de esta forma, disponen de dos tipos de interacciones: las de fase reversa y las de intercambio iónico. Las interferencias y los analitos pueden eluirse así de forma separada durante las etapas de lavado y elución, respectivamente, seleccionando adecuadamente el pH y el disolvente en cada una de ellas. La compañía Waters fue pionera en el desarrollo de adsorbentes poliméricos en modo mixto con las fases Oasis MCX y Oasis MAX, basadas en la fase Oasis HLB pero modificadas químicamente con grupos sulfónico y amina cuaternaria, respectivamente, y clasificadas como intercambiadores catiónico (MCX) y aniónico (MAX) fuerte. Los primeros fueron utilizados en la presente tesis doctoral para aislar drogas de abuso en aguas residuales. Posteriormente, la misma compañía comercializó la versión de intercambio catiónico débil (Oasis WCX, con grupos carboxílico) y aniónico débil (Oasis WAX, con grupos piperacina). La **Tabla II.5** recoge las características más relevantes de algunos de los adsorbentes poliméricos comerciales más utilizados [94].

Tabla II.5. Características más relevantes de diferentes adsorbentes poliméricos comerciales [94].

| Clase | Adsorbente | Proveedor | Estructura | Área Superficial (m ² g ⁻¹) |
|---------------------------------|-----------------|---------------------|--------------------|--|
| Macroporoso | Amberlite XAD-2 | Applied Separations | PS-DVB | 300 |
| | Amberlite XAD-4 | Applied Separations | PS-DVB | 750 |
| | PLRP-S-10 | Polymer Lab. | PS-DVB | 500 |
| | Strata SDB-L | Phenomenex | PS-DVB | 500 |
| Hypercrosslinked | Chromabond HR-P | Macherey-Nagel | PS-DVB | 1200 |
| | Envi-Chrom P | Supelco | PS-DVB | 800-950 |
| | LiChrolut EN | Merck | PS-DVB | 1200 |
| Monómero | Amberlite XAD-7 | Applied Separations | MA-DVB | 450 |
| hidrofílico | Amberlite XAD-8 | Applied Separations | MA-DVB | 310 |
| | Oasis HLB | Waters | PVP-DVB | 830 |
| | Porapak RDX | Waters | PVP-DVB | 550 |
| Químicamente modificados | Isolute ENV+ | IST | PS-DVB-OH | 1100 |
| | Strata X | Phenomenex | PS-DVB-Pirrolidona | 800 |
| | Bond Elut Plexa | Varian&Polymer Lab. | PS-DVB-OH | 450 |

Las fases descritas anteriormente permiten la separación y preconcentración de grupos amplios de compuestos orgánicos, por lo que no siempre es fácil la determinación final de los analitos de interés. Entre los adsorbentes más selectivos se encuentran los materiales de acceso restringido, los adsorbentes de afinidad y los polímeros impresos molecularmente.

Los *materiales de acceso restringido* presentan poros de pequeño diámetro cuya superficie interna contiene una fase ligada, afin al analito. La superficie externa de las partículas es no adsorbente y compatible con la matriz de la muestra (hidrofílica en el caso de matrices acuosas) de forma que, cuando ésta se pasa a través de la fase sólo las moléculas de menor tamaño entran en los poros y sólo aquéllas afines a la fase ligada quedan retenidas. Se utilizan habitualmente en la purificación de extractos biológicos para separar los analitos de las macromoléculas presentes en la muestra [91].

Los *adsorbentes de afinidad* están constituidos por un soporte inerte sobre el que se inmovilizan enzimas, anticuerpos u hormonas capaces de interactuar selectivamente con sus correspondientes sustratos, antígenos o receptores (los analitos). Los más comunes, los inmunoadsorbentes, se basan en interacciones reversibles antígeno-anticuerpo en las que pueden verse involucradas diferentes tipos de fuerzas: puentes de hidrógeno, enlaces iónicos, fuerzas hidrofóbicas o fuerzas de Van der Waals. La elución se realiza, como en el caso de los intercambiadores iónicos, con agentes desplazantes o variando el pH del medio. La extraordinaria selectividad de estos materiales permite llevar a cabo la extracción, la

concentración y la purificación de los analitos en un solo paso, originando extractos más limpios que los obtenidos con otras fases estacionarias y resultando especialmente útiles para procesar matrices muy complejas. Sus principales desventajas radican en su baja estabilidad y en la dificultad y el coste de su síntesis, que limitan su diversidad comercial y, con ello, sus aplicaciones prácticas [91].

Los *polímeros impresos molecularmente (MIPs)* son polímeros altamente entrecruzados y muy estables que poseen propiedades de reconocimiento molecular selectivo. Se obtienen mediante la polimerización de monómeros en presencia de una molécula plantilla, de forma que, al separarla, se crean sitios vacantes en la matriz polimérica susceptibles de retener moléculas muy similares a ella [91].

Los principales componentes implicados en la síntesis de un MIP son el agente entrecruzante, la molécula plantilla y el monómero. De la elección de este último dependerá la posterior habilidad del polímero para interaccionar selectivamente con el analito, interacción generalmente establecida a través de enlaces de hidrógeno o fuerzas iónicas; los más comunes son el ácido metacrílico (para la retención de compuestos básicos) y la 4-vinilpiridina (para compuestos ácidos) [92]. El agente entrecruzante proporciona estabilidad mecánica y controla la porosidad de la fase resultante. Aunque hay varios disponibles, el más ampliamente utilizando es el etilenglicol-dimetacrilato. Entre los métodos de síntesis se distinguen fundamentalmente cuatro: la coordinación metal-ión, la impresión covalente, la semicovalente y la impresión no-covalente. La diferencia entre ellos radica en el tipo de unión que se establece entre la molécula plantilla y los monómeros durante la polimerización (y, por ende, entre el analito y el polímero durante el proceso de extracción) siendo la impresión no covalente la utilizada con mayor frecuencia [95].

La primera aplicación de los MIPs a SPE (abreviada como MISPE) data del año 1994 y corresponde a la determinación de pentamidina en orina [96]. Desde entonces, se han desarrollado nuevos materiales para la extracción de diferentes compuestos que han sido aplicados a matrices de naturaleza biológica y medioambiental. En esta última línea, en la presente tesis doctoral se ha utilizado una fase disponible comercialmente para la determinación de drogas anfetamínicas en aguas residuales. Los principios de operación son los mismos que con cualquier otro adsorbente clásico de SPE: se acondiciona, se pasa la muestra, se realiza un lavado para eliminar impurezas y se eluyen los analitos. El lavado es clave en la extracción desde muestras acuosas: dado que en este medio las interacciones polares son más débiles y la adsorción menos selectiva que en un disolvente orgánico, el paso de un disolvente aprótico mantiene los puentes de hidrógeno que ligan el analito al polímero a la vez que arrastra las demás impurezas orgánicas. La adición de una pequeña cantidad de

ácido puede facilitar la ruptura de interacciones entre algunas impurezas y la matriz polimérica, pero es un proceso delicado porque puede implicar también la ruptura de las uniones con el analito.

Algunos MIPs tienen selectividades y constantes de afinidad muy altas, semejantes a las de los sistemas de reconocimiento natural antígeno-anticuerpo; sin embargo, comparados con los inmunoabsorbentes presentan una serie de ventajas: menor coste y tiempo de preparación, mayor reproducibilidad de síntesis y mayor duración. Sus principales inconvenientes radican en la escasa variedad comercial de la que se dispone y en su baja capacidad; adicionalmente, su extrema selectividad los hace inadecuados en procedimientos multianalito [91,92].

2.2.4. Ventajas e inconvenientes de SPE

La SPE supera muchas de las limitaciones de la LLE: permite llevar a cabo la extracción y concentración de los analitos en un solo paso, reduce el consumo de disolventes orgánicos, evita la formación de emulsiones, es aplicable a un gran número de analitos y matrices diferentes, debido a la extensa variedad de adsorbentes disponibles, y es fácilmente automatizable. Además, los porcentajes de recuperación y los factores de preconcentración conseguidos son generalmente elevados, es posible llevar a cabo una purificación simultánea a la extracción y es compatible con el análisis cromatográfico. Sin embargo, el precio de los cartuchos incrementa el coste total del análisis, el volumen de muestra a procesar es considerable y, en comparación con las técnicas de microextracción, el consumo de disolventes no es despreciable.

2.2.5. Aplicaciones de SPE a la determinación de agentes antimicrobianos en agua

La SPE se encuentra entre las técnicas más utilizadas para la extracción de agentes antimicrobianos en matrices acuosas. En la **Tabla II.6** se recogen algunos de los estudios que han empleado esta metodología para aislar parabenos, triclosán, metil triclosán y triclocarbán en muestras de agua.

Los cartuchos-jeringa Oasis HLB en sus múltiples tamaños (60, 200, 500 mg), son los más recurridos para este conjunto de compuestos, tanto en métodos desarrollados para pocos analitos [20,82] como en métodos multicomponente [52]. Los volúmenes de rotura con este tipo de fase son más bajos para las especies más polares, aspecto que puede limitar el volumen de muestra a concentrar cuando se determinan metil o etil parabén. Con aguas residuales, este volumen está limitado además por la posible obstrucción que puede experimentar el cartucho debido a la retención de materia orgánica disuelta; algunos autores proponen reducir esta retención ajustando el pH de la muestra a 7 [97]. Adicionalmente, las

interferencias retenidas pueden eliminarse parcialmente mediante una etapa de lavado del adsorbente previa a la elución de los analitos (con agua, para eliminar sales, y/o metanol o hexano, para compuestos orgánicos) [17] o mediante una purificación posterior del extracto obtenido (e.g. con sílice) [82]. Entre los disolventes empleados para la elución se encuentran el metanol, el diclorometano o el acetato de etilo.

Además de Oasis HLB, otros de los materiales poliméricos utilizados para la extracción en fase sólida de agentes antimicrobianos en aguas son los copolímeros de PS-DVB (SDB-XC y Bio Beads SM-2), los polímeros químicamente modificados Isolute ENV+, Strata X y Bond Elut Plexa, y los adsorbentes en modo mixto Oasis MCX y Oasis MAX. Pedrouzo et al. [17] compararon la efectividad de los cartuchos de reciente aparición Bond Elut Plexa (200 mg) con los Oasis HLB de 500 mg en la extracción de 4 parabenos, triclosán, triclocarbán y 5 filtros UV en muestras de agua; encontraron que las recuperaciones obtenidas con el primer adsorbente era superiores para todos los compuestos (especialmente para los más polares, metil y etil parabén) al extraer 500 mL de agua fluvial. Al reducir el volumen de muestra para el análisis de aguas residuales, sin embargo, no detectaron diferencias significativas en los porcentajes de recuperación alcanzados con ambas fases, tan sólo una pequeña variación en el flujo de paso de muestra, inferior con Bond Elut Plexa debido al menor tamaño de partícula (45 frente a 60 μm).

Entre los adsorbentes no poliméricos, el octadecilsilano (sílice enlazada a C_{18}) es el más empleado en la extracción de los agentes antimicrobianos incluidos en la presente memoria, fundamentalmente en el caso del triclosán y su derivado O-metilado. Los disolventes de elución utilizados son el acetato de etilo, el metanol, el diclorometano o la acetona, entre otros. Mc Avoy et al. [34] combinaron esta fase con Florisil para aislar triclosán, metil triclosán y tres difenil éteres polibromados hidroxilados (closanos) en aguas residuales, obteniendo, en el caso del triclosán y el metil triclosán, recuperaciones del 79 y el 70%, respectivamente.

Recientemente, Beltrán et al. [98] desarrollaron dos polímeros impresos molecularmente para la extracción de parabenos: sintetizaron el primero mediante la estrategia de impresión semicovalente empleando el metil parabén como molécula plantilla; para el segundo utilizaron el butil parabén y la impresión convencional no-covalente como estrategia de síntesis. Ambos adsorbentes demostraron mayor afinidad por esta clase de bactericidas que un polímero control no impreso molecularmente. Posteriormente, desarrollaron un procedimiento de SPE con el segundo MIP para la extracción de parabenos en aguas y compararon los resultados con los obtenidos utilizando un adsorbente comercial (Oasis HLB): extrayendo 500 mL de agua fluvial e incluyendo una etapa posterior de lavado con 1 mL de 2-propanol, el MIP consiguió cromatogramas más limpios que la fase HLB y porcentajes de recuperación superiores para el butil parabén.

Tabla II.6. Determinación de agentes antimicrobianos en aguas mediante extracción en fase sólida.

| Análitos | Muestras | Pretratamiento | Extracción | Postratamiento | Detección | %R | Ref. |
|---|--|--|--|--|--------------------------|---|------|
| MeP, EtP, <i>n</i> -PrP, BzP, TCS | 500 mL (río) 250 mL (ef.) 100 mL (inf.) Puntuales | Filtración | Bond Elut Plexa 200 mg (río) Oasis HLB 500 mg (inf., ef.) Acond.: 5 mL MeOH, 2 mL H ₂ O Lavado: 5 mL 15% MeOH/H ₂ O Elución: 5 mL MeOH, 5 mL DCM | Conc. a 3-4 mL Dilución a 5 mL con H ₂ O Filtración | UPLC- (ESI)- MS/MS | 69-101 (río) 20-92 (ef.) 27-89 (inf.) | [17] |
| MeP, EtP, <i>n</i> -PrP, <i>n</i> -BuP, BzP (+ otros compuestos) | 250 mL (río) 200 mL (ef.) 100 mL (inf.) Integr., 24 h | Filtración Ad. 5% MeOH | Oasis HLB 200 mg Acond.: HX, MTBE:2-PrOH (1:1), MeOH, H ₂ O Elución: 3 mL MTBE:2-PrOH (1:1), 3 mL MeOH | Conc. a 0.25 mL Ad. 0.25 mL MeOH:H ₂ O (1:1) Conc. a 0.25 mL | LC- (ESI)- MS/MS | 86-126 (no específica) | [15] |
| MeP, EtP, <i>n</i> -PrP, <i>n</i> -BuP, TCS (+ otros PPCPs, y drogas) | 1000 mL (río) 250 mL (ef., inf.) Puntuales | A pH 2 Ad. 500 mg Na ₂ EDTA Filtración | Oasis MCX 60 mg Acond.: 2 mL MeOH, 2 mL 2% HCOOH/H ₂ O Lavado: 2 mL 2% HCOOH/H ₂ O Elución: 2 mL MeOH, 2 mL 5% NH ₄ OH/MeOH | Evap. a sequedad Reconstitución en 0.5 mL H ₂ O (con TrBA y HOAc) | UPLC- (ESI)- MS/MS | 40-126 (río) 8-85 (ef.) 6-72 (inf.) | [51] |
| MeP, <i>n</i> -PrP, <i>n</i> -BuP, TCS (+ otros PCPs) | 500-1000 mL (río) Puntuales | Filtración A pH 7 | Supelclean ENVI-18 500 mg Acond.: 3 x 2 mL EtOAc, MeOH, H ₂ O Lavado: 2 mL 5% MeOH/H ₂ O Elución: 3 x 2 mL EtOAc:MeOH (1:1) | Evap. a sequedad Derivat. con MSTFA Evap. a sequedad Reconstitución en 0.1 mL EtOAc | GC-(EI)- MS | 74-110 (agua grifo) | [22] |
| MeP, EtP, <i>n</i> -PrP, <i>n</i> -BuP, TCS (+ otros PPCPs) | 1000 mL (ef., inf.) Integr., 24 h | Filtración A pH 3 | Oasis MAX 150 mg Acond.: 4 mL MeOH, 10 mL H ₂ O pH 3 Lavado: 5 mL MeOH:NaOAc _(aq) Elución: 5 mL MeOH | Ad. 25 µL NaOAc _(aq) Conc. a 0.5 mL Ad. 1 mL <i>tert</i> -butil-éter Conc. a 0.1 mL Derivat. con PFPA | GC-(EI)- MS | 87-99 (agua destilada) | [16] |
| <i>n</i> -PrP, <i>i</i> -BuP, TCS, TCC (+ 20 compuestos) | 50-100, 500 mL (ef., inf.) Integr., 24 h | Filtración | Oasis HLB 200 mg Acond.: 5 mL DCM, 5 mL MTBE, 5 mL MeOH, 5 mL H ₂ O Lavado: 5 mL H ₂ O Elución: 5 mL MeOH:MTBE (1:9), 5 mL DCM | Conc. a 2 mL Ad. 1 mL isoctano Conc. a 0.5 mL | LC- (ESI)- MS/MS | 58 (ef.) | [52] |

Tabla II.6 (continuación). Determinación de agentes antimicrobianos en aguas mediante extracción en fase sólida.

| Análitos | Muestras | Pretratamiento | Extracción | Postratamiento | Detección | %R | Ref. |
|-----------------------------|--|--|---|--|---|--|------|
| TCS, TCC | 1000 mL, río, ef., inf, subterránea, grifo. Puntuales | Centrifugación | Oasis HLB 60 mg Elución: 4 mL MeOH:Acetona (1:1) 10 mM HOAC | Evap. a sequedad Reconstitución en 1 mL MeOH:Acetona | LC-(ESI)- MS/MS | 95-103 (sólo TCC) | [20] |
| TCS (+ otros clorofenoles) | 1000 mL (río, inf.) Puntuales (río) Integr., 12 h (inf.) | A pH 2.5 Filtración | Oasis HLB 60 mg Elución: 2 mL EtOAc | Purificación con sílice Derivat. con MTBSTFA | GC-(EI)- MS | 88 (río) 102 (ef.) 98 (inf.) | [82] |
| TCS, MTCS (+ 3 closanos) | 100 mL (ef.) 50 mL (inf.) Integr. 24 h | Ad. 20 mL EtOH A pH < 2 Sonicación | C ₁₈ 1 g - Florisil 0.5 g Acond.: 6 mL HX, 6 mL EtOH, 10 mL 20% EtOH/H ₂ O Lavado: 10 mL 20% EtOH/H ₂ O Elución: 1 mL EtOAc, 1 mL tolueno, 8 mL HX | Conc. a 0.5 mL Derivat. con TMSDEA | GC-(EI)- MS | 79-88 (inf.) 36-87 (ef.) | [34] |
| TCS (+ bifeníol) | 50 mL (ef., inf.) Puntuales | Filtración A pH 4 | Sep-Pak C ₁₈ 500 mg, Isolute Env+ 200 mg, Oasis HLB 200 mg Acond.: 7 mL MeOH, 6 mL H ₂ O (C ₁₈) 2 mL EtOAc, 2 mL acetona, 4 mL H ₂ O (Env+) 5 mL EtOAc, 5 mL MeOH, 4 mL H ₂ O (HLB) Elución: 2 x 4 mL MeOH, 2 x 5 mL Acetona | Evap. a sequedad Reconstitución en 1 mL EtOAc (GC), 1 mL ACN:H ₂ O (LC) | GC-(EI)- MS GC-(NCI)- MS LC-(ESI)- MS/MS | 84 (C ₁₈) 69 (Env+) 68 (HLB) | [30] |
| TCS, MTCS | 1000 mL (río, lago) Puntuales | A pH 2 | Bio Beads SM-2 Elución: MeOH:DCM | Conc. Derivat. con diazoetano Purificación con sílice | GC-(EI)- MS | 50-90 | [14] |
| TCS (+ otros PPCPs, Y EDCs) | 4000 mL (canal) Puntuales | Filtración A pH 2 | SDB-XC Empore disk Acond.: 50 mL MeOH, 50 mL DCM, 50 mL MeOH, 50 mL H ₂ O Elución: 50 mL MeOH, 50 mL DCM, 50 mL MeOH | Conc. a 0.5 mL Purificación con sílice Derivat. con BSTFA | GC-(EI)- MS | 60 | [99] |

PCPs: productos de cuidado personal; PPCPs: fármacos y PCPs; EDCs: disruptores endocrinos; ef.: efluente; inf.: influente; integr.: integradas; Ad.: adición; Acond.: acondicionamiento; Evap.: evaporación; Conc.: concentración; Derivat.: derivatización; MeOH: metanol; EtOH: etanol; 2-PrOH: 2-propanol; DCM: diclorometano; HX: hexano; MTBE: metil-*tert*-butil-éter; HCOOH: ácido fórmico; HOAC: ácido acético; EtOAc: acetato de etilo; NaOAc: acetato sódico; NH₄OH: hidróxido amónico; ACN: acetonitrilo; TrBA: tributilamina; PFPA: anhídrido pentafluoropropiónico; MSTFA: *N*-metil-*N*-(trimetilsilil)trifluoroacetamida; MTBSTFA: *N*-metil-*N*-(*tert*-butildimetilsilil)trifluoroacetamida; TMSDEA: *N*-(*tert*-butildimetilsilil)-*N*-dietilamina; BSTFA: *N*,*O*-bis(trimetilsilil)trifluoroacetamida.

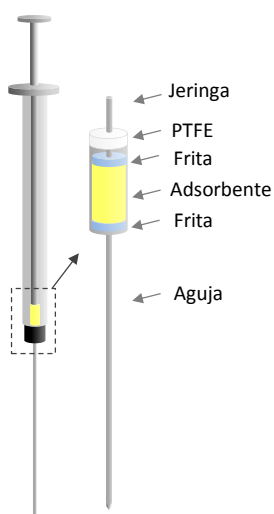
2.3. MICROEXTRACCIÓN CON ADSORBENTES EMPAQUETADOS

La microextracción con adsorbentes empaquetados (*microextraction by packed sorbents*, MEPS) es una miniaturización de la SPE originalmente desarrollada por Abdel-Rehim en 2004 [100]. Dado que se basa en sus mismos principios, los métodos tradicionales de SPE pueden transferirse fácilmente a MEPS realizando un reajuste en los volúmenes de muestra y eluyente utilizados. Los objetivos perseguidos son reducción en el consumo de disolventes y muestra (requerimiento típico en bioanálisis), disminución del tiempo de extracción, automatización, simplicidad y reducción de costes [101].

2.3.1. Formato y adsorbentes

En MEPS, se utiliza una pequeña cantidad de adsorbente (desde 1 a 4 mg) empaquetada entre dos filtros de polipropileno (20 μm de tamaño de poro) en el cuerpo de una jeringa de vidrio (100-250 μL) o, alternativamente, acomodada en un pequeño cartucho situado entre el cuerpo y la aguja (**Figura II.2**). Al tratarse de una jeringa cromatográfica, la extracción puede llevarse a cabo de forma totalmente automatizada mediante el empleo de un inyector automático convencional, sin necesidad de utilizar un robot adicional como ocurre con la SPE *on-line*. Además, el volumen de disolvente necesario para la elución es suficientemente bajo como para poder ser inyectado directamente en el cromatógrafo, permitiendo la integración de las etapas de preparación de muestra y determinación [102]. El dispositivo comercial es suministrado por SGE Analytical Science y está disponible para preparación de muestra *off-line* y *at-line* [103].

Figura II.2. Esquema de un sistema de MEPS.

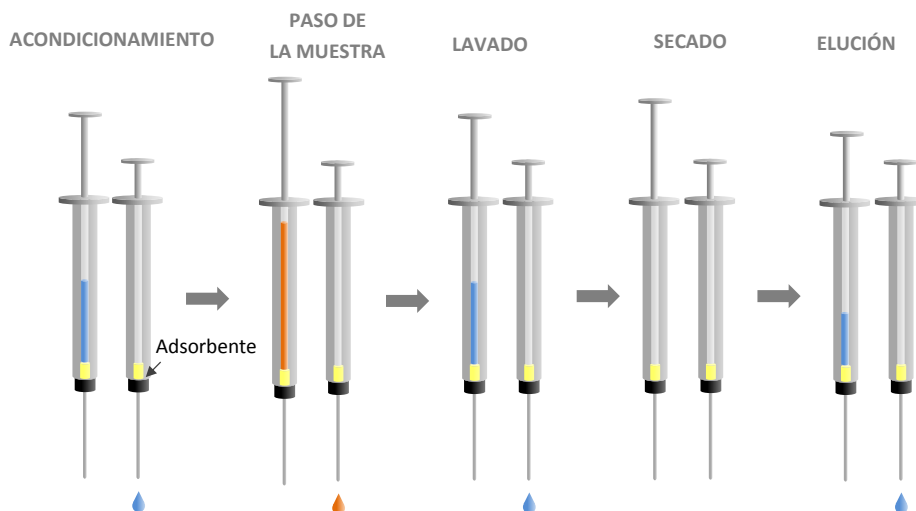


Los adsorbentes empleados son los mismos que se utilizan en SPE clásica. En las aplicaciones publicadas hasta la fecha, los más habituales han sido las sílices enlazadas en fase reversa (C_2 , C_8 , C_{18}), pero también se han utilizado intercambiadores catiónicos fuertes (sílice enlazada a grupos sulfónicos), adsorbentes con base de carbón, polímeros de PS-DVB o polímeros impresos molecularmente. Para evitar sobrepresiones, se prefieren adsorbentes formados por partículas relativamente grandes (40-60 μm); la cantidad empaquetada suele ser 1 mg, aunque dependiendo de las condiciones pueden emplearse cantidades superiores (hasta 4 mg) [104].

2.3.2. Etapas

Las etapas de un procedimiento de MEPS (**Figura II.3**) son análogas a las descritas en SPE [102,104]:

Figura II.3. Etapas típicas de un procedimiento de microextracción con adsorbentes empaquetados.



- *Acondicionamiento de la fase estacionaria.* Aunque no es imprescindible (debido probablemente a la pequeña cantidad de fase utilizada o a que la muestra suele ser bombeada repetidamente a través de ella) sí es frecuente acondicionar el adsorbente con metanol y agua o con el mismo disolvente utilizado para su posterior lavado.
- *Paso de la muestra.* Previamente al paso de la muestra, deben eliminarse las partículas que contenga (por centrifugación, filtración), ajustar el pH de la misma para favorecer la adsorción de los analitos (o dificultar la retención de interferencias) y, en el caso de muestras biológicas, reducir su viscosidad para prevenir la oclusión de la fase. La carga puede llevarse a cabo de dos formas: aspirando un pequeño volumen (entre 25 y 250 μL) a través del adsorbente y expulsándolo al desecho (realizando esta operación con una o múltiples porciones de disolución fresca) o aspirando y expulsando repetidas veces la muestra dentro del mismo vial que la contiene. En este segundo modo (*multiple draw-eject cycles*) la concentración de los analitos en la muestra se va diluyendo a medida que avanza el proceso de carga, de forma que para algunos puede superarse el volumen de corte antes de que su extracción haya sido completada; como contrapartida, el volumen de muestra requerido puede ser tan bajo como permita la jeringa, resultando especialmente útil para algunas aplicaciones bioanalíticas. En ambos casos, la aspiración se realiza lentamente ($10\text{-}20 \mu\text{L s}^{-1}$) para favorecer el contacto entre la matriz y el adsorbente.

- *Lavado*. Las interferencias débilmente retenidas pueden eliminarse pasando un pequeño volumen (50-100 μL) de agua pura o de agua conteniendo un pequeño porcentaje de un disolvente orgánico (5-10% de metanol, 2-propanol o acetonitrilo). Este porcentaje, el pH y el número de ciclos deben establecerse cuidadosamente para evitar pérdidas de analitos.
- *Secado*. Antes de la elución, se recomienda incluir una etapa de secado de la fase estacionaria, e.g. bombeando aire repetidamente.
- *Elución de los analitos*. La elución se lleva a cabo con un pequeño volumen de disolvente (20-50 μL) que puede ser introducido directamente en el inyector del cromatógrafo. La naturaleza y el pH de este disolvente deben fijarse de manera tal que se consigan las mayores recuperaciones con el menor volumen posible. Es necesario además que sea miscible con la muestra (ya que es difícil que el adsorbente se seque por completo) y compatible con el sistema de medida utilizado (miscible con la fase móvil si la determinación posterior se lleva a cabo mediante cromatografía de líquidos, y volátil si se lleva a cabo mediante cromatografía de gases). Entre los utilizados con mayor frecuencia para la elución de compuestos orgánicos se encuentran el metanol, el 2-propanol y el acetonitrilo (puros o combinados con disoluciones ácidas o básicas), en el caso de determinaciones mediante cromatografía de líquidos y el acetato de etilo para la combinación de MEPS con cromatografía de gases.

2.3.3. Lavado del adsorbente tras la inyección

Una característica que diferencia a MEPS de la SPE clásica es que los adsorbentes pueden (y deben) reutilizarse en múltiples ocasiones antes de ser desechados. La pequeña cantidad de fase sólida empleada puede lavarse fácil y eficientemente entre inyecciones de forma totalmente automática mientras que, por el contrario, este proceso de lavado no resulta simple ni efectivo con los cartuchos de SPE convencionales, generalmente desechables. En la bibliografía se han descrito aplicaciones en las que el MEP ha sido reutilizado más de 100 veces con matrices como plasma u orina y más de 400 con muestras de agua, aunque la extensión de esta reutilización debe evaluarse para cada aplicación concreta [105].

Para el proceso de lavado, se recomienda aspirar y desechar repetidamente (4-5 ciclos) dos disoluciones diferentes. La primera (disolución fuerte) pretende limitar el efecto memoria derivado de la reutilización del adsorbente y debe ser un disolvente afín a los analitos (o incluso el propio disolvente empleado en la elución); e.g. metanol o acetona, puros o conteniendo entre un 10 y un 20% de 2-propanol o un porcentaje menor (0.2%) de una disolución ácida o básica (dependiendo de la naturaleza de los analitos). La segunda (disolución débil) puede ser agua pura o conteniendo un 5% de metanol [102,104]. De esta forma, se ha conseguido reducir el efecto memoria a menos de un 0.1%.

2.3.4. Ventajas e inconvenientes de MEPS

En comparación con SPE, MEPS reduce el tiempo de preparación de muestra de varias horas a minutos, disminuye el consumo de muestra y disolventes orgánicos, puede ser completamente automatizada y las fases adsorbentes pueden ser reutilizadas hasta más de 100 veces. Frente a otras técnicas de microextracción, presenta también algunas ventajas: su automatización es más sencilla que en el caso de la extracción con barras agitadoras (SBSE) o SPE con puntas de pipeta, y es más robusta y menos sensible a la naturaleza de la matriz que la microextracción en fase sólida (SPME), resultando más adecuada para muestras complejas como plasma u orina. Entre sus inconvenientes, se incluyen la posibilidad de formación de burbujas de aire y las dificultades derivadas de su utilización *off-line*, ya que una velocidad de aspiración baja y constante es crucial para conseguir recuperaciones cuantitativas y reproducibles [101,105].

2.3.5. Aplicaciones de MEPS a la determinación de contaminantes orgánicos en aguas

MEPS ha demostrado ser una técnica prometedora para la extracción de diversos compuestos (principalmente drogas y metabolitos) en matrices biológicas: orina [106-108], plasma [109-111], sangre [112,113], saliva [114,115] y extractos de pelo [116]. En menor extensión, se ha utilizado también para aislar contaminantes orgánicos en muestras en agua, aplicaciones entre las que se incluye un trabajo desarrollado en la presente tesis doctoral para la determinación de parabenos, triclosán y metil triclosán. A continuación se presenta un resumen de las condiciones empleadas en varios estudios medioambientales y las principales características de los métodos analíticos optimizados.

El-Beqqali et al. [117] aislaron diversos hidrocarburos aromáticos policíclicos en aguas mediante MEPS acoplada *at-line* con GC-MS. Para la extracción utilizaron 60 ciclos de aspiración-expulsión de 50 μL de muestra, realizados a una velocidad de 20 $\mu\text{L s}^{-1}$; la elución se llevó a cabo con 30 μL de metanol (inyectados directamente en el cromatógrafo) calentando simultáneamente el adsorbente (C_8 , 1 mg) a 40 $^\circ\text{C}$. Las recuperaciones obtenidas se situaron en torno al 70% y los límites de detección oscilaron entre 1 y 5 ng L^{-1} . Comparada con SPME y SBSE, MEPS consiguió reducir el tiempo de preparación de muestra entre 20 y 100 veces.

Morales-Cid et al. [118] estudiaron la viabilidad de la combinación de MEPS con la espectrometría de masas con Transformada de Fourier-resonancia de ion ciclotrón (FT-ICR-MS) para la determinación de materia orgánica disuelta en agua fluvial y de mar. En este caso, la fase sólida utilizada fue C_{18} (4 mg) y el proceso de extracción consistió en aspirar y desechar 2.210 mL de muestra acidificada a una velocidad de 0.1 mL min^{-1} , eluyendo los analitos con

260 μL de metanol e introduciendo 50 μL directamente en la interfase de electrospray (ESI) del MS. Este procedimiento *at-line* demostró ser más reproducible que el procedimiento análogo de extracción manual (*off-line*).

Möder et al. [119] desarrollaron un método de MEPS acoplado a GC-MS para la determinación de filtros UV, fragancias policíclicas y cafeína en aguas. Compararon la eficacia de dos adsorbentes (C_8 y C_{18} , 1 mg) extrayendo 800 μL de muestra (8 ciclos de 100 μL) y realizando la elución con 2 porciones de 25 μL de acetato de etilo, inyectadas consecutivamente en el inyector de grandes volúmenes (LVI) del cromatógrafo; obtuvieron porcentajes de recuperación de entre 46 y 114% en el primer caso y entre 65 y 109% en el segundo, con límites de detección (34-96 ng L^{-1}) inferiores a los niveles de concentración de los analitos en el medio ambiente.

Prieto et al. [120] determinaron 41 contaminantes orgánicos en muestras de agua residual tratada y nieve mediante MEPS-LVI-GC-MS. Utilizaron cartuchos de MEPS de 2 mg de C_{18} y llevaron a cabo la extracción con $8 \times 100 \mu\text{L}$ de muestra (aspirados y expulsados al desecho, $10 \mu\text{L s}^{-1}$); los analitos fueron eluidos con 2 porciones de acetato de etilo:hexano 1:1, la primera de 50 μL y la segunda de 25 μL , inyectadas consecutivamente en el cromatógrafo. Comparada con un procedimiento de SPE utilizando 200 mg de adsorbente (C_{18}), 100 mL de muestra y 30 mL de disolvente de elución (acetato de etilo:hexano 1:1), MEPS proporcionó límites de detección más bajos (0.2-266 ng L^{-1} frente a 0.2-736 ng L^{-1}) y porcentajes de recuperación y desviaciones estándar relativas similares (superiores al 75 e inferiores al 21%, respectivamente, para cada parámetro).

Estos mismos autores [121] sintetizaron un polímero impreso molecularmente para aislar compuestos relacionados con la fluoroquinolona y lo utilizaron como adsorbente en un procedimiento de MEPS y en otro de SPE convencional (MISPE) para el análisis de aguas residuales. En ambos métodos, el MIP demostró una alta selectividad hacia los compuestos objetivo. MEPS permitió además su cuantificación a niveles de los ng L^{-1} utilizando un volumen de muestra de 1600 μL (aspirados y expulsados al desecho en ciclos de 100 μL , $5 \mu\text{L s}^{-1}$) y $2 \times 25 \mu\text{L}$ de metanol:ácido acético 1:1 como disolvente de elución. En este caso, la etapa de extracción no se integró con la de determinación, realizada mediante cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS).

Similarmente, en un estudio posterior [122], desarrollaron un método de MEPS acoplado a LVI con derivatización en el inyector-GC-MS para la determinación de diversos disruptores endocrinos en aguas. Compararon la eficacia de dos adsorbentes (C_{18} y MIP) en la extracción de $8 \times 100 \mu\text{L}$ de muestra ($10 \mu\text{L s}^{-1}$) realizando la elución con una única porción de 50 μL de acetato de etilo:diclorometano 7:3. Para la mayoría de analitos, los porcentajes de

recuperación oscilaron entre 75 y 109% con C_{18} y entre 81 y 103% con el MIP; los límites de detección entre 0.02 y 87 ng L⁻¹ y entre 1.3 y 22 ng L⁻¹, respectivamente, y las desviaciones estándar relativas entre 4 y 22% para ambas fases.

2.4. OTRAS TÉCNICAS DE MICROEXTRACCIÓN

Además de la microextracción con adsorbentes empaquetados, aplicada a la determinación de agentes antimicrobianos por vez primera en la presente tesis doctoral, se han utilizado otras técnicas de microextracción para aislar algunos de estos compuestos en muestras de agua. Una característica común a muchas de estas metodologías es que están basadas en procesos de equilibrio y, en consecuencia, sólo un porcentaje de los analitos es extraído a la fase aceptora. Esto contrasta con lo que ocurre en SPE o MEPS, donde, idealmente, todas las moléculas de analito quedan retenidas en el adsorbente.

Atendiendo a la naturaleza la fase aceptora, las técnicas de microextracción se pueden englobar en dos grandes grupos: microextracciones con fase aceptora líquida y microextracciones con fase aceptora sólida.

2.4.1. Técnicas de microextracción con fase aceptora líquida

La microextracción en fase líquida surge como resultado de aplicar los principios de la microextracción en fase sólida (SPME), miniaturización y extracción de los analitos mediante procesos de equilibrio, a la extracción líquido-líquido (LLE). En la práctica hay varias formas de alcanzar estos objetivos, dependiendo de si la disolución extractante se pone directamente en contacto con la muestra o de si ambas se separan por medio de una membrana polimérica que puede adoptar diferentes configuraciones [123].

La *microextracción con gota suspendida* (*single drop microextraction*, SDME) se incluye dentro de las técnicas de microextracción en fase líquida sin membrana. En este caso, la fase extractante es una microgota de disolvente orgánico (1-3 μ L) suspendida en el extremo de la aguja de una microjeringa y expuesta a la muestra acuosa (por inmersión o en espacio de cabeza). Una vez alcanzado el equilibrio de distribución entre las dos fases, la gota se retrae en el interior de la jeringa y se inyecta en el cromatógrafo. Desde su introducción en 1996, la SDME ha experimentado un rápido desarrollo justificado por su bajo coste y su elevada capacidad de preconcentración. Sus principales inconvenientes son la posible formación de emulsiones, que conducen a la disolución de la gota, y la limitada estabilidad de esta última [123].

Esta técnica fue utilizada por Fiamegos et al. [124] para extraer varios disruptores endocrinos fenólicos, entre ellos el metil parabén y el triclosán, en matrices acuosas. Para ello suspendieron 2 μL de una fase extractante (cloroformo:octanol 1:1 con etilcloroformiato) en 4 mL de una muestra acuosa conteniendo una disolución tampón (pH 10.5) y un agente formador de pares iónicos. En estas condiciones, los fenoles se desprotonan y se combinan con el catión del par iónico añadido, formando un nuevo par iónico que difunde a través de la interfase líquido-líquido hacia la fase orgánica; los fenolatos reaccionan entonces con el etilcloroformiato formando un compuesto neutro que queda retenido en esta fase. Tras 12 minutos, la gota de disolvente se retrae en la jeringa y se inyecta en el cromatógrafo. Las recuperaciones relativas oscilaron entre 75 y 108% y los límites de detección entre 0.2 y 1.3 ng mL^{-1} para GC-MS y entre 8.5 y 26.5 ng mL^{-1} para GC-FID (detección por ionización en llama).

Saraji et al. [125] aplicaron también la SDME seguida de GC-MS a la determinación de cinco parabenos en aguas. Utilizaron un volumen de muestra de 3 mL, 3 μL de disolvente orgánico extractante y 20 minutos de tiempo de muestreo; finalizada la extracción, retrajeron la gota y la mezclaron, en la propia jeringa, con 0.4 μL de BSA (*N,O*-bis(trimetilsilil)acetamida), como agente derivatizante. Las recuperaciones relativas variaron entre 73 y 99% y los límites de detección entre 1 y 15 ng L^{-1} .

Otra modalidad de microextracción en fase líquida sin membrana es la *microextracción líquido-líquido dispersiva* (*dispersive liquid-liquid microextraction*, DLLME). Desarrollada por Rezaee et al. en 2006 [126], está basada en la adición rápida a una muestra líquida de una mezcla binaria de dos disolventes: un extractante inmisible con ella (y preferiblemente más denso) y un dispersante miscible. Como consecuencia de esta adición, la fase extractante forma una niebla de finísimas gotitas que se dispersan en la disolución, acelerando el proceso de transferencia de analitos entre la muestra y el disolvente. La centrifugación de esta dispersión permite que las microgotas se agreguen y se depositen (si son más densas) en el fondo de la muestra; esta fase sedimentada se recoge con una microjeringa y se inyecta en el cromatógrafo.

Montes et al. [47] desarrollaron un procedimiento de DLLME para extraer triclosán y metil triclosán en aguas de diversa naturaleza. Como agente extractante-dispersante utilizaron una mezcla ternaria de metanol (1 mL), 1,1,1-tricloroetano (40 μL) y *N*-metil-*N*-(*tert*-butildimetilsilil)trifluoroacetamida (derivatizante, MTBSTFA, 40 μL), que adicionaron a un volumen de muestra de 10 mL. Tras la centrifugación (3 minutos) analizaron la fase sedimentada por GC-MS/MS, obteniendo un rendimiento de extracción en torno al 90% y límites de cuantificación entre 2 y 5 ng L^{-1} .

Farajzadeh et al. [127] aislaron diversos agentes conservantes, entre ellos metil, etil y *n*-propil parabén, en diferentes matrices mediante DLLME combinada con GC-FID. En este caso, la mezcla binaria se obtuvo combinando 20 μL de octanol con 0.5 mL de acetona, y la centrifugación se llevó a cabo durante 10 minutos a 6000 rpm. Para los parabenes considerados, las recuperaciones relativas oscilaron entre 25 y 72% y los límites de detección entre 5 y 15 ng mL^{-1} .

La *microextracción por emulsión asistida por ultrasonidos* (*ultrasound assisted emulsification-microextraction*, USAEME) combina los principios de DLLME con los de la extracción líquido-líquido asistida por ultrasonidos: prescinde del disolvente dispersante y emulsiona el extractante mediante la aplicación de radiación de ultrasonidos para favorecer el contacto con la muestra. Tras un tiempo controlado de irradiación, la disolución se centrifuga y se obtiene una fase sedimentada [128].

Esta técnica fue empleada por Regueiro et al. [41] para extraer triclosán, dos clorofenoles relacionados y cuatro parabenes en aguas de diversa naturaleza. Utilizaron un volumen de muestra de 10 mL y 100 μL de 1,1,1-tricloroetano como agente extractante (combinado con 200 μL de anhídrido acético para la derivatización in situ de los analitos). La extracción se llevó a cabo bajo sonicación (40 kHz, 100 W) durante 5 minutos, y la centrifugación (3 minutos, 3000 rpm) permitió sedimentar la fase extractante para su análisis por GC-MS/MS. Los porcentajes de recuperación relativa alcanzados oscilaron entre 85 y 100% y los límites de detección entre 4 y 28 ng L^{-1} .

La *microextracción en fase líquida con fibra hueca* (*hollow-fiber liquid phase microextraction*, HF-LPME) emplea membranas porosas en forma de varilla o en forma de U para separar la muestra acuosa del disolvente orgánico extractante; este disolvente impregna completamente los poros de la membrana facilitando el paso de los analitos a través de ella y permitiendo su concentración. Las fibras son baratas y normalmente se desechan después de cada uso, evitando problemas de contaminación cruzada entre muestras; además, su pequeño tamaño de poro impide la extracción de moléculas de elevado peso molecular y facilita la obtención de extractos limpios. Su principal desventaja radica en los tiempos de extracción, que pueden llegar a ser bastante largos para algunas aplicaciones [123].

Zhao et al. [129] desarrollaron un procedimiento de HF-LPME para extraer triclosán en agua de grifo y en agua de río. Empleando una jeringa, llenaron el interior de una fibra hueca con 5 μL de *n*-dodecano y la sumergieron en 10 mL de muestra durante 20 minutos bajo agitación constante. A continuación retrajeron el disolvente en la jeringa y lo transfirieron a un vial para su análisis por GC-MS, obteniendo unos porcentajes de recuperación relativa de 84% (agua de grifo) y 114% (agua fluvial) y un límite de detección de 20 ng L^{-1} .

La *microextracción líquido-líquido asistida por membranas* (*membrane-assisted liquid-liquid extraction*, MALLE) utiliza una membrana hidrofóbica no porosa para separar la muestra y la fase extractante; esta membrana actúa como una barrera de difusión que permite el paso de los analitos pero no de otras moléculas, proporcionando un alto grado de selectividad al proceso de extracción. Resulta especialmente adecuada para muestras muy complejas con un elevado contenido en materia orgánica, aunque no es aplicable a compuestos altamente hidrofílicos con baja capacidad para difundirse a través de la membrana. Las cinéticas de extracción pueden ser incluso inferiores a las de HF-LPME, por lo que se recomienda la utilización de temperaturas superiores a la ambiental [130].

Villaverde de Súa et al. [42] aplicaron esta técnica combinada con GC-LVI-MS/MS a la determinación de triclosán y siete parabenos (incluyendo los isómeros *iso*- del propil y el butil parabén) en muestras de agua. Utilizaron un volumen de muestra de 18 mL, 400 μ L de cloroformo como disolvente extractante y llevaron a cabo la extracción a 35 °C, agitando (500 rpm), durante 90 min. En estas condiciones, obtuvieron unas eficacias de extracción de entre 46 y 110% y unos límites de detección de entre 0.1 y 1.4 ng L⁻¹.

2.4.2. Técnicas de microextracción con fase aceptora sólida

La *microextracción en fase sólida* (*solid-phase microextraction*, SPME) utiliza un dispositivo a modo de jeringa que posee una fibra de sílice fundida recubierta de una fase estacionaria absorbente, adsorbente o una combinación de ambas. La fibra se expone a la muestra durante un tiempo controlado y, a continuación, los analitos se desorben térmicamente en el inyector de un cromatógrafo de gases o por disolución en un disolvente adecuado para su acoplamiento a cromatografía de líquidos. Es una técnica simple, de moderado coste (la fibra es reutilizable) y que alcanza un elevado poder de concentración. Entre sus inconvenientes destacan las bajas eficacias de extracción, la fragilidad de la fibra y la necesidad de incluir una etapa de pretratamiento en el caso de matrices muy complejas [87].

Canosa et al. [131] desarrollaron un método de SPME combinado con GC-MS/MS para aislar cinco parabenos (MeP, EtP, *n*-PrP, *n*-BuP y BzP) en aguas de diversa procedencia. Emplearon poliacrilato como fase extractante y realizaron el muestreo por inmersión directa de la fibra en 10 mL de muestra (pH 6, 150 mg mL⁻¹ de NaCl), durante 40 minutos, a temperatura ambiente y bajo agitación constante. Posteriormente, derivatizaron los parabenos absorbidos exponiendo la propia fibra a vapores de MTBSTFA durante 10 min. Las recuperaciones relativas (respecto a agua ultrapura) oscilaron entre 87 y 114% y los límites de cuantificación entre 1 y 25 ng L⁻¹.

Estos mismos autores optimizaron un procedimiento muy similar para la extracción de triclosán, metil triclosán y otros dos clorofenoles relacionados en muestras de agua [132]. Ajustaron las muestras (de 22 mL) a pH 4.5, sumergieron en ellas una fibra de poliacrilato durante 30 minutos y llevaron a cabo una derivatización en fibra de los grupos fenólicos mediante exposición de la misma a vapores de MTBSTA (temperatura ambiente, 10 min). Los porcentajes de recuperación relativos variaron entre 73 y 109% y los límites de cuantificación se mantuvieron por debajo de 10 ng L⁻¹.

La *extracción con barras agitadoras* (*stir-bar sorptive extraction*, SBSE) se basa en los mismos principios que la SPME pero utiliza un sistema de extracción diferente: una barra magnética recubierta del elemento adsorbente o absorbente (generalmente polidimetilsiloxano, PDMS). La extracción tiene lugar mientras la muestra líquida se agita con la barra; posteriormente la barra se retira y los analitos se desorben térmicamente o con un disolvente. SBSE emplea volúmenes de fase extractante entre 50 y 200 veces superiores a los empleados en SPME, lo que le permite alcanzar mayores eficacias de extracción y, en consecuencia, límites de detección inferiores. Por otro lado, el proceso de desorción térmica es más lento y requiere la utilización de una interfase especial para su acoplamiento a cromatografía de gases [123].

Silva et al. [133] aplicaron SBSE seguida de LC con detección con red de diodos (LC-DAD) para la determinación de triclosán en muestras de diversa naturaleza. Sumergieron las barras de PDMS en 25 mL de muestra (60 minutos, 1000 rpm) y posteriormente las sonicaron en acetonitrilo (60 minutos) para desorber el analito. El rendimiento de la extracción alcanzó el 78% y el límite de detección se situó en 100 ng L⁻¹.

Kawaguchi et al. [134] extrajeron este mismo compuesto en agua de río exponiendo las barras de PDMS a 10 mL de muestra durante 120 minutos y desorbiéndolas térmicamente en un cromatógrafo de gases acoplado a un espectrómetro de masas. Obtuvieron porcentajes de recuperación de entre 92 y 108% y un límite de detección de 5 ng L⁻¹.

Análogamente, Ferreira et al. [135] desarrollaron un procedimiento de SBSE con acilación in situ y detección por GC-MS para determinar triclosán, metil triclosán y cinco parabenos en muestras de agua (5 mL). Extendiendo la extracción a 60 minutos, las recuperaciones superaron el 79% y los límites de detección se mantuvieron entre 0.6 y 4 ng L⁻¹.

3. PREPARACIÓN DE MUESTRAS DE LODO, SUELO Y SEDIMENTO

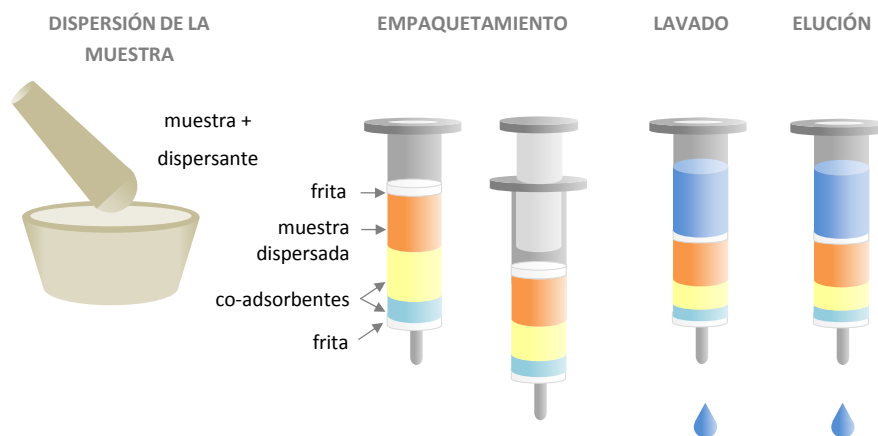
3.1. DISPERSIÓN DE LA MATRIZ EN FASE SÓLIDA

Barker et al. introdujeron en 1989 una nueva metodología de extracción: la dispersión de la matriz en fase sólida (*matrix solid-phase dispersion*, MSPD). Los principios operacionales son muy simples: la muestra (líquida o, más comúnmente, sólida) se dispersa en un mortero con un adsorbente sólido y la mezcla resultante se transfiere a una columna o cartucho para la elución de los analitos con un disolvente apropiado. La dispersión provoca la rotura mecánica de la estructura de la matriz y su distribución sobre las partículas de adsorbente, incrementando el área superficial expuesta al proceso de extracción; si el adsorbente consta, además, de una fase orgánica enlazada, su función es doble, ya que disuelve a los componentes de la muestra facilitando la disrupción completa de la misma [136].

3.1.1. Etapas

Un procedimiento clásico de MSPD consta de las siguientes etapas, descritas gráficamente en la **Figura II.4** [136]:

Figura II.4. Etapas típicas de un procedimiento de dispersión de la matriz en fase sólida.



- *Dispersión de la muestra.* La muestra líquida, semisólida o sólida se coloca en un mortero con el adsorbente y se dispersa mecánicamente hasta obtener una mezcla de aspecto homogéneo. El mortero y el pistilo han de ser de vidrio o ágata, ya que se ha demostrado que la porcelana y otros materiales porosos pueden producir pérdidas de analito. La

cantidad de muestra suele ser bastante pequeña (en torno a 0.5 g) y su relación con respecto a la masa de adsorbente suele estar comprendida entre 1/4 y 1/1. En algunas aplicaciones se ha empleado (adicionalmente o como propio agente abrasivo) un agente desecante como la sílice o el sulfato sódico anhidro, obteniéndose una mezcla seca muy adecuada si la etapa de elución se lleva a cabo con disolventes apolares.

- *Empaquetamiento.* Una vez que la muestra ha sido dispersada, el material resultante se transfiere a una columna o un cartucho-jeringa vacío de SPE provisto de una frita de acero inoxidable o polipropileno, un filtro de celulosa o un tapón de lana de vidrio en su parte inferior. Generalmente se coloca una segunda frita en la parte superior de la muestra y, a continuación, se procede a su compresión utilizando el émbolo de una jeringa. En esta etapa son aplicables los principios de una buena cromatografía: evitar la formación de canales preferentes en la columna y no comprimir el material en exceso.
- *Limpieza.* Antes de la elución o de forma simultánea a ella, es posible introducir una etapa de limpieza (descrita en detalle en el apartado 3.1.3) para eliminar interferencias.
- *Elución.* Finalmente, se lleva a cabo la elución de los analitos. Dado que toda la muestra está presente en la columna, es posible llevar a cabo una elución fraccionada de la misma, aislando diferentes compuestos o familias de compuestos mediante la utilización de disolventes diferentes. La mayoría de eluciones se lleva a cabo por gravedad, aunque es posible iniciar el flujo mediante la aplicación de presión positiva o negativa o controlarlo durante todo el proceso mediante el empleo de un sistema de vacío.

3.1.2. Variables que afectan a la eficacia de extracción

Agente dispersante. Las aplicaciones clásicas de MSPD emplean como agente dispersante un adsorbente con base de sílice. Estos materiales presentan la ventaja adicional de contener grupos silanoles libres capaces de formar enlaces de hidrógeno con las moléculas de agua, actuando simultáneamente como agentes dispersantes y desecantes. Dentro de las sílices, las utilizadas con mayor frecuencia han sido las sílices enlazadas de fase reversa (particularmente C₁₈ y C₈) que provocan la disolución de los componentes de la matriz sobre su superficie favoreciendo la completa disrupción de la muestra. Para aislar analitos más polares se han empleado adsorbentes de fase normal como sílices enlazadas a grupos polares (e.g. aminas) u óxidos inorgánicos no enlazados (la propia sílice, la alúmina, el Florisil). Estos últimos interaccionan con los componentes de la matriz solamente por adsorción y, en consecuencia, no producen su disolución; no obstante, sus propiedades adsorbentes pueden modificarse dependiendo de su contenido en agua y de su carácter ácido o básico [136,137].

Otra tendencia reflejada en la bibliografía apunta a la utilización de materiales inertes (arena, tierra de diatomeas, Celite), originando métodos más baratos pero a expensas de una

selectividad limitada, ya que solo está controlada por la solubilidad de los diferentes componentes de la muestra en el disolvente de elución. Finalmente, en algunos trabajos se han empleado dispersantes menos comunes como carbón grafitizado, adsorbentes poliméricos (XAD-7 HP, Oasis HLB), polímeros impresos molecularmente o nanotubos de carbono (*multiwalled carbon nanotubes*) [137,138].

Matriz. En MSPD, la muestra dispersada forma parte del sistema cromatográfico implicado en la separación de los analitos y, por ende, su composición afecta directamente a la eficacia y selectividad de dicha separación. Se ha observado que ciertas clases de analitos co-eluyen con los componentes de la matriz en una fracción dada, diferente a la prevista por su distribución relativa entre el soporte sólido y el disolvente de elución. En muchos casos, sin embargo, las polaridades relativas de los analitos y los co-eluyentes son bastante diferentes y estas potenciales interferencias pueden eliminarse mediante la introducción de una etapa de limpieza (apartado 3.1.3) previa o simultánea a la elución de los analitos [136,138].

Adicionalmente, puede ser necesario alterar el estado de ionización de los componentes de la muestra para garantizar ciertas interacciones con el soporte sólido o con el disolvente de elución. Esto puede conseguirse mediante la adición de ácidos, bases, sales, agentes quelatantes, antioxidantes, etc. durante la etapa de dispersión de la muestra o en el propio disolvente de elución [137].

Disolvente de elución. Durante la elución, la distribución de los analitos entre la fase sólida y el disolvente se rige por procesos de partición y/o adsorción similares a los que ocurren en una columna cromatográfica. Una adecuada estrategia debería recuperar eficazmente los analitos, evitando, en lo posible, la coelución de interferencias. Para la extracción de analitos apolares se han utilizado disolventes apolares como hexano, diclorometano o mezclas de ambos; para compuestos de polaridad media o alta, los más recurridos han sido el acetonitrilo, la acetona, el acetato de etilo o el metanol. Adicionalmente, el agua caliente también se ha aplicado con éxito para la extracción de contaminantes polares y moderadamente polares en matrices sólidas, fundamentalmente alimentos [137].

Temperatura y presión. Algunos autores han empleado sus equipos de PLE para elevar la temperatura y la presión de los disolventes extractantes en MSPD y, con ello, conseguir extracciones automáticas más rápidas y exhaustivas. Ambas técnicas comparten principios operacionales y, a veces, es difícil establecer una frontera entre ambas. De hecho, en PLE es frecuente dispersar la muestra con arena o tierra de diatomeas e incluso se han publicado aplicaciones que utilizan adsorbentes no inertes.

3.1.3. Etapa de limpieza

Incluso tras una cuidadosa selección del agente dispersante y del disolvente de elución, los extractos obtenidos con MSPD pueden no estar suficientemente limpios como para ser analizados de manera directa. En estos casos, es posible integrar una etapa de purificación mediante la colocación de un co-adsorbente en el fondo de la columna de MSPD o, alternativamente, en una segunda columna acoplada en serie. La función de este adsorbente es retener las interferencias permitiendo el paso de los analitos, por lo que su naturaleza dependerá de las propiedades de ambos: los materiales de fase normal (alúmina, sílice, Florisil) se utilizan habitualmente para retener interferencias polares en muestras dispersadas con C₁₈ u otros adsorbentes de fase reversa; para analitos con una elevada estabilidad química puede emplearse sílice acidificada (conteniendo hasta un 44% de ácido sulfúrico), que destruye los lípidos y proporciona extractos extraordinariamente limpios con matrices grasas. Por otro lado, los principales inconvenientes de este material son el riesgo de retención de los analitos en la capa de carbón originada por la oxidación de la muestra y la restricción que conlleva para seleccionar el disolvente de elución, ya que éste no debe reaccionar con el ácido sulfúrico. En la práctica, esto limita la elección a disolventes de polaridad baja o media, como alcanos, diclorometano o cloroformo [136,137].

Otra posibilidad para incrementar la selectividad de la extracción, alternativa o complementaria a la inclusión de una co-columna, consiste en introducir una etapa de lavado de la fase sólida (mezcla de adsorbente y muestra) antes de la elución de los analitos. Como ejemplo, el agua se ha utilizado para eliminar azúcares, sales y compuestos polares en matrices dispersadas con C₁₈, mientras que las interferencias apolares, como los lípidos, se han extraído con disolventes apolares como el hexano. Obviamente, si el lavado y la elución se llevan a cabo con disolventes inmiscibles, es necesario secar el adsorbente por completo entre estas dos etapas.

3.1.4. Ventajas e inconvenientes de MSPD

La MSPD es una técnica simple que, comparada con otras metodologías de extracción sólido-líquido, reduce considerablemente la manipulación de la muestra y, con ello, el tiempo total del análisis, limita el consumo de muestra y de disolventes orgánicos y no requiere el empleo de instrumentación costosa y específica, ya que las condiciones de extracción utilizadas (temperatura, presión) son generalmente suaves. Una adecuada combinación de agente dispersante y disolvente de elución proporciona, en general, resultados analíticos similares o incluso mejores que los obtenidos con otros métodos tradicionales. Su principal desventaja radica, sin embargo, en que no es automatizable y requiere la presencia del analista, como mínimo, durante las etapas de dispersión y empaquetamiento.

3.1.5. Aplicaciones de MSPD a la determinación de agentes antimicrobianos en muestras ambientales

La MSPD ha sido aplicada en la presente tesis doctoral para extraer triclosán y metil triclosán en muestras de lodo y sedimento.

Simultáneamente a la realización de este trabajo, Sánchez-Brunete et al. [37] desarrollaron un método muy similar para aislar estos dos mismos compuestos en lodos y suelos. Las muestras (1 g de lodo o, alternativamente, 2 g de suelo) se combinaron con 2 g de C_{18} y 1 g de sulfato sódico anhidro y, una vez dispersadas, se transfirieron a una columna provista de una capa de Florisil (1 g) en su parte inferior. La elución se llevó a cabo con 13 mL de acetonitrilo y la determinación posterior se realizó mediante GC-MS. Las recuperaciones estuvieron comprendidas entre 98 y 101% y los límites de detección entre 0.1 y 0.12 $ng\ g^{-1}$ para lodo y entre 0.05 y 0.08 $ng\ g^{-1}$ para las muestras de suelo.

Anteriormente, Canosa et al. [62] habían aplicado la MSPD, combinada con GC-MS/MS, a la determinación de triclosán y cuatro parabenes en polvo: tomaron 0.5 g de muestra, los mezclaron con la misma cantidad de sulfato sódico anhidro y los dispersaron con 1.25 g de C_{18} . Para evitar la coelución de interferencias polares incluyeron un co-adsorbente (Florisil, 2 g) en la parte inferior del cartucho; las interferencias apolares fueron eliminadas mediante un lavado con 10 mL de diclorometano y, posteriormente, los analitos se eluyeron con 10 mL de acetonitrilo. Los porcentajes de recuperación obtenidos oscilaron entre 80 y 114% y los límites de cuantificación, concentrando el extracto a 1 mL, entre 0.6 y 2.6 $ng\ g^{-1}$.

Estos mismos autores desarrollaron un procedimiento similar para aislar triclosán y su derivado O-metilado en biota y alimentos [139]. En este caso, las muestras (0.5 g) se mezclaron con 2 g de sulfato sódico anhidro y se dispersaron con 1.5 g de sílice; el material resultante fue transferido a un cartucho conteniendo 3 g de sílice impregnada en ácido sulfúrico (10%, p/p) y los analitos se eluyeron con 10 mL de diclorometano. Las recuperaciones variaron entre 77 y 120% y los límites de cuantificación entre 1 y 2 $ng\ g^{-1}$.

3.2. OTRAS TÉCNICAS DE EXTRACCIÓN

En la bibliografía, la extracción de triclosán y metil triclosán en muestras de lodo, suelo y sedimentos ha sido abordada mediante el empleo de técnicas diferentes a la MSPD: la extracción con fluidos supercríticos, la extracción con líquidos presurizados, la extracción asistida por microondas y la extracción asistida por ultrasonidos, entre otras [87]. En la **Tabla II.7** se recogen algunas de las aplicaciones de estas técnicas a la determinación de agentes antimicrobianos en las matrices anteriormente citadas.

La **extracción con fluidos supercríticos** (*supercritical fluid extraction, SFE*) emplea como agente extractante un fluido en condiciones supercríticas. Las propiedades de estos fluidos, intermedias entre las de un gas y un líquido, incrementan su poder de solvatación y penetración, favoreciendo la extracción selectiva de los analitos. Su principal desventaja radica en la dificultad de extraer compuestos polares o iónicos y en la complejidad a la hora de optimizar los métodos (es necesario controlar un elevado número de parámetros).

La **extracción con líquidos presurizados** (*pressurized liquid extraction, PLE*) combina la utilización de temperaturas y presiones elevadas con disolventes en estado líquido para proporcionar una extracción rápida y eficaz. Como contrapartida, su selectividad es limitada y generalmente es necesario introducir etapas de limpieza, que pueden integrarse o no en el proceso de extracción. Requiere la utilización de instrumentación específica.

La **extracción asistida por microondas** (*microwave assisted extraction, MAE*) utiliza la energía de microondas para elevar la temperatura de la muestra y, con ello, facilitar el proceso de extracción. La aplicación de esta energía puede llevarse a cabo en vasos abiertos (a presión atmosférica) o en recipientes sellados (bajo control de presión y temperatura). Es una técnica rápida que implica un consumo moderado de disolventes; sin embargo, no es especialmente selectiva y los equipos empleados son relativamente costosos.

En la **extracción asistida por ultrasonidos** (*ultrasound assisted extraction, USAE*), la muestra se sumerge en un disolvente adecuado y se somete a radiación ultrasónica. Como consecuencia de esta irradiación, se elevan la temperatura y la presión de la suspensión, incrementándose paralelamente la solubilidad de los analitos en el disolvente y favoreciendo la penetración de este último en los poros de la matriz sólida. Comparada con la extracción Soxhlet, los tiempos de extracción son más reducidos, pero es menos reproducible y el consumo de disolventes sigue siendo elevado.

Tabla II.7. Extracción de agentes antimicrobianos en lodo y sedimentos mediante SFE, PLE, MAE y USAE.

| Analitos | Muestras | Pretratamiento | Extracción | Postratamiento | Detección | %R | Ref. |
|---|-------------------|---|---|--|--------------------------|--|-------|
| TCS, MTCS (+ 3 closanos) | Lodo | Liofilización | SFE 1 g sílice + 0.5 g lodo liofilizado + 0.5 g sílice CO ₂ , 380 bar, 40 °C, elu.: 1 mL HX 10-20 °C | Derivat. con TMSDEA | GC-(EI)- MS | 84-108 | [34] |
| TCS, MTCS (+ otros compuestos) | Lodo | Liofilización | PLE 4-6 g lodo liofilizado + 10 g tierra de diatomeas EtOAc, 150 bar, 90 °C | Purificación con sílice Cromatografía de exclusión por tamaños Fraccionamiento con sílice | GC-(EI)- MS | 55-114 | [140] |
| TCS (+ otros PPCPs y fármacos) | Suelo Lodo | - | PLE 2.7 g suelo / 1 g lodo liofilizado + arena de mar MeOH:H ₂ O 1:1, 1500 psi, 60 °C | Purificación mediante SPE (HLB) Elu.: 10 mL EtOAc:Acetona 1:1 | LC- (ESI)- MS/MS | 110 (suelo) | [36] |
| MeP, EtP, n-PrP, BzP, TCS, TCC, (+ otros PPCPs) | Lodo | Liofilización | PLE 1 g lodo liofilizado + Al ₂ O ₃ MeOH, MeOH:H ₂ O pH 7, 140 bar, 100 °C | Filtración 0.45 µm | UPLC- (ESI)- MS/MS | 72-106 | [35] |
| TCS (+ 2 clorofenoles) | Sedimento Lodo | Liofilización Tamizado | MAE 1 g sedimento / 0.5 g lodo liofilizado 30 mL MeOH:Acetona 1:1, 130 °C, 20 min | Centrifugación Adición 100 mL H ₂ O/0.2 M NaOH Lavado con 2 x 15 mL HX Purificación mediante SPE (HLB, sílice) Derivat. con MTBSTFA | GC-(EI)- MS/MS | 99-100 (sedimento) 82-97 (lodo) | [32] |
| TCS (+ 4 EDCs) | Lodo | Secado (40 °C) Trituración Secado (60 °C) | USAE 0.02 g lodo seco 8 mL MeOH:H ₂ O 2.7:1, 50 °C, 30 min | Centrifugación Purificación mediante SPE (C ₁₈) Derivat. con BSTFA | GC-(EI)- MS | 71-86 | [141] |
| TCS (+ 7 fármacos) | Biosólidos | Liofilización | USAE 0.5 g biosólidos MeOH:HOAc (0.1 M):Na ₂ EDTA (5%) 2:1:1 | Purificación mediante SPE (Strata-X) | LC- (ESI)- MS/MS | 53-68 | [142] |

PPCs: productos de cuidado personal; PPCPs: fármacos y PPCPs; EDCs: disruptores endocrinos; Elu.: elución; Derivat.: derivatización; HX: hexano; EtOAc: acetato de etilo; MeOH: metanol; HOAc: ácido acético; NaOAc: acetato sódico; NaOAc: acetato sódico; MTBSTFA: N-metil-N-(*tert*-butildimetilsilil)trifluoroacetamida; TMSDEA: N-(trimetilsilil)-N-dietilamina; BSTFA: N,O-bis(trimetilsilil)trifluoroacetamida.

4. DETERMINACIÓN

Las dos técnicas más utilizadas hasta la fecha para la determinación de agentes antimicrobianos en muestras ambientales han sido la cromatografía de gases (*gas chromatography*, GC) y la cromatografía de líquidos (*liquid chromatography*, LC) acopladas a la espectrometría de masas simple (*mass spectrometry*, MS) o en tándem (MS/MS). En menor medida, se han empleado otros sistemas de detección como los detectores de captura electrónica (*electron capture detector*, ECD) y emisión atómica (*atomic emission detector*, AED), en el caso de la cromatografía de gases, o los detectores de absorbanza ultravioleta-visible combinados con cromatografía de líquidos.

En la presente tesis doctoral se han utilizado ambas modalidades cromatográficas acopladas a la espectrometría de masas como sistema de detección. El siguiente apartado describe las características más destacadas de ambas técnicas a la vez que recoge una breve revisión bibliográfica de sus aplicaciones a la determinación de agentes antimicrobianos en muestras ambientales.

4.1. CROMATOGRFÍA DE GASES

La cromatografía gas-líquido, abreviada normalmente como cromatografía de gases (*gas chromatography*, GC), se basa en la distribución de los analitos entre una fase móvil gaseosa (el gas portador) y una fase líquida inmovilizada sobre la superficie de un sólido (la columna cromatográfica). Su rango de aplicación se limita a sustancias volátiles y térmicamente estables, ya que la muestra suele introducirse en la columna en fase gas y, para ello, las muestras líquidas deben experimentar una etapa previa de volatilización a elevadas temperaturas.

4.1.1. Derivatización

Los compuestos polares y/o termosensibles se pueden determinar mediante GC si previamente se transforman en otras especies más apolares y/o térmicamente más estables; las reacciones de derivatización tienen como objetivo incrementar la estabilidad térmica y la volatilidad de las especies transformadas, mejorar la resolución entre picos cromatográficos e, incluso, incrementar la respuesta de los analitos en el sistema de detección [87].

La sililación constituye una de las reacciones de derivatización más populares: consiste en la sustitución de un hidrógeno activo (perteneciente a un grupo hidroxilo, carboxilo, amida,

etc.) por un grupo sililo mediante la formación de un enlace silicio-oxígeno o silicio-nitrógeno. La reacción debe realizarse en medio aprótico (de lo contrario el agente derivatizante se consumiría con el propio disolvente) y, entre los agentes sililantes utilizados con mayor frecuencia se encuentran el *N*-metil-*N*-(trimetilsilil)trifluoroacetamida (MSTFA), el *N*-metil-*N*-(*tert*-butildimetilsilil)trifluoroacetamida (MTBSTFA) y el *N,O*-bis-(trimetilsilil)trifluoroacetamida (BSTFA).

4.1.2. Sistemas de inyección

La introducción de muestras líquidas en la columna cromatográfica puede llevarse a cabo de forma directa o, más comúnmente, incluyendo una etapa previa de volatilización de la misma. Los *inyectores convencionales* presentan una cámara de volatilización (*liner*) que se mantiene a temperatura elevada (250-350 °C) para facilitar el proceso de evaporación de la muestra. Estos inyectores permiten dos modos de trabajo diferentes: el modo *split*, en el que sólo una fracción de la muestra introducida alcanza la columna cromatográfica (el resto es eliminado a través de la válvula de purga) y el modo *splitless*, en el que dicha válvula permanece cerrada durante el tiempo en el que tiene lugar la transferencia de la muestra desde el inyector a la columna cromatográfica (tiempo de *splitless*); transcurrido este tiempo, la válvula se abre y a través de ella se hace circular un flujo elevado de gas portador (flujo de limpieza) que prepara el *liner* para la siguiente inyección. Este modo es el utilizado con mayor frecuencia en el análisis de trazas (permite alcanzar mejores límites de detección) y ha sido el empleado en dos de los tres trabajos basados en cromatografía de gases que se incluyen en la presente tesis doctoral.

El tercero de los estudios se llevó a cabo utilizando un *inyector de temperatura programada* (*programmed temperature vaporizer*, PTV) operando en modo *solvent vent*. El PTV consta de los mismos elementos que un inyector convencional *split/splitless* pero, adicionalmente, presenta un sistema de enfriamiento y calentamiento muy eficiente gracias al cual la temperatura del *liner* se puede controlar de forma programada durante el proceso de inyección. Esta característica le permite operar en los clásicos modos *split* o *splitless* en frío o en caliente, pero, además, ofrece la posibilidad de introducir grandes volúmenes de muestra mediante la inyección con eliminación de disolvente (*solvent vent*). En este caso, la inyección se realiza manteniendo el *liner* a una temperatura ligeramente inferior al punto de ebullición del disolvente y con la válvula solenoidal en posición de *split*; como consecuencia, el disolvente se elimina a través de dicha válvula mientras que los analitos, menos volátiles, permanecen retenidos en el *liner*. A continuación, la válvula se pone en posición de *splitless* y los analitos se transfieren a la columna cromatográfica mediante un rápido calentamiento del

inyector. Concluida la transferencia, la válvula pasa de nuevo a modo *split* para preparar la cámara para la siguiente inyección.

Con el objetivo de incrementar la retención de los analitos durante la eliminación de disolvente y minimizar la pérdida de los mismos, se han utilizado *liners* con diferentes materiales de relleno: Tenax, lana de vidrio, poliimida, etc. Sin embargo, estos materiales pueden originar una retención excesiva o irreversible de los analitos o dar lugar a problemas de contaminación cruzada entre inyecciones. Alternativamente, existen *liners* comerciales con configuraciones diferentes a la recta convencional: configuración recta con muesca o configuración en zig-zag (la utilizada en el trabajo citado anteriormente y desarrollado en la presente tesis doctoral).

4.1.3. Acoplamiento a espectrometría de masas

La espectrometría de masas es una técnica de detección polivalente, sensible y extremadamente específica, proporcionando información acerca de la naturaleza, la composición y la estructura de las especies individuales detectadas. En un espectrómetro de masas, los compuestos pasan por las siguientes etapas: *ionización* (las especies neutras se transforman en iones que, adicionalmente, pueden experimentar algún tipo de fragmentación), *aceleración* (los iones son acelerados y focalizados hacia el analizador), *separación* (el analizador separa los iones en función de su relación masa/carga, m/z) y *detección*.

El acoplamiento GC-MS data de la década de 1970 y revolucionó el análisis de mezclas complejas de compuestos orgánicos debido a que combina el elevado poder de resolución de la cromatografía de gases con la alta sensibilidad y la información estructural aportadas por la espectrometría de masas. La combinación de ambas técnicas se consigue fácilmente debido a que ambas operan en fase gas y requieren un volumen muy pequeño de muestra para realizar el análisis. Adicionalmente, el bajo flujo de gas portador utilizado en las columnas capilares hace posible la conexión directa de la columna cromatográfica (a presión atmosférica) con la fuente de ionización del espectrómetro de masas (mantenida a alto vacío).

Entre las fuentes de ionización en fase gas, la *fente de impacto electrónico* (*electron impact*, EI) es la más común y ha sido la utilizada en la presente memoria en todos los análisis llevados a cabo por GC-MS. En ella, los electrones emitidos por un filamento caliente de renio o wolframio son acelerados mediante un potencial de aproximadamente 70 eV que se aplica entre el filamento y el ánodo. La trayectoria de estos electrones de elevada energía es perpendicular al camino de entrada de las moléculas en la fuente, de forma que, cuando ambos se acercan lo suficiente, cada molécula pierde un electrón por repulsión electrostática. Adicionalmente, alcanza un estado rotacional o vibracional excitado desde el cual la

subsecuente relajación se produce vía fragmentación en un gran número de iones positivos de diversas masas menores (y en ocasiones mayores) que la del ion molecular. Los complejos espectros de masas resultantes permiten la identificación de los compuestos detectados. Sin embargo, la extensa fragmentación puede ser también un inconveniente cuando da lugar a la desaparición del pico del ion molecular, perdiéndose la información más importante para establecer el peso molecular [143].

Una vez generados, los iones son conducidos al analizador para su separación en función de su relación m/z . El analizador ideal debería ser capaz de distinguir diferencias de masa muy pequeñas y, simultáneamente, permitir el paso de un número suficiente de iones para producir corrientes iónicas fáciles de medir. A lo largo de la presente tesis doctoral se han utilizado dos configuraciones GC-MS basadas en dos analizadores diferentes: el analizador de masas cuadrupolar (*quadrupole*, Q) y la trampa de iones (*ion trap*, IT)

El **analizador de masas cuadrupolar** utiliza un conjunto de cuatro barras cilíndricas paralelas que actúan como electrodos. A cada par se le aplica una combinación dada de potenciales de corriente continua y corriente alterna de forma que, para una combinación dada, todos los iones excepto aquéllos con una determinada relación m/z inciden en las barras y se convierten en moléculas neutras. En el modo SIM (*selected ion monitoring*) estos potenciales se mantienen constantes y sólo los iones con un valor específico de m/z consiguen atravesar completamente el analizador. En el modo *scan* o de barrido, los potenciales se incrementan simultáneamente desde cero hasta un valor máximo manteniéndose su relación ligeramente inferior a 6; el resultado es la filtración continua de iones con valores de m/z crecientes. Los espectrómetros de masas cuadrupolares convencionales resuelven fácilmente iones que difieren en una unidad de m/z , llegando hasta 3000 o 4000 m/z . Presentan una elevada velocidad de barrido que les permite obtener un espectro de masas completo en menos de 100 ms [143].

La **trampa de iones** consta de un electrodo anular y un par de electrodos colectores que forman una cavidad en la que tiene lugar el proceso completo de fragmentación, almacenamiento y filtración de los iones formados (la ionización puede ser externa o interna). El electrodo anular está sometido a un potencial de radiofrecuencia variable que crea un campo eléctrico hiperbólico tridimensional en el que los iones son atrapados en órbitas circulares. A medida que se incrementa este potencial, los iones se desestabilizan en orden creciente de m/z y son expulsados hacia el detector. Los espectrómetros de trampa de iones son robustos y compactos. Una versión comercial corriente es capaz de resolver iones que difieren en una unidad de m/z con un intervalo de trabajo habitual de entre 10 y 650 m/z . Son fácilmente configurables para el trabajo en MS/MS e incluso en MSⁿ pero, como desventaja,

no permiten la selección de iones discontinuos como los cuadrupolos en modo SIM. Alternativamente, pueden operar en modo *pseudo*-SIM, en el que el rango de m/z seleccionado es tan bajo que se aproxima a una unidad [143].

4.1.4. Aplicación de GC-MS a la determinación de agentes antimicrobianos en muestras ambientales

Entre los agentes antimicrobianos considerados en la presente memoria, el triclocarbán es el único compuesto que no se ha determinado mediante cromatografía de gases debido a su baja estabilidad térmica y a que es difícilmente derivatizable. El triclosán, el metil triclosán y los parabenos, por el contrario, sí han sido determinados por GC-MS en múltiples ocasiones, incluidos diversos estudios ambientales centrados en el análisis de muestras de agua y muestras de lodo, suelo y sedimento (**Tabla II.8**).

Aunque en algunas de estas aplicaciones el triclosán ha sido determinado sin derivatizar [30,140], en la mayoría de los casos este compuesto y los parabenos se han hecho reaccionar con algún agente sililante (MSTFA [22], MTBSTFA [32,37,82], TMSDEA [34], BSTFA [141]) previamente a su introducción en el cromatógrafo de gases. La principal ventaja de la sililación de fenoles es que es una reacción rápida y cuantitativa y da lugar a productos estables con buenas características cromatográficas [144]. Alternativamente, algunos autores han recurrido a opciones de derivatización diferentes como la formación de ésteres por reacción con anhídrido pentafluoropropiónico (PFPA) [16], la acilación de los grupos fenólicos *in situ* (en medio acuoso) [41,42] o la etilación del triclosán con diazoetano [14] (en sustitución de la metilación con diazometano para permitir la distinción entre la especie nativa y su anisol metilado).

Generalmente, la inyección de muestras líquidas se ha llevado a cabo en modo *splitless* (con o sin pulso de presión) y para la separación se han empleado columnas cromatográficas típicas de fase 5% difenil 95% polidimetilsiloxano, 30 m de longitud, diámetros internos de 0.25 mm o 0.32 mm y espesores de fase de 0.25 μm [16,30,37,82,145].

Para la detección mediante GC-MS se han utilizado principalmente fuentes de ionización por impacto electrónico y analizadores cuadrupolares simples operando en modo SIM [16,22,34,140,141]. Agüera et al. [30] compararon la sensibilidad de la ionización por impacto electrónico con la ionización química negativa (*negative chemical ionisation*, NCI) para la determinación de triclosán y encontraron que, en este último caso, los límites de detección eran 50 veces más bajos. Alternativamente, algunos autores han utilizado trampas de iones para llevar a cabo análisis mediante espectrometría de masas en tándem [32].

Tabla II.8. Condiciones empleadas para la determinación de parabenos, TCS y MTCS en muestras ambientales mediante GC-MS.

| Análitos | Matriz | Extracción | Derivatización | Inyección | Columna | Detección | LOD o LOQ (ng L ⁻¹) | Ref. |
|---|---------------------|------------|-----------------------------------|--|--|-------------------------------------|---|-------|
| MeP, <i>n</i> -PrP, <i>n</i> -BuP, TCS (+ otros PCPs) | río | SPE | MSTFA 100% 70 °C, 2 h | - | DB-5 MS (30 m × 0.25 mm; 0.25 µm) | (EI)-Q-MS modo SIM | 0.1-0.5 LOD | [22] |
| MeP, EtP, <i>n</i> -PrP, <i>n</i> -BuP, TCS (+ otros PPCPs) | efluente, influente | SPE | PFPA 1:1 T.a., 20 min | Splitless con pulso de presión (30 psi, 1 min) | Rtx-5Sil MS (30 m × 0.25 mm; 0.25 µm) | (EI)-Q-MS modo SIM | 10 LOD | [16] |
| TCS (+ otros clorofenoles) | río | SPE | MTBSTFA 1:25 T.a., 5 min | Splitless | HP-5 MS (30 m × 0.25 mm; 0.25 µm) | (EI)-IT-MS modo <i>full scan</i> | 4 LOQ | [82] |
| TCS, MTCS (+ 3 closanos) | efluente | SPE | TMSDEA 1:1 T.a., 10 min | On-column o splitless | DB-5 (30 m × 0.25 mm; 0.25 µm) | (EI)-Q-MS modo SIM | 10 LOD | [34] |
| TCS (+ bifenilol) | efluente | SPE | - | Splitless con pulso de presión (30 psi, 1.5 min) | HP-5 MS (30 m × 0.25 mm; 0.25 µm) | (EI)-Q-MS (NCI)-Q-MS modo SIM | 1000 (EI) 20 (NCI) LOD | [30] |
| TCS, MTCS | río | SPE | Diazoetano | Splitless | DB-5 MS (25 m × 0.32 mm; 0.25 µm) | (EI)-B-MS modo SIM | < 0.4 LOD | [14] |
| TCS, MTCS | suelo | MSPD | MTBSTFA 1:2 60 °C, 10 min | Splitless con pulso de presión (45 psi, 1.5 min) | ZB-5 MS (30 m × 0.25 mm; 0.25 µm) | (EI)-Q-MS modo <i>full scan</i> | 0.05-0.08 (suelo) 0.1-0.12 (lodo) LOD | [37] |
| TCS, MTCS (+ otros compuestos) | lodo | PLE | - | Splitless | DB-5 MS (15 m × 0.25 mm; 0.25 µm) | (EI)-Q-MS modo SIM | 3-30 LOD | [140] |
| TCS (+ 2 clorofenoles) | sedimento | MAE | MTBSTFA 1:10 T.a., 5 min | Splitless | HP-5 MS (30 m × 0.25 mm; 0.25 µm) | (EI)-IT-MS/MS | 0.4 (sedimento) 0.8 (lodo) LOQ | [32] |
| TCS (+ otros compuestos) | lodo | USAE | BSTFA + Piridina 65 °C, 20 min | Splitless | DB-5 MS (60 m × 0.32 mm; 0.25 µm) | (EI)-Q-MS modo SIM | 150 LOD | [141] |

PCPs: productos de cuidado personal; PPCPs: fármacos y PCPs; T.a.: temperatura ambiente; MSTFA: *N*-metil-*N*-(trimetilsilil)trifluoroacetamida; MTBSTFA: *N*-metil-*N*-(*tert*-butildimetilsilil)trifluoroacetamida; TMSDEA: *N*-(trimetilsilil)-*N*-dietilamina; BSTFA: *N*,*O*-bis(trimetilsilil)-*N*-dietilamina; STFA: *N*,*O*-bis(trimetilsilil)trifluoroacetamida; PFPA: anhídrido pentafluoropropiónico; B: analizador de sector magnético.

4.2. CROMATOGRAFÍA DE LÍQUIDOS

La cromatografía de líquidos (*liquid chromatography*, LC) se basa en la distribución de los analitos entre una fase estacionaria sólida y una fase móvil líquida y es la técnica por excelencia para separar compuestos no volátiles, polares y/o térmicamente inestables.

4.2.1. Acoplamiento a espectrometría de masas

El acoplamiento LC-MS nace en la década de 1970, centrándose en los veinte años posteriores en resolver los dos problemas fundamentales derivados de la combinación de ambas técnicas: transformar las moléculas en disolución en iones en fase gaseosa sin que se produzca su degradación térmica y eliminar la gran cantidad de gas y vapor procedente de la fase líquida antes de entrar en la región de alto vacío del espectrómetro de masas. Con tal objetivo, se han diseñado diferentes fuentes de ionización.

Las fuentes de ionización a presión atmosférica combinan los procesos de volatilización e ionización en una sola etapa. Suministran energía a la muestra sólida o líquida en condiciones de presión atmosférica, provocando la formación de iones gaseosos y dando lugar a espectros muy simplificados (técnicas blandas de ionización). Dentro de ellas, la *ionización por electrospray* (*electrospray ionisation*, ESI) se ha convertido en una de las más importantes y ha sido empleada en todos los análisis llevados a cabo por LC-MS en la presente tesis doctoral. En ESI, la disolución de la muestra es nebulizada a través de una aguja capilar que se mantiene a un potencial de varios kV con respecto a un electrodo cilíndrico circundante. Las microgotas cargadas resultantes se desolvatan por repulsiones coulombicas y con la ayuda adicional de flujos de gas y calor, originando iones en fase gas con una o múltiples cargas. Este sistema permite controlar la fragmentación (presencia/ausencia) variando los potenciales de los electrodos y es compatible con MS/MS. Su principal inconveniente radica en que es muy sensible a los efectos de matriz: los componentes presentes en ella pueden intervenir en reacciones ácido-base, disminuyendo o incrementando la ionización de los analitos; adicionalmente, pueden competir con ellos en su acceso a la superficie de las gotas del spray, dificultando su desolvatación, o variar (reduciendo o aumentando) la tensión superficial de las gotas [143].

En la presente tesis doctoral, la cromatografía líquida se ha utilizado en combinación con la espectrometría de masas en tándem. Mediante esta técnica, los iones generados en la fuente se hacen colisionar con un gas inerte (generalmente argón o nitrógeno) para inducir su fragmentación y llevar a cabo la detección de los fragmentos cargados resultantes. Los experimentos de MS/MS pueden realizarse en una trampa de iones o en espectrómetros de masas provistos de más de un analizador (habitualmente tres, de los cuales el intermedio

actúa como celda de colisión). En concreto, en la presente tesis doctoral se han utilizado un instrumento de triple cuadrupolo (QqQ) y un sistema híbrido cuadrupolo-tiempo de vuelo (QTOF).

La configuración de *triple cuadrupolo* permite operar en MS/MS en cuatro modos de trabajo diferentes:

- *Product ion scanning*: el primer analizador opera en modo SIM seleccionando una relación m/z característica del compuesto a determinar; el tercero opera en modo *scan* generando el espectro de fragmentación (*full-product ion spectrum*) del ion anterior.
- *Precursor ion scanning*: al revés; el primer analizador hace un barrido de iones precursores y el tercero sólo deja pasar al detector aquellos fragmentos comunes con una determinada relación m/z .
- *Neutral loss scanning*: ambos cuadrupolos operan en modo *scan*.
- *Selective reaction monitoring* (SRM): ambos analizadores trabajan en modo SIM, seleccionando un único ion precursor y, tras su fragmentación, un único ion producto. De esta forma se minimizan y, en algunos casos, se eliminan por completo las interferencias, reduciéndose el ruido químico de los cromatogramas y alcanzándose selectividades y sensibilidades excelentes [146].

La posibilidad de operar en modo SRM, junto con la robustez y el amplio rango dinámico del triple cuadrupolo lo convierten en uno de los sistemas más utilizados en análisis cuantitativo. Su principal inconveniente radica en su bajo poder de resolución, que obliga a monitorizar dos transiciones por compuesto (ion precursor \rightarrow ion producto) para evitar falsos positivos cuando se analizan muestras complejas [147]. Consecuentemente, el número de analitos incluidos en un método se limita todavía más, ya que un incremento en el número de transiciones monitorizadas conlleva un descenso en el tiempo de adquisición de cada transición (*dwell time*) y, con ello, un descenso del número de puntos obtenidos por pico cromatográfico. Las medidas en masa nominal, que no permiten distinguir entre compuestos isobáricos coeluyentes, y la necesidad de patrones para optimizar las transiciones en SRM son otras de las desventajas de estos sistemas. Desde un punto de vista cualitativo, el trabajo del triple cuadrupolo en los modos *Product ion scanning* y *Neutral loss scanning* puede proporcionar información estructural relevante, pero la baja sensibilidad en *scan* y la baja resolución de los instrumentos convencionales no permiten la determinación de fórmulas moleculares. Las trampas de iones, más sensibles en modo *scan* y capaces de llevar a cabo experimentos en MS^n , resultan útiles para ciertos propósitos identificativos, pero su resolución es similar a la de los analizadores cuadrupolares [148].

A.4. DETERMINACIÓN

En los *analizadores de tiempo de vuelo* (*time of flight*, TOF) los iones son acelerados mediante un potencial de 10^3 a 10^4 V e introducidos en un tubo analizador de aproximadamente un metro de longitud que no está sometido a ningún campo eléctrico. Debido a que todos los iones que entran en el tubo tienen, idealmente, la misma energía cinética, sus velocidades dentro de él varían inversamente con sus masas, llegando antes al detector las partículas más ligeras (para igual carga). Los tiempos de vuelo típicos son del orden de 1 μ s. Clásicamente, la sensibilidad y la resolución de los espectrómetros de masas con analizadores de tiempo de vuelo eran inferiores a las de sus homólogos cuadrupolares. Sin embargo, los instrumentos de nueva generación han superado estas limitaciones, lo que unido a la robustez, la elevada velocidad de barrido y la posibilidad de registrar un intervalo de masas virtualmente ilimitado los convierte en analizadores con enormes posibilidades [143]. Su elevado poder resolutivo y su alta exactitud de masas les permiten establecer fórmulas moleculares. Adicionalmente, la elevada sensibilidad de adquisición que presentan en modo *scan* hace posible evaluar la presencia de compuestos no preseleccionados originalmente como analitos una vez que el análisis ha finalizado (*post-target analysis*). De esta forma, se puede detectar un número teóricamente ilimitado de especies sin necesidad de disponer de sustancias patrón.

Los sistemas híbridos *cuadrupolo-tiempo de vuelo* permiten llevar a cabo análisis confirmatorios seleccionando un ion en el primer cuadrupolo y generando el espectro de masas exactas de los productos de fragmentación resultantes (*full-product ion spectrum*). Los experimentos QTOF-MS/MS resultan especialmente útiles para elucidar la estructura de compuestos desconocidos y/o confirmar potenciales positivos revelados mediante triples cuadrupolos o TOF simples. Cuantitativamente, aunque el funcionamiento inherente del tubo de tiempo de vuelo impide la selección de un único ion producto, como en el modo *Product ion scanning*, la elevada sensibilidad de adquisición en *scan* de los nuevos TOF permite realizar la cuantificación de una especie a partir de su espectro de fragmentación. La elevada resolución y exactitud de masas contribuyen, adicionalmente, a reducir el riesgo de interferencias derivadas de compuestos isobáricos.

4.2.2. Aplicación de LC-MS a la determinación de agentes antimicrobianos en aguas

Contrariamente a lo que le ocurre al metil triclosán, de marcado carácter apolar, el triclosán y los parabenos pueden determinarse también mediante cromatografía de líquidos, evitando de esta forma la introducción de una etapa previa de derivatización. Por su parte, el triclocarbán es determinado mediante LC. En la **Tabla II.9** se presentan las condiciones de LC-MS empleadas en algunos estudios para la determinación de los citados agentes antimicrobianos en muestras de agua.

En la mayoría de los casos, la separación se ha llevado a cabo mediante cromatografía de líquidos de alta eficacia (*high-performance liquid chromatography*, HPLC, abreviada habitualmente como LC), aunque algunos trabajos se han basado en la cromatografía de líquidos de ultra eficacia (*ultra-performance liquid chromatography*, UPLC) para conseguir separaciones más rápidas y eficientes [17,51].

En general, se han utilizado fases estacionarias apolares como el C₁₈ [15,17,51,149,150], el C₈ [30] o el fenil-hexil [151], y fases móviles agua/metanol [15,17,51,52,150,151] o agua/acetonitrilo [30,149] conteniendo algún modificador como el ácido acético [17], el acetato amónico [15,52], el hidróxido amónico [30,150], el formiato amónico [149] o la tributilamina [151].

La detección por espectrometría de masas se ha realizado principalmente mediante espectrometría de masas en tándem con sistemas de triple cuadrupolo [15,17,51,52,150,151] o trampa iónica [30,149] y con ionización por electrospray en modo negativo.

Tabla II.9. Condiciones empleadas para la determinación de parabenos, TCS y TCC en muestras ambientales mediante LC-MS.

| Analitos | Matriz | Extracción | Separación | Columna | Fase móvil (modificador) | Detección | LOD o LOQ (ng L ⁻¹) | Ref. |
|--|----------------------------------|------------|------------|--|--|----------------------------------|---|-------|
| MeP, EtP, <i>n</i> -PrP, BzP, TCS, TCC (+ 5 filtros UV) | río, efluente, influyente | SPE | UPLC | Zorbax Eclipse XDB C ₁₈ (50 mm × 4.6 mm; 1.8 µm) | H ₂ O (HOAc, pH 2.8) / MeOH | (ESI -) QqQ-MS/MS modo SRM | 3-10 (ef.) 5-10 (inf.) LOD | [17] |
| MeP, EtP, <i>n</i> -PrP, <i>n</i> -BuP, BzP (+ otros fenoles) | río, efluente, influyente | SPE | LC | Lichrospher RP 18ec | H ₂ O (NH ₄ OAc 4 mM) / MeOH | (ESI -) QqQ-MS/MS modo SRM | 0.2-1.6 LOQ | [15] |
| MeP, EtP, <i>n</i> -PrP, <i>n</i> -BuP, TCS (+ otros PPCPs y drogas) | río, efluente, influyente | SPE | UPLC | ACQUITY UPLC BEH C ₁₈ (100 mm × 1 mm; 1.7 µm) | H ₂ O:MeOH (HOAc 0.5%, NH ₄ OH 5 mM) / H ₂ O:MeOH (HOAc 0.5%, NH ₄ OH 5 mM) | (ESI -) QqQ-MS/MS modo SRM | 0.1-4 (río) 0.6-72 (ef.) 2-97 (inf.) LOQ | [51] |
| <i>n</i> -PrP, <i>i</i> -BuP, TCS, TCC (+ 20 compuestos) | efluente, influyente | SPE | LC | Synergi Max-RP (250 mm × 4.6 mm) | H ₂ O (NH ₄ OAc 100 mM) / MeOH | (ESI -) QqQ-MS/MS modo SRM | 0.25-1 LOQ | [52] |
| MeP, EtP, <i>n</i> -PrP, BzP | río, efluente, | SPE | LC | Luna C ₁₈ (100 mm × 2 mm; 3 µm) | H ₂ O:ACN (NH ₄ OCOH 1 mM) / ACN | (ESI -) IT-MS/MS | 0.2-0.5 LOD | [149] |
| TCS (+ bifenilol) | efluente, influyente | SPE | LC | X Terra MS C ₈ (100 mm × 2.1 mm; 3.5 µm) | H ₂ O (NH ₄ OH 0.02%) / ACN | (ESI -) IT-MS/MS | 350 LOD | [30] |
| TCS | río, efluente | SPE | LC | Genesis C ₁₈ (150 mm × 2.1 mm; 4 µm) | H ₂ O (NH ₄ OH 1 M) / MeOH (NH ₄ OH 1 M) | (ESI -) QqQ-MS/MS modo SRM | 4 (río) 10 (ef.) LOQ | [150] |
| TCS (+ fármacos) | lago, efluente, influyente | SPE | LC | Luna Phenyl-Hexyl (150 mm × 2 mm; 3 µm) | H ₂ O:MeOH (TrBA 10 mM, HOAc 0.5%) / H ₂ O:MeOH (TrBA 10 mM, HOAc 0.5%) | (ESI -) QqQ-MS/MS modo SRM | 11 (lago) 24 (ef.) LOQ | [151] |

PCPPs: fármacos y productos de cuidado personal; MeOH: metanol; ACN: acetonitrilo; HOAc: ácido acético, NH₄OAc: acetato amónico, NH₄OH: hidróxido amónico; NH₄OCOH: formiato amónico; TrBA: tri-*n*-butilamina; ef.: efluente; inf.: influyente.

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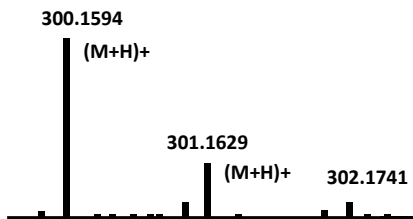
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B. DROGAS DE ABUSO

1. ASPECTOS GENERALES

1.1. DEFINICIÓN, CLASIFICACIÓN Y CONSUMO EN LA UNIÓN EUROPEA. METABOLISMO

El término drogas ilícitas presenta cierta ambigüedad porque no se limita exclusivamente a drogas ilegales (sin uso médico permitido) sino que pretende abarcar sustancias legales reconocidas y/o registradas como fármacos pero que pueden ser utilizadas de forma ilegal. Por este motivo, la Oficina de las Naciones Unidas para las Drogas y el Crimen (*United Nations Office of Drugs and Crime, UNODC*) no clasifica una droga como lícita o ilícita en función de su identidad química, sino que se centra en el modo en que esa sustancia es manufacturada, formulada, distribuida, adquirida y/o consumida. Esta definición permite la inclusión de fármacos legales como drogas ilícitas, esto es, cuando son manufacturados, formulados, distribuidos, adquiridos y/o utilizados de forma ilegal y sin supervisión médica [1].

Las principales sustancias ilícitas determinadas en matrices ambientales son precisamente aquéllas de mayor consumo y sus principales metabolitos de excreción urinaria. Atendiendo a una clasificación general, se distinguen los siguientes grupos:

1.1.1. Opioides

De acuerdo con el Informe Anual del 2011 del Observatorio Europeo de las Drogas y Toxicomanías (*European Monitoring Centre for Drugs and Drug Addiction, EMCDDA*) [2], la prevalencia media anual del abuso de opiáceos en la Unión Europea se sitúa entre 3.6 y 4.4 casos por cada 1000 habitantes de entre 15 y 64 años, lo que significa 1.3-1.4 millones de consumidores en 2009, incluyendo aquéllos bajo tratamientos de sustitución.

Este grupo de sustancias abarca los constituyentes naturales del opio (como la morfina, utilizada legalmente como potente analgésico) y sus derivados semisintéticos (la codeína, analgésico legal, y la heroína, el opiáceo ilegal de mayor consumo a nivel mundial, entre otros) [3]. La morfina es parcialmente excretada en la orina como conjugados glucurónidos [4] que, por la acción de β -glucuronidasas presentes en las bacterias fecales, son rápidamente hidrolizados dando lugar a la molécula de partida [5]. La determinación de estos compuestos en orina y, por ende, en aguas residuales, se centra en la morfina (residuo metabólico común a la heroína, 4% de una dosis, la codeína y la propia morfina, 10% de una dosis), y en la 6-acetilmorfina, metabolito específico de la heroína (en torno a 1.3% de una dosis) e indicador clave de su abuso [6].

Entre los opiáceos sintéticos, diferentes estructuralmente a la morfina pero que actúan sobre sus mismos receptores, se encuentran el fentanilo y la metadona, ambos utilizados en la práctica clínica: el fentanilo como analgésico (es 100 veces más potente que la morfina) y la metadona como sustituto de la heroína en tratamientos de drogodependencia [4]. El fentanilo es metabolizado por *N*-dealquilación principalmente a norfentanilo e hidroxinorfentanilo [4]. La metadona es excretada como tal en la orina (5-50% de la dosis) o metabolizada por *N*-demetilación seguida de ciclación espontánea dando lugar a la 2-etiliden-1,5-dimetil-3,3-difenilpirrolidina (EDDP, 3-25% de una dosis) [6].

1.1.2. Cannabinoides

El cannabis es la droga ilegal más extendida en Europa. Estudios recientes sugieren una tendencia a la estabilización e incluso a la baja en su consumo tras años de continuo aumento, si bien los niveles notificados siguen siendo elevados en términos históricos: se estima que unos 22.5 millones de europeos consumieron cannabis en el último año, lo que representa una media del 6.7 % de toda la población de entre 15 y 64 años [2].

El principal constituyente activo de la planta *Cannabis sativa* es el Δ^9 -tetrahidrocannabinol (THC). Cuando se fuma, se absorbe a través de los pulmones y se metaboliza en el hígado a 11-hidroxi-THC, que posteriormente se oxida originando decenas de compuestos diferentes entre los que se incluye el 11-nor-9-carboxi- Δ^9 -THC (THCCOOH), el principal metabolito de excreción urinaria; alrededor del 0.6% del THC ingerido se excreta en la orina en forma de conjugados glucurónidos del THCCOOH [7] que son hidrolizados por la acción de β -glucuronidasas a la forma libre.

1.1.3. Alcaloides

La cocaína es el principal alcaloide presente en la planta *Erythroxylum coca* y constituye uno de los estimulantes del sistema nervioso central más potentes que existen. Su consumo e incautaciones aumentaron considerablemente en el último decenio, convirtiéndola en la segunda droga ilegal más consumida en Europa, por detrás del cannabis. Aunque las cifras varían mucho de un país a otro, se estima que, de media, un 1.2% de la población de entre 15 y 64 años (unos 4 millones de europeos) consumieron esta sustancia en el último año [2].

En el cuerpo, la cocaína se metaboliza rápidamente a benzoilecgonina y ecgonina metil ester, que se excretan a través de la orina en porcentajes que varían del 40 al 50% de una dosis. Entre un 1 y un 9%, dependiendo del pH de la orina, es excretado sin metabolizar. Cuando se consume combinada con etanol, la cocaína puede transesterificarse originando un metabolito urinario exclusivo de tal combinación, el cocaetileno (0.7% de una dosis) [6].

La escopolamina, también conocida como hioscina, es otro alcaloide que se encuentra de forma natural en numerosas plantas. En medicina, presenta tres usos fundamentales: (1) se utiliza en muy pequeñas cantidades (parches cutáneos) para prevenir y tratar el mareo, las náuseas y los vómitos [8]; (2) como agente antiespasmódico y (3) para dilatar la pupila en exámenes de fondo de ojo. Ilegalmente, es empleada por criminales para anular la voluntad de las víctimas en casos de robo, violación, etc., aunque no se dispone de datos concretos de su abuso en la Unión Europea.

El metabolismo de la escopolamina no ha sido evaluado rigurosamente y, en base a los estudios existentes, se puede concluir que la farmacocinética y la farmacodinámica de esta sustancia dependen significativamente de la forma de administración (oral, dérmica, intravenosa) [9].

1.1.4. Derivados anfetamínicos

Las anfetaminas (término genérico que incluye tanto a la anfetamina como a la metanfetamina) y el éxtasis se encuentran entre las drogas ilegales más consumidas en Europa. En términos de cifras absolutas, el consumo de cocaína puede ser más elevado, pero su concentración geográfica significa que en muchos países son las sustancias ilícitas, tras el cannabis, consumidas en mayor proporción. Las estimaciones indican que entre 1.5 y 2 millones de europeos consumieron anfetaminas en el último año (el 0.5% de la población entre 15 y 64 años), mientras que 2.5 millones (0.7%) recurrieron al consumo de éxtasis [2].

El término éxtasis hace referencia a sustancias sintéticas químicamente relacionadas con las anfetaminas pero cuyos efectos son algo diferentes. La más conocida es la 3,4-metilen-dioxi-metanfetamina (MDMA), aunque en ocasiones también pueden encontrarse otras como la 3,4-metilen-dioxi-anfetamina (MDA) y la *N*-etil-3,4-metilen-dioxi-etil-anfetamina (MDEA) [2]. A excepción de esta última, que es eliminada en la orina como MDMA (19%), MDA (28%) y 4-hidroxi-etil-anfetamina (HMEA, 32%), el resto de los derivados de la anfetamina son mayoritariamente excretados en su forma nativa [6].

1.1.5. Derivados de la piperacina

El carácter ilegal de las anfetaminas promovió la búsqueda de sustancias con efectos similares que pudiesen ser comercializadas de forma legal. Con este propósito, los derivados de la piperacina empezaron a venderse en forma de pastillas ("*party pills*"), solos o combinados con otras drogas como la anfetamina o el MDMA [3,4]. Aunque la disponibilidad de la *N*-bencilpiperacina (BZP), una de las piperacinas más populares, parece haber disminuido tras la decisión tomada por el Consejo de someterla a medidas de control en toda la Unión Europea, algunos países siguen informando de importantes operaciones de incautación de

esta sustancia. Por otra parte, la 1-(3-clorofenil)-piperacina (mCPP) es, desde hace ya algunos años, la “nueva droga sintética” más disponible en el mercado ilegal del éxtasis, tanto sola como combinada con MDMA [10].

Se conoce relativamente muy poco acerca del metabolismo de las piperacinas en el cuerpo. Para la BZP se ha observado hidroxilación, seguida de formación de conjugados sulfatados en las posiciones 3 y 4 del anillo aromático, así como en uno de los nitrógenos del grupo piperacina [4].

1.1.6. Alucinógenos

La droga alucinógena sintética más conocida en Europa es la dietilamida del ácido lisérgico (LSD). Su consumo, relativamente estable, se asocia a los adultos jóvenes, con una prevalencia de entre el 0 y el 5.5% de la población comprendida entre 15 y 34 años que admite haber consumido esta sustancia en algún momento de su vida. Las estimaciones para el consumo durante el último año son mucho menores [2].

El LSD se ingiere generalmente en dosis muy pequeñas (50-100 µg) y se metaboliza en gran extensión, por lo que es difícil de detectar en orina. Su principal metabolito en esta matriz, detectado en mayor cantidad que el compuesto nativo, es el 2-oxo-3-hidroxi-LSD (O-H-LSD) [4].

1.1.7. Anestésicos disociativos

La fenciclidina (PCP) es un anestésico disociativo retirado de la práctica clínica en la década de 1960 por sus efectos alucinógenos. En su forma sólida (“*Polvo de Ángel*”) es extremadamente lipofílica y puede absorberse a través de la piel en cantidades farmacológicamente activas. Se metaboliza extensamente originando varios derivados hidroxilados que pueden permanecer en el cuerpo hasta 28 días, eliminándose finalmente a través de las heces y la orina [4].

La ketamina fue sintetizada en 1962 como alternativa al PCP; relegada a emergencias específicas en el ámbito clínico, todavía es utilizada como anestésico en la práctica veterinaria [4]. Como droga de abuso, la prevalencia de su consumo entre la población en general es baja, pero puede ser mucho mayor en algunos grupos, contextos, o zonas geográficas concretas [2]. En el cuerpo humano, se metaboliza principalmente por *N*-demetilación dando lugar a la norketamina [4].

1.2. ESTRUCTURA Y PROPIEDADES FÍSICO-QUÍMICAS

Dentro de la presente tesis doctoral se han considerado las drogas de abuso y metabolitos recogidos en la **Tabla II.10**. Se presentan sus estructuras, fórmulas empíricas, pesos moleculares monoisotópicos y propiedades físico-químicas más relevantes desde una perspectiva analítica: pK_a , logaritmo de la constante de partición octanol-agua ($\log K_{ow}$) y presión de vapor (P_v). Todos los datos son experimentales (recogidos en la base de datos PhysProp, Syracuse Research Corporation [11]) excepto los marcados con (^a) y (^b), que corresponden a valores calculados mediante *software* proporcionados por la base de datos SciFinder Scholar [12] y Chemicalize [13], respectivamente.

El THC y su metabolito son las drogas más apolares consideradas en la presente memoria y las únicas con carácter ácido: el THC se puede catalogar como ácido débil, mientras que el grupo carboxilo del THCCOOH le confiere un carácter de ácido moderado.

Los opioides naturales y semisintéticos y la 6-acetilmorfina se encuentran entre los compuestos más polares estudiados. Los derivados sintéticos (el fentanilo y la metadona) son más apolares y ligeramente más básicos.

Entre los alcaloides, la escopolamina es la segunda droga más polar considerada después de la morfina, mientras que la cocaína y sus metabolitos son ligeramente menos polares. En relación a su carácter ácido-base, es necesario remarcar que la benzoilecgonina contiene, además del grupo básico amino (común a todos ellos) un grupo ácido carboxilo.

Las anfetaminas y los derivados de la piperacina son polares y constituyen las familias más básicas estudiadas, mientras que el ácido lisérgico es también polar pero ligeramente menos básico.

Por último, los anestésicos disociativos presentan una polaridad intermedia (en comparación con el resto de drogas) y, nuevamente, carácter básico.

Tabla II.10. Estructura y propiedades físico-químicas de las drogas de abuso consideradas. Datos experimentales recogidos en la base de datos PhysProp, Syracuse Research Corporation [11], o calculados mediante *software* proporcionados por la base de datos Scifinder Scholar ^(a) [12] o Chemicalize ^(b) [13]).

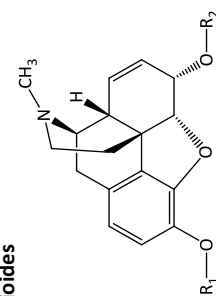
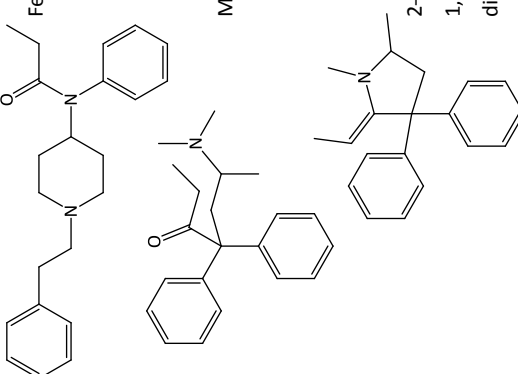
| Clasificación y estructura | Compuesto | Abreviatura | R ₁ | R ₂ | Fórmula Empírica | Pm monoisotópico | pK _a grupo básico | pK _a grupo ácido | log K _{ow} | P _v (mm Hg) |
|--|--|-------------|-------------------|-------------------|--|------------------|------------------------------|-----------------------------|---------------------|------------------------|
| Opioides  | Morfina | MOR | H | H | C ₁₇ H ₁₉ NO ₃ | 285.14 | 8.25 ^a | 9.48 ^a | 0.89 | 1.69 E-9 |
| | Codeína | COD | CH ₃ | H | C ₁₈ H ₂₁ NO ₃ | 299.15 | 8.23 ^a | 13.40 ^a | 1.19 | 4.15 E-9 |
| | Heroína | HER | COCH ₃ | COCH ₃ | C ₂₁ H ₂₃ NO ₅ | 369.16 | 7.93 | - | 1.58 | 7.59 E-10 |
| | 6-Acetil morfina | 6-AM | H | COCH ₃ | C ₁₉ H ₂₁ NO ₄ | 327.15 | 8.03 ^a | 9.41 ^a | 1.56 ^a | 1.83 E-9 ^a |
| Opioides sintéticos  | Fentanilo | FEN | - | - | C ₂₂ H ₂₈ N ₂ O | 336.22 | 8.92 ^a | - | 4.05 | 5.29 E-9 |
| | Metadona | MET | - | - | C ₂₁ H ₂₇ NO | 309.21 | 8.94 | - | 3.93 | 1.12 E-6 |
| | 2-Etilideno-1,5-dimetil-3,3-difenilpirrolidina | EDDP | - | - | C ₂₀ H ₂₃ N | 277.18 | 7.71 ^a | - | 5.36 ^a | 2.06 E-6 ^a |

Tabla II.10 (continuación). Estructura y propiedades físico-químicas de las drogas de abuso consideradas. Datos experimentales recogidos en la base de datos PhysProp, Syracuse Research Corporation [11], o calculados mediante *software* proporcionados por la base de datos SciFinder Scholar ^(a) [12] o Chemicalize ^(b) [13].

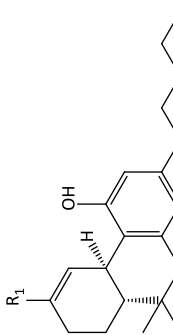
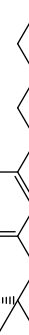
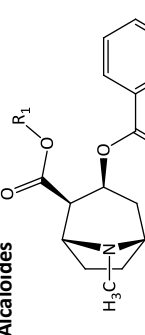
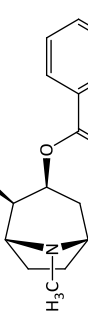
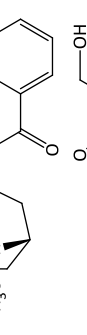
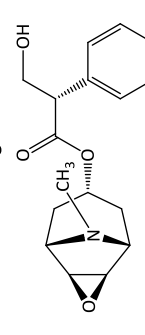
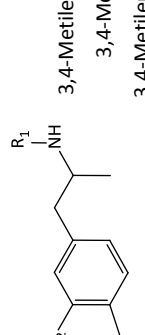
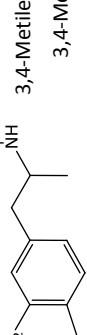
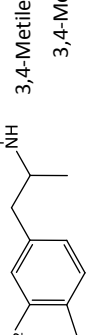
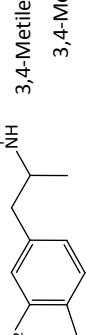
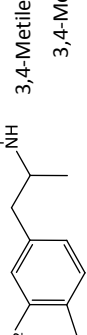
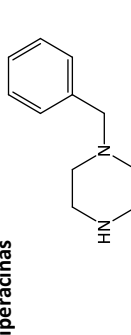
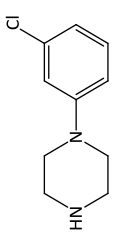
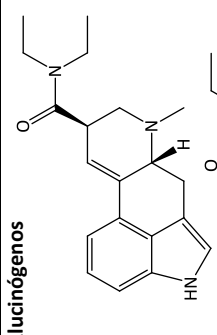
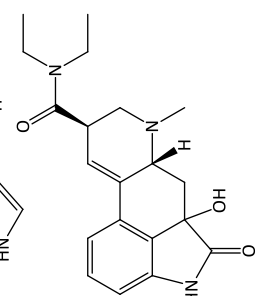
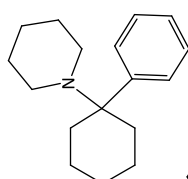
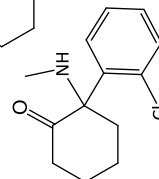
| Clasificación y estructura | Compuesto | Abreviatura | R ₁ | R ₂ | Fórmula Empírica | Pm monoisotópico | pK _a grupo básico | pK _a grupo ácido | log K _{ow} | P _v (mm Hg) |
|---|---|-------------|----------------------------------|----------------------|---|------------------|------------------------------|-----------------------------|---------------------|------------------------|
| Cannabinoides | | | | | | | | | | |
|  | Δ ⁹ -Tetrahydrocannabinol | THC | CH ₃ | - | C ₂₁ H ₃₀ O ₂ | 314.22 | - | 10.60 | 7.60 | 4.63 E-8 |
|  | 11-Nor-9-carboxi-Δ ⁹ -Tetrahydrocannabinol | THCCOOH | COOH | - | C ₂₁ H ₂₈ O ₄ | 344.20 | - | 4.66 ^a 10.60 | 5.25 ^a | 1.62 E-9 ^a |
| Alcaloides | | | | | | | | | | |
|  | Cocaina | COC | CH ₃ | - | C ₁₇ H ₂₁ NO ₄ | 303.15 | 8.61 | - | 2.30 | 1.91 E-7 |
|  | Benzoilecgonina | BE | H | - | C ₁₆ H ₁₉ NO ₄ | 289.13 | 10.8 ^a | 3.35 ^a | 2.26 ^a | 1.32 E-8 ^a |
|  | Cocaetileno | COE | CH ₂ -CH ₃ | - | C ₁₈ H ₂₃ NO ₄ | 317.16 | 9.04 ^a | - | 2.78 ^a | 6.80 E-7 ^a |
|  | Escopolamina | SCO | - | - | C ₁₇ H ₂₁ NO ₄ | 303.15 | 8.01 ^a | 14.11 ^a | 0.98 | 7.18 E-9 |
| Anfetaminicos | | | | | | | | | | |
|  | Anfetamina | AMP | H | H | C ₉ H ₁₃ N | 135.10 | 10.10 | - | 1.76 | 0.24 |
|  | Metanfetamina | MAMP | CH ₃ | H | C ₁₀ H ₁₅ N | 149.12 | 9.87 | - | 2.07 | 0.163 |
|  | 3,4-Metilendioxi metanfetamina | MDMA | CH ₃ | O-CH ₂ -O | C ₁₁ H ₁₅ NO ₂ | 193.11 | 10.32 ^a | - | 2.05 ^a | 3.17 E-3 ^a |
|  | 3,4-Metilendioxi anfetamina | MDA | H | O-CH ₂ -O | C ₁₀ H ₁₃ NO ₂ | 179.09 | 9.67 | - | 1.64 | 1.69 E-3 |
|  | 3,4-Metilendioxi etil anfetamina | MDEA | CH ₂ -CH ₃ | O-CH ₂ -O | C ₁₂ H ₁₇ NO ₂ | 207.13 | 10.34 ^a | - | 2.56 ^a | 1.17E-3 ^a |
| Piperacinas | | | | | | | | | | |
|  | N-Bencilpiperacina | BZP | - | - | C ₁₁ H ₁₆ N ₂ | 176.13 | 9.25 ^a | - | 1.08 ^a | 6.19E-3 ^a |

Tabla II.10 (continuación). Estructura y propiedades físico-químicas de las drogas de abuso consideradas. Datos experimentales recogidos en la base de datos PhysProp, Syracuse Research Corporation [11], o calculados mediante *software* proporcionados por la base de datos SciFinder Scholar ^(a) [12] o Chemicalize ^(b) [13].

| Clasificación y estructura | Compuesto | Abreviatura | R ₁ | R ₂ | Fórmula Empírica | Pm monoisotópico | pK _a grupo básico | pK _a grupo ácido | log K _{ow} | P _v (mm Hg) | |
|---|---------------------------------|-------------|----------------|----------------|---|------------------|------------------------------|-----------------------------|---------------------|------------------------|--|
|  | 1-(3-Clorofenil)-piperacina | mCPP | - | - | C ₁₀ H ₁₃ ClN ₂ | 196.08 | - | - | 2.19 ^b | 2.13 E-3 ^b | |
| Alucinógenos | | | | | | | | | | | |
|  | Dietilamida del ácido lisérgico | LSD | - | - | C ₂₀ H ₂₅ N ₃ O | 323.20 | 7.80 | - | 2.95 | 9.03 E-10 | |
|  | 2-Oxo-3-hidroxi-LSD | O-H-LSD | - | - | C ₂₀ H ₂₅ N ₃ O ₃ | 355.19 | - | - | - | - | |
| Anestésicos disociativos | | | | | | | | | | | |
|  | Fenciclidina | PCP | - | - | C ₁₇ H ₂₅ N | 243.20 | 8.29 | - | 4.69 | 1.91 E-4 | |
|  | Ketamina | KET | - | - | C ₁₃ H ₁₆ ClNO | 237.09 | 6.46 ^a | - | 3.12 | 5.15 E-5 | |

1.3. DISTRIBUCIÓN EN EL MEDIO AMBIENTE

Las aguas residuales constituyen la principal vía de entrada de las drogas de abuso en el medio ambiente, fundamentalmente como resultado de su excreción urinaria en forma libre, conjugada o como metabolitos. En menor medida, la descarga directa de sustancias ilícitas sobre desagües y sumideros puede incrementar la concentración de drogas en forma libre, mientras que los residuos procedentes de laboratorios clandestinos pueden contribuir a la presencia de agentes precursores [14]. Muchos de estos compuestos no son completamente eliminados de las aguas tras los tratamientos de depuración aplicados en las estaciones depuradoras [15,16], alcanzando las aguas superficiales [17] e, incluso, las aguas potables [17,18]. Debido a su carácter relativamente polar, las drogas de abuso y sus metabolitos tienden a distribuirse en la fase acuosa; sin embargo, algunas de ellas han sido determinadas en lodos [19,20] y material particulado suspendido [21,22] a niveles de muy pocos ng g^{-1} . Finalmente, y a pesar de su baja volatilidad, también han sido detectadas asociadas a material particulado en aire [23,24], especialmente aquéllas cuyos patrones específicos de consumo (fumadas o por inhalación en forma de polvo sólido) favorecen la liberación de vapores y partículas a la atmósfera.

1.3.1. Aguas

1.3.1.1. Aguas residuales

En la **Tabla II.11** se recogen los niveles encontrados en diversos países en agua residual sin tratar (influyente), tratada (efluente) y superficial (río) para las drogas de abuso y metabolitos considerados en la presente tesis doctoral. La escopolamina y la 1-(3-clorofenil)-piperacina (mCPP) no fueron determinadas en ningún caso.

En general, la cocaína y la benzoilecgonina fueron las sustancias encontradas a mayor concentración, y con mayor frecuencia, en los influentes analizados: hasta $4.7 \mu\text{g L}^{-1}$ y $7.5 \mu\text{g L}^{-1}$, respectivamente, en uno de los estudios llevados a cabo en España [25]. Aunque menores, también se determinaron valores altos para estas sustancias en Suíza [26], Canadá [22], o Bélgica [27] mientras que el resto de países notificaron niveles más bajos. Para el cocaetileno, las concentraciones encontradas fueron sensiblemente inferiores. Como reflejo de sus elevados niveles en las aguas residuales sin tratar, la cocaína y la benzoilecgonina resultaron ser, nuevamente, las drogas más abundantes en las aguas residuales tratadas, hasta 0.5 y $2.2 \mu\text{g L}^{-1}$, respectivamente [28].

B.1. ASPECTOS GENERALES

Entre los opioides, la codeína y la morfina presentaron los valores máximos más elevados: hasta 2.7 y 2 $\mu\text{g L}^{-1}$, respectivamente, entre los influentes, y 1.2 $\mu\text{g L}^{-1}$ entre los efluentes [26,29]. La metadona y su principal metabolito de excreción urinaria, el EDDP, superaron en uno de los estudios la concentración de 1 $\mu\text{g L}^{-1}$ en agua residual sin tratar, y los valores de 0.7 y 1 $\mu\text{g L}^{-1}$, respectivamente, en agua residual tratada [17]. La heroína y el fentanilo sólo fueron detectados en un caso a niveles próximos al límite de detección [15,29], mientras que la 6-acetilmorfina se mantuvo por debajo de los 82 ng L^{-1} en todas las muestras consideradas.

A pesar de su carácter moderadamente hidrofóbico, los cannabinoides también fueron detectados en aguas residuales. El THC sólo fue incluido en estudios llevados a cabo en España, encontrándose valores en torno a los pocos ng L^{-1} [15,17]. El THCCOOH, más abundante, fue determinado también en otros países, y aunque en algún caso aparece como no detectado/no cuantificado es necesario tener en cuenta que los límites de detección y cuantificación obtenidos para estos dos compuestos suelen ser generalmente elevados.

Las concentraciones determinadas para la anfetamina fueron excepcionalmente altas en algunos de los estudios considerados (hasta 1 $\mu\text{g L}^{-1}$ y 2.3 $\mu\text{g L}^{-1}$ en aguas residuales sin tratar de España y Reino Unido, respectivamente, y hasta 0.3 $\mu\text{g L}^{-1}$ en el agua tratada en España [28,29]). La metanfetamina y el éxtasis fueron cuantificados también a niveles relativamente altos (hasta 0.7 y 0.6 $\mu\text{g L}^{-1}$ en influentes [25,28]) mientras que el MDA y el MDEA mostraron en general valores inferiores.

Entre los anestésicos disociativos, el PCP sólo fue incluido, y no detectado, en el estudio llevado a cabo en Reino Unido [29]; la ketamina fue determinada en cuatro estudios, tres en España [25,28,30] y uno en Reino Unido [29], mostrando los valores más elevados en este último caso (hasta 160 y 228 ng L^{-1} en influente y efluente, respectivamente). Finalmente, la *N*-bencilpiperacina sólo se determinó en el Reino Unido [29], y el alucinógeno LSD y su metabolito, el O-H-LSD, sólo fueron cuantificados a niveles muy bajos en España [15].

En general, las concentraciones de drogas de abuso en los efluentes resultaron ser un orden de magnitud inferiores a las medidas en los influentes, lo que pone de manifiesto su eliminación parcial de las aguas tras los tratamientos aplicados en las estaciones depuradoras.

En base a seis de los estudios considerados en la **Tabla II.11**, que aportaron datos de porcentajes de eliminación [17,22,25,27,28,31], la cocaína y sus metabolitos fueron los compuestos que se eliminaron con mayor eficacia, generalmente en porcentajes superiores al 90%.

La eliminación de opioides resultó menos efectiva (en torno a un 50-70%), encontrándose en algunos casos concentraciones más elevadas en las aguas tratadas que en las aguas residuales sin tratar para la morfina o la codeína [17,32]. Este hecho puede atribuirse a que estos compuestos son excretados en la orina como conjugados glucurónidos [4] que, posteriormente, se hidrolizan dando lugar a la molécula de partida [33].

Esta misma tendencia fue observada para algunos derivados anfetamínicos como la metanfetamina, el MDMA y el MDA [15,25,29]; en el caso de este último, el incremento de su concentración en los efluentes ha sido atribuido a la *N*-demetilación del MDMA durante los tratamientos de depuración [25], mientras que los procesos implicados en la formación de metanfetamina y MDMA no han sido completamente elucidados (algunos autores sugieren que podrían tratarse de procesos de desorción, [34]). En general, los porcentajes de eliminación notificados para las anfetaminas variaron significativamente, tanto entre compuestos como entre estaciones depuradoras.

Entre los cannabinoides, la eliminación del THC resultó más efectiva (>90%) que la del THCCOOH (<50%), otra de las sustancias que pueden ver incrementada su concentración en los efluentes como consecuencia de la hidrólisis de formas conjugadas menos estables.

1.3.1.2. Aguas superficiales

La cocaína y los opioides codeína, morfina y metadona, junto con los metabolitos EDDP y benzoilecgonina, fueron las drogas de abuso detectadas con mayor frecuencia en aguas fluviales. Las concentraciones más elevadas dentro de los estudios considerados correspondieron a la codeína ($0.3 \mu\text{g L}^{-1}$, Reino Unido [29]) y la benzoilecgonina ($0.5 \mu\text{g L}^{-1}$, España [28]). La morfina, el EDDP y la cocaína se mantuvieron a valores inferiores o en torno a 100 ng L^{-1} , mientras que la metadona no superó los 18 ng L^{-1} . La heroína, la 6-acetilmorfina y el fentanilo o no fueron detectados o se detectaron a valores muy bajos.

El THCCOOH fue cuantificado a concentraciones de hasta 80 ng L^{-1} en uno de los estudios realizados en España [17], mostrando valores sensiblemente inferiores en el resto de los casos considerados.

Entre las anfetaminas, el MDMA fue la sustancia detectada con mayor frecuencia, si bien a niveles inferiores a los 25 ng L^{-1} . El resto de derivados anfetamínicos mostraron valores más bajos, con excepción de la anfetamina, que sorprendentemente se detectó a 300 ng L^{-1} en aguas fluviales españolas [28]. La *N*-bencilpiperacina y la ketamina se determinaron a niveles de hasta 65 y 51 ng L^{-1} , respectivamente, en el Reino Unido [29], mientras que el LSD y su principal metabolito no superaron el límite de detección en ningún caso.

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Tabla II.11. Rango de concentraciones, concentración media o mediana* / máxima (ng L⁻¹) de las drogas de abuso consideradas en aguas residuales y fluviales. N: número de estaciones depuradoras (residual) o puntos de muestreo (fluvial) incluidos en el estudio; nd: inferior al límite de detección; nc: inferior al límite de cuantificación.

| Concentración (ng L ⁻¹) | N | Influyente | Efluente | N | Río | País | Ref. |
|-------------------------------------|----|------------|----------|----|---------|-------------|------|
| Opioides | | | | | | | |
| Morfina | 4 | 63-196 | 12-30 | - | - | España | [15] |
| | 15 | 26-278 | 12-81 | 16 | nd-31 | España | [17] |
| | 1 | 90-275 | 60-155 | 6 | 12-19 | España | [28] |
| | 1 | 83 | nc | - | - | Italia | [33] |
| | 7 | 481/819 | 131/244 | 6 | 36/36 | Reino Unido | [29] |
| | 5 | nc-1970 | 84-1270 | 22 | nc-14 | Suíza | [26] |
| | 11 | 310*/820 | 40*/110 | 7* | 10/78 | Alemania | [35] |
| Codeína | 15 | 6-120 | 3-397 | 16 | nd-251 | España | [17] |
| | 1 | 234-1556 | 289-786 | 6 | 32-174 | España | [28] |
| | 7 | 2121/2703 | 437/1206 | 6 | 128/342 | Reino Unido | [29] |
| | 5 | nc-389 | 94-274 | 22 | nc-18 | Suíza | [26] |
| | 11 | 220*/540 | 85*/260 | 7 | 38*/94 | Alemania | [35] |
| Heroína | 4 | nd-2 | nd-1 | - | - | España | [15] |
| | 15 | nd | nd | 16 | nd | España | [17] |
| | 1 | nd | nd | 6 | nd | España | [28] |
| | 7 | nd | nd | 6 | nd | Reino Unido | [29] |
| 6-Acetilmorfina | 4 | 6-19 | 2-5 | - | - | España | [15] |
| | 15 | nd | nd | 16 | nd | España | [17] |
| | 1 | nd | nd | 6 | nd | España | [28] |
| | 1 | 12 | nc | - | - | Italia | [33] |
| | 7 | 22/70 | nd | 6 | nd | Reino Unido | [29] |
| | 5 | nc-82 | nc-7 | 22 | nc-1 | Suíza | [26] |
| Metadona | 15 | 3-1531 | 3-732 | 16 | nd-18 | España | [17] |
| | 1 | 19-127 | 15-80 | 6 | 2-14 | España | [28] |
| | 1 | 12 | 9 | - | - | Italia | [33] |
| | 7 | 88/171 | 50/69 | 6 | 10/18 | Reino Unido | [29] |
| | 5 | 42-202 | 44-128 | 22 | nc-5 | Suíza | [26] |
| EDDP | 15 | 3-1029 | 3-1150 | 16 | 2-64 | España | [17] |
| | 1 | 64-542 | 49-90 | 6 | 31-40 | España | [28] |
| | 1 | 20 | 23 | - | - | Italia | [33] |
| | 7 | 193/342 | 89/162 | 6 | 19/38 | Reino Unido | [29] |
| | 5 | 153-634 | 151-442 | 22 | 0.6-12 | Suíza | [26] |
| Fentanilo | 15 | nd | nd | 16 | nd-2 | España | [17] |
| | 7 | 1.7/2.2 | nd | 6 | nd | Reino Unido | [29] |
| Cannabinoides | | | | | | | |
| THC | 4 | nd-39 | nd-21 | - | - | España | [15] |
| | 15 | 11-127 | 21 | 16 | nd | España | [17] |

Tabla II.11 (continuación). Rango de concentraciones, concentración media o mediana* / máxima (ng L⁻¹) de las drogas de abuso consideradas en aguas residuales y fluviales.

| Concentración (ng L ⁻¹) | N | Influyente | Efluente | N | Río | País | Ref. |
|-------------------------------------|----------|------------|----------|-------|---------|-------------|------|
| THCCOOH | 4 | nd-33 | 4-19 | - | - | España | [15] |
| | 15 | 24-402 | 15-72 | 16 | nd-80 | España | [17] |
| | 1 | 63 | nc | - | - | Italia | [33] |
| | 5 | nc | nc | 22 | nc | Suiza | [26] |
| Alcaloides | | | | | | | |
| Cocaína | 4 | 316-1120 | 3-105 | - | - | España | [15] |
| | 16 | 79/225 | 17/47 | 1 | 6/10 | España | [30] |
| | 42 | 4-4700 | 1-100 | | | España | [25] |
| | 1 | 40-820 | 12-496 | 6 | 5-87 | España | [28] |
| | 1 | 421 | nc | - | - | Italia | [33] |
| | 7 | 71/109 | 29/65 | 6 | 6/14 | Reino Unido | [29] |
| | 5 | nc-1920 | nc-106 | 22 | nc-4 | Suiza | [26] |
| | 4 | 5-282 | nc-21 | - | - | Francia | [31] |
| | 3 | 209-823 | nc-530 | - | - | Canadá | [22] |
| | 7 | 92-753 | nc-8 | 10 | 2-115 | Bélgica | [27] |
| Benzoilecgonina | 4 | 1020-5980 | 9-318 | - | - | España | [15] |
| | 16 | 810/2307 | 216/928 | 1 | 77/111 | España | [30] |
| | 42 | 9-7500 | 1-1500 | - | - | España | [25] |
| | 1 | 851-4094 | 487-2221 | 6 | 10-530 | España | [28] |
| | 1 | 1132 | nc | - | - | Italia | [33] |
| | 7 | 243/368 | 116/293 | 6 | 27/53 | Reino Unido | [29] |
| | 5 | nc-1860 | 37-425 | 22 | nc-11 | Suiza | [26] |
| | 4 | 64-849 | 8-149 | - | - | Francia | [31] |
| | 1 | -*/78 | -*/49 | 3 | 3*/3 | Alemania | [35] |
| | 3 | 287-2624 | 62-775 | - | - | Canadá | [22] |
| 7 | 322-2258 | 4-23 | 10 | 8-520 | Bélgica | [27] | |
| Cocaetileno | 4 | 44-125 | 0.9-7 | - | - | España | [15] |
| | 1 | 12 | nc | - | - | Italia | [33] |
| | 7 | nd | nd | 6 | nd | Reino Unido | [29] |
| Derivados anfetamínicos | | | | | | | |
| Anfetamina | 4 | 7-53 | 0.4-3 | - | - | España | [15] |
| | 16 | 15/15 | nc | 1 | nd | España | [30] |
| | 42 | 3-688 | 4-210 | - | - | España | [25] |
| | 1 | 212-1021 | 215-325 | 6 | 309 | España | [28] |
| | 1 | 15 ± 11 | nc | - | - | Italia | [33] |
| | 7 | 830/2300 | 8/24 | 6 | 3/4 | Reino Unido | [29] |
| | 5 | nc-93 | nc | 22 | nc | Suiza | [26] |
| | 3 | nc-25 | nd-14 | - | - | Canadá | [22] |

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Tabla II.11 (continuación). Rango de concentraciones, concentración media o mediana^{*} / máxima (ng L⁻¹) de las drogas de abuso consideradas en aguas residuales y fluviales.

| Concentración (ng L ⁻¹) | N | Influyente | Efluente | N | Río | País | Ref. |
|-------------------------------------|----|------------|----------|----|-------|-------------|------|
| Metanfetamina | 4 | 3-28 | 2-7 | - | - | España | [15] |
| | 42 | 3-277 | 3-90 | - | - | España | [25] |
| | 1 | 475-700 | nd | 6 | nd | España | [28] |
| | 1 | 16 | 4 | - | - | Italia | [33] |
| | 7 | 2/3.8 | 1/1.2 | 6 | nd | Reino Unido | [29] |
| | 5 | nc-27 | nc-11 | 22 | nc | Suíza | [26] |
| | 3 | nc-65 | nd-95 | - | - | Canadá | [22] |
| MDMA | 4 | 47-245 | 30-376 | - | - | España | [15] |
| | 16 | 49/91 | 41/67 | 1 | 3/3.5 | España | [30] |
| | 42 | 2-598 | 2-267 | - | - | España | [25] |
| | 1 | nd | nd | 6 | nc | España | [28] |
| | 1 | 14 | 4 | - | - | Italia | [33] |
| | 7 | 39/138 | 38/156 | 6 | 9/25 | Reino Unido | [29] |
| | 5 | nc-108 | nc-29 | 22 | nc-1 | Suíza | [26] |
| | 4 | nc-28 | nc-10 | - | - | Francia | [31] |
| | 3 | 9-35 | nd-32 | - | - | Canadá | [22] |
| MDA | 42 | 3-266 | 1-200 | - | - | España | [25] |
| | 1 | 266 | nd | 6 | nd | España | [28] |
| | 1 | 5 | 1 | - | - | Italia | [33] |
| | 7 | 10/15 | 15/25 | 6 | nd | Reino Unido | [29] |
| | 3 | nc | nc | - | - | Canadá | [22] |
| MDEA | 16 | 28/28 | nc | 6 | nd | España | [30] |
| | 42 | 6-114 | 12 | - | - | España | [25] |
| | 1 | nd | nd | 6 | nd | España | [28] |
| | 1 | 1.5 | nc | - | - | Italia | [33] |
| | 7 | nd | nd | 6 | nd | Reino Unido | [29] |
| Derivados de la piperacina | | | | | | | |
| BZP | 7 | 25/38 | 31/66 | 6 | 26/65 | Reino Unido | [29] |
| Alucinógenos | | | | | | | |
| LSD | 4 | 1-5 | 0.2-2 | - | - | España | [15] |
| | 7 | nd | nd | 6 | nd | Reino Unido | [29] |
| O-H-LSD | 4 | nd-9 | nd-1 | - | - | España | [15] |
| | 7 | nd | nd | 6 | nd | Reino Unido | [29] |
| Anestésicos disociativos | | | | | | | |
| PCP | 7 | nd | nd | 6 | nd | Reino Unido | [29] |
| Ketamina | 16 | 41/50 | 19/49 | 1 | nd | España | [30] |
| | 42 | 7-50 | 5 | - | - | España | [25] |
| | 1 | nd | nd | 6 | nd | España | [28] |
| | 7 | 79/160 | 130/228 | 6 | 21/51 | Reino Unido | [29] |

1.3.1.3. Aguas potables

Como consecuencia de su distribución en aguas superficiales, las drogas de abuso y sus metabolitos pueden alcanzar las aguas destinadas al consumo humano si no son previamente eliminadas de manera conveniente. Bajo esta premisa, el grupo de F. Ventura [17,36] evaluó la efectividad de los tratamientos aplicados en una planta potabilizadora española para la eliminación de diversas sustancias ilícitas detectadas previamente en el agua de entrada a la planta. Concluyeron que mientras la morfina, la codeína, los derivados anfetamínicos y los cannabinoides eran eliminados por completo tras todo el tratamiento, en torno a un 9%, un 12% y un 10% de la metadona, el EDDP y la benzoilecgonina, respectivamente, resistían el proceso de potabilización.

Recientemente, estos mismos autores evaluaron la presencia de diversas drogas de abuso en aguas potables procedentes de Japón y de diversos países de Europa y América del Sur [18]. Para la cocaína y la benzoilecgonina las concentraciones medias globales fueron de 0.3 y 1.8 ng L⁻¹, respectivamente, con valores especialmente altos en América Latina (hasta 15 ng L⁻¹ para la benzoilecgonina). Entre los opioides, la morfina y la codeína no fueron detectadas en ningún caso, mientras que la metadona y el EDDP pudieron ser cuantificados en el 19 y el 58% de las muestras alcanzando valores medios globales de 0.1 y 0.3 ng L⁻¹, respectivamente. Los derivados anfetamínicos fueron detectados de manera muy ocasional, y los cannabinoides, el LSD, la ketamina, el fentanilo y el PCP no superaron los límites de detección en ninguna de las muestras analizadas.

1.3.2. Lodos, biosólidos y material particulado suspendido

Los primeros trabajos que evidencian la presencia de alguna de las drogas de abuso consideradas en la presente tesis doctoral en lodos procedentes de estaciones depuradoras aparecieron en 2006: Kaleta et al. [20] analizaron muestras procedentes de 12 depuradoras diferentes y encontraron niveles de anfetamina de entre 5 y 50 ng g⁻¹ en 10 casos, y en torno a 300 ng g⁻¹ en los dos restantes. Posteriormente, Jones-Lepp y Stevens [19] detectaron metanfetamina en muestras de esta misma naturaleza a niveles de hasta 4 ng g⁻¹. Kinney et al. [37] determinaron las concentraciones de diversos fármacos en suelos irrigados con agua residual reutilizada en Estados Unidos notificando valores máximos de 22 ng g⁻¹ para la codeína.

Finalmente, sólo dos estudios publicados hasta la fecha han determinado drogas de abuso en material particulado suspendido en aguas residuales: Metcalfe et al. [22] detectaron cocaína y benzoilecgonina a concentraciones máximas de 16 y 10 ng g⁻¹, respectivamente, lo que representa una proporción del 3% con respecto a los niveles que encontraron en agua

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residual. Entre las anfetaminas consideradas en su estudio, anfetamina, MDMA, metanfetamina y MDA, sólo estas dos últimas fueron detectadas en material particulado suspendido, a valores máximos de 0.3 y 2.7 ng g⁻¹, respectivamente (que representan un 6 y un 18% de adsorción).

Baker y Kasprzyk-Hordern [21] compararon las concentraciones de diversas sustancias ilícitas y metabolitos en agua residual y en material particulado suspendido de 3 estaciones depuradoras del Reino Unido. Encontraron que los porcentajes medios de adsorción sobre las partículas, con respecto a la fase acuosa, eran inferiores al 5% para la cocaína, la benzoilecgonina, el cocaetileno, el MDMA, la codeína, la morfina y la ketamina, mientras que superaban el 10% para la metadona, el EDDP, la *N*-bencilpiperacina y el fentanilo. Para estos últimos compuestos, concluyeron que descartar la fracción adsorbida sobre el material particulado (como ocurre habitualmente al filtrar las muestras de agua antes de analizarlas) podría implicar un error en los niveles ambientales notificados.

1.3.3. Atmósferas exteriores

Técnicamente, la primera vez que se detectó una droga de abuso en una matriz ambiental fue precisamente en aire: un análisis no dirigido (*non-target*) reveló la presencia de cocaína asociada a partículas atmosféricas en ambientes exteriores de la ciudad de Los Ángeles [38]. Desde entonces, la monitorización de drogas en atmósferas se ha extendido a otras sustancias ilícitas como cannabinoides, anfetaminas y opioides [23,39] y a otros países como Italia, Portugal, España, Chile o Brasil. Los niveles detectados no superan por lo general los pocos pg m⁻³, aunque estos valores pueden oscilar dos o tres órdenes de magnitud dentro de la misma área geográfica y son especialmente dependientes de las condiciones meteorológicas [14,23].

1.4. CONSIDERACIONES ECO-TOXICOLÓGICAS

La información disponible acerca del impacto tóxico de las drogas de abuso en el medio ambiente es muy escasa, especialmente en el caso de las drogas sintéticas. La mayor parte de los estudios realizados hasta la fecha evalúan la toxicidad aguda en humanos o mamíferos modelo, principalmente ratas y ratones; poco se conoce en cambio acerca de los posibles efectos adversos de estas sustancias en los organismos acuáticos, principales receptores de sus concentraciones ambientales.

Aunque propiamente no existen ensayos con cocaína, Suzuki et al. [40,41] descubrieron que varios anestésicos locales sintéticos derivados de ella (e.g. la lidocaína) promueven el

crecimiento de diferentes especies de algas y cianobacterias a niveles de exposición de entre 0.01 y 1000 mg L⁻¹.

En crustáceos, la codeína y la morfina mostraron valores de concentración efectiva (EC₅₀) tras 24 horas de exposición de 83.5 y 88.3 mg L⁻¹, respectivamente, para la especie *Daphnia pulex* [42]. Alternativamente, la concentración letal (LC₅₀) de anfetamina determinada para *Daphnia magna* en tests a 24 horas varía de 60.4 a 265.3 mg L⁻¹ [43,44]. Para esta misma especie, la EC₅₀ del THC tras 48 horas de exposición es de 24.5 mg L⁻¹ [45].

Finalmente, varios estudios recogen los efectos de diversas drogas ilegales en peces. Darland y Dowling [46] observaron que una exposición repetida a cocaína induce en el pez cebra (*Danio rerio*) ralentización de movimientos, disminución de la sensibilidad visual, excitación e incremento de la agresividad. En embriones de esta misma especie, concentraciones superiores a 2 mg L⁻¹ de THC provocan la muerte de un significativo número de individuos tras 24 horas de exposición. Tal y como se recoge en la Hoja de Seguridad de Cerilliant para esta sustancia, el LC₅₀ para la trucha arcoíris (*Onchorhynchus mykiss*, 96-h) y la carpa europea (*Cyprinus carpio*, 48-h) es de 19 y 36 mg L⁻¹, respectivamente [45].

En base a los trabajos existentes con *Daphnia magna*, *Daphnia pulex*, *Danio rerio*, *Onchorhynchus mykiss* y *Cyprinus carpio*, Domingo et al. [47] estimaron los valores de concentración prevista sin efectos (PNEC) para la morfina, la anfetamina, la cocaína y el THC y los compararon con los valores de concentración ambiental medida (MEC) proporcionados por diversos estudios ambientales en agua superficial; concluyeron que ninguna de las sustancias consideradas implicaba un riesgo potencial para los organismos estudiados a las concentraciones a las que se habían detectado en el medio acuático, mostrando valores de cociente de riesgo (RQ = MEC/PNEC) sensiblemente inferiores a 1 en todos los casos. No obstante, estos mismos autores destacaban que eran necesarios estudios adicionales para poder establecer con mayor claridad el riesgo medioambiental real de las drogas de abuso. Muchas de estas sustancias y sus metabolitos coexisten en las aguas superficiales, pudiendo dar lugar efectos sinérgicos y/o aditivos sobre los organismos acuáticos que, además, están expuestos a ellas durante las diferentes etapas de su desarrollo.

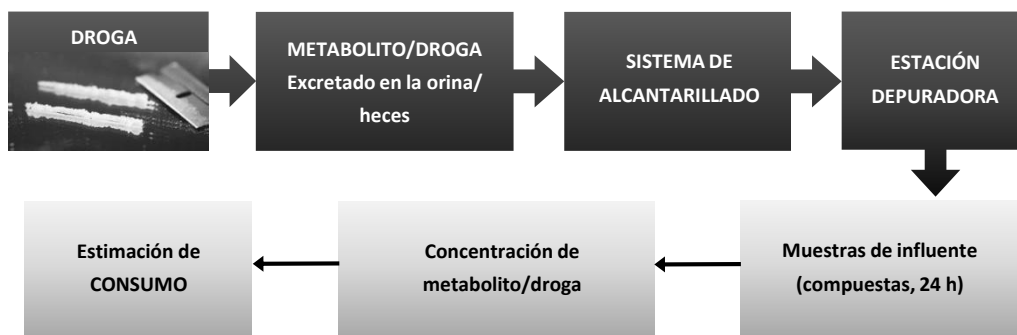
Finalmente, y aunque algunos estudios sugieren que estas sustancias se degradan con gran rapidez en el medio ambiente, las reacciones en las que se ven implicadas pueden dar lugar a productos de transformación potencialmente más tóxicos. El único trabajo publicado hasta la fecha en relación a esta materia evalúa la fototransformación de la metadona en el medio acuático y concluye que, en este caso, la toxicidad aguda de los compuestos generados no es relevante [48].

1.5. POTENCIAL DEL ANÁLISIS DE AGUAS COMO APROXIMACIÓN PARA MONITORIZAR EL CONSUMO DE DROGAS

En la actualidad, los métodos oficiales existentes para estimar la prevalencia del abuso de drogas en un país o región determinada están basados en estudios socio-epidemiológicos clásicos que integran encuestas a la población, historiales médicos, índices de incautación y producción de sustancias ilegales y estadísticas criminológicas. Una parte considerable de la información recabada en estos estudios procede de los propios consumidores, por lo que los métodos resultantes se consideran sesgados en la medida en que los datos que utilizan no son objetivos y tienden a subestimar la extensión del problema de la drogadicción [49]. Adicionalmente, la realización de encuestas a la población es tediosa e implica una importante cantidad de recursos económicos y humanos, por lo que es difícil que permita detectar a tiempo real cambios en las pautas de abuso [50].

En 2001, C.G. Daughton [51] sugirió que, dado que las drogas y sus metabolitos son excretados a través de la orina y liberados a las aguas residuales, su concentración en esta matriz podría correlacionarse con la prevalencia de su consumo dentro de una población determinada. Aplicada por primera vez en 2005, esta nueva metodología, referida como *Epidemiología de Aguas Residuales*, permitió a Zuccato et al. estimar el consumo local de cocaína en diversas ciudades italianas a partir del análisis de sus aguas residuales [52]. Desde entonces, se ha extendido a otras sustancias (cannabis, anfetaminas, heroína) y ha sido aplicada con éxito en diversos países [16,17,25,53-55].

Figura II.5. Esquema general de la *Epidemiología de Aguas Residuales*.



La **Figura II.5** presenta el esquema general del fundamento de esta metodología [56,57]. Se toman muestras de influente, preferiblemente muestras integradas de manera proporcional al flujo de entrada de la estación depuradora durante un período de tiempo conocido, e.g. 24 horas; se determina la concentración de los compuestos objetivo (los

métodos de análisis desarrollados hasta la fecha serán discutidos en siguientes apartados de la presente memoria) y se evalúan las cargas de excreción de cada compuesto multiplicando los valores de su concentración por el flujo medio de entrada de agua en la estación depuradora:

$$Carga\ de\ excreción\ (g\ día^{-1}) = Concentración\ (g\ L^{-1}) \times Flujo\ (L\ día^{-1})$$

Las cargas de excreción se transforman en cargas de consumo aplicando un factor de corrección que consiste en la inversa de la fracción de sustancia que es excretada como tal en orina. Para aquellas drogas que se metabolizan en gran extensión, generalmente se determina el producto o productos mayoritarios de excreción urinaria, siempre que sean estables en agua residual e, idealmente, exclusivos de esa sustancia. En estos casos el factor de corrección se calcula como la relación de los pesos moleculares dividida por la fracción molar de droga nativa que es excretada en esa forma metabólica.

$$Carga\ de\ consumo\ (g\ día^{-1}) = Carga\ de\ excreción\ (g\ día^{-1}) \times Factor\ de\ corrección$$

Donde,

$$Factor\ de\ corrección = \frac{Pm_{Droga}/Pm_{Metabolito}}{Fracción\ molar\ de\ droga\ excretada\ como\ metabolito}$$

Finalmente, dividiendo la carga de consumo por la masa de sustancia que constituye habitualmente una dosis y por el número de usuarios de la estación depuradora (EDAR) considerada se obtiene una estimación aproximada de la prevalencia de su consumo en esa región:

$$Consumo\ (dosis\ usuario^{-1}\ día^{-1}) = \frac{Carga\ de\ consumo\ (g\ día^{-1})}{masa\ dosis\ (g\ dosis^{-1}) \times n^{\circ}\ usuarios\ EDAR\ (usuarios)}$$

Habitualmente expresada como número de dosis por cada 1000 usuarios (o habitantes).

En la **Tabla II.12** se recogen las principales drogas de abuso y residuos metabólicos monitorizados en *Epidemiología de Aguas Residuales* junto con los factores de corrección aplicados en cada caso [50].

El consumo de cocaína y cannabis se estima a partir de las cargas de excreción de sus metabolitos mayoritarios y más estables, la benzoilecgonina y el THCCOOH, respectivamente; para los derivados anfetamínicos, excretados principalmente sin metabolizar, se utilizan las cargas del compuesto en su forma nativa. El caso de la heroína es más complicado: su producto urinario mayoritario, la morfina, es un residuo metabólico común a la codeína y la propia morfina, utilizadas legalmente con fines médicos; la contribución de estas aplicaciones

B.1. ASPECTOS GENERALES

puede estimarse en base a informes clínicos y sustraerse para el cálculo global, tal y como se ha llevado a cabo con la morfina en un estudio reciente [56]. Alternativamente, podría estimarse la carga de la 6-acetilmorfina, un metabolito minoritario (1.3%) pero exclusivo de la heroína [6]; sin embargo, su concentración en aguas residuales es generalmente muy baja y difícil de monitorizar.

Tabla II.12. Drogas de abuso, residuos metabólicos y factores de corrección aplicados en *Epidemiología de Aguas Residuales*.

| Droga | Residuo | % Droga excretada como residuo | $Pm_{Droga}/Pm_{Residuo}$ | Factor de Corrección |
|---------------|-----------------|--------------------------------|---------------------------|----------------------|
| Cocaína | Benzoilecgonina | 45 | 1.05 | 2.33 |
| | Cocaína | 10 | 1.00 | 10.00 |
| Heroína | Morfina | 42 | 1.29 | 3.07 |
| | 6-acetilmorfina | 1.3 | 1.13 | 86.92 |
| Anfetamina | Anfetamina | 30 | 1.00 | 3.33 |
| Metanfetamina | Metanfetamina | 43 | 1.00 | 2.33 |
| Éxtasis | MDMA | 65 | 1.00 | 1.54 |
| Cannabis | THCCOOH | 0.6 | 0.91 | 151.67 |

La evaluación del consumo de heroína pone de manifiesto que la *Epidemiología de Aguas Residuales* no constituye una herramienta alternativa, sino complementaria, a los métodos clásicos de estimación de consumo de drogas. Comparada con los estudios socio-epidemiológicos, es más objetiva, más barata, menos intrusiva y facilita datos cuantitativos y cualitativos casi a tiempo real, permitiendo detectar cambios en las pautas de consumo en el mismo momento (días) en que se están produciendo [56,57]. No obstante, presenta algunas fuentes de gran variabilidad que deben ser controladas en un futuro para poder mejorar la exactitud de los cálculos obtenidos. Entre ellas, destacan las siguientes:

- metabolismo, farmacocinética y ruta de administración de la droga (los porcentajes de sustancia excretada como tal o como metabolito están dentro de unos rangos y no responden a valores exactos)
- procesos de adsorción/degradación que pueda experimentar desde su excreción hasta su llegada a la estación depuradora
- posibilidad de que cantidades de sustancia pura sean desechadas a través de los sistemas de alcantarillado
- fugas en dichos sistemas
- fluctuaciones temporales en el número de usuarios de las estaciones depuradoras

2. PREPARACIÓN DE MUESTRAS DE AGUA

En la presente tesis doctoral, la determinación de drogas de abuso ha sido abordada únicamente en muestras de agua utilizando la SPE como técnica de extracción. Los aspectos generales de esta metodología han sido detallados en la sección II.A.2, por lo que el siguiente apartado pretende resumir las condiciones descritas en la bibliografía para la extracción en fase sólida (*off-line* y *on-line*) de sustancias ilícitas en aguas. Adicionalmente, se presentan otras de las técnicas que han sido utilizadas para la determinación ambiental de estos compuestos: la inyección directa en el cromatógrafo de líquidos (sin etapa previa de preconcentración) [26,28,58,59] y el empleo de muestreadores pasivos, combinados con el respectivo proceso de desorción [60,61]. Como primer apartado se incluye una discusión de las condiciones de almacenamiento y los procesos de pretratamiento (filtración, acidificación) aplicados a las muestras para incrementar la estabilidad de este grupo de analitos.

2.1. ALMACENAMIENTO Y PRETRATAMIENTO DE MUESTRAS

Varios de los estudios publicados en relación a la determinación de drogas de abuso en aguas almacenan las muestras durante un tiempo (días) antes de proceder a su extracción y/o análisis. En algún caso, el almacenamiento se lleva a cabo a $-20\text{ }^{\circ}\text{C}$, si bien es más frecuente mantener las muestras a $4\text{ }^{\circ}\text{C}$ durante un máximo de 3 días [22,33,62,63]. En estas condiciones, la fiabilidad de los datos de concentración obtenidos puede verse cuestionada por la inestabilidad de los analitos. La mayor parte de los compuestos utilizados como drogas de abuso son bioactivos y pueden ser metabolizados por las bacterias presentes en el agua, (especialmente en el agua residual) originando una sub- o una sobrestimación de la cantidad de sustancia o metabolito contenida originalmente en las muestras. Adicionalmente, tanto las drogas como sus metabolitos pueden experimentar diversas reacciones de transformación (e.g. hidrólisis) o procesos de adsorción sobre el material particulado suspendido.

En esta línea, Castiglioni et al. [33] evaluaron la estabilidad de varias drogas de abuso y metabolitos en agua residual sin tratar (dopada y mantenida a $4\text{ }^{\circ}\text{C}$ durante 3 días) y encontraron una disminución significativa en las concentraciones de cocaína y cocaetileno (-36% y -13%, respectivamente) acompañada por un aumento en la correspondiente a la benzoilecgonina (+14%). Este mismo comportamiento se observó en el caso de la 6-acetilmorfina, hidrolizada en un 14%. Los niveles de los derivados anfetamínicos no variaron en gran extensión (entre -4 y +5%) y el del THCCOOH disminuyó en un 8%.

Gheorge et al. [64] monitorizaron a 24, 72 y 120 horas la señal de la cocaína y la benzoilecgonina en agua superficial a diferentes valores de pH (2 y 6) y temperatura (-20, 4 y 20 °C); la benzoilecgonina demostró ser estable durante todo el período de estudio y bajo todas las condiciones consideradas, mientras que la concentración de cocaína sólo se mantuvo constante en las muestras conservadas a -20 °C (con independencia del pH) o en las muestras acidificadas (con independencia de la temperatura).

En el mayor estudio de estabilidad de drogas de abuso realizado hasta la fecha, Baker y Kasprzyk-Hordern [65] incluyeron, además de la temperatura y el pH, una nueva variable: la filtración. En principio, la filtración de las muestras previa a su almacenamiento parece susceptible de promover la constancia en la concentración de los analitos en disolución al prevenir su adsorción sobre el material particulado suspendido y eliminar parte de las bacterias y microorganismos implicados en los procesos de biodegradación. Entre los compuestos estudiados, la benzoilecgonina demostró una gran estabilidad bajo todas las condiciones de almacenamiento consideradas: agua residual filtrada o sin filtrar, a pH 2 o 7.4 y conservada a 2 o a 19 °C. Para la cocaína, el factor de mayor relevancia resultó ser el pH (incrementándose su estabilidad con el descenso del mismo) mientras que para el cocaetileno la filtración tuvo un papel significativamente positivo. Exceptuando la anfetamina, cuya concentración experimentó un apreciable aumento en agua sin filtrar, conservada a pH 7 y a 2 °C, los restantes derivados anfetamínicos no sufrieron grandes cambios en sus niveles bajo ninguna condición. La estabilidad de la heroína descendió dramáticamente con el incremento del pH y con la presencia de sólidos en suspensión: en muestras sin filtrar, mantenidas a pH neutro y a 2 °C, su concentración descendió un 66% en tan sólo 12 horas, cuestionando la validez de los resultados obtenidos para esta sustancia con las muestras integradas a 24 horas, de uso tan habitual en *Epidemiología de Aguas Residuales*. Finalmente, y coincidiendo con las observaciones realizadas por Castiglioni et al. [33], la morfina y su conjugado glucurónico demostraron ser estables sólo a pH ácido.

Además de la acidificación y la congelación, en la presente tesis doctoral se ha demostrado que el método más eficaz para evitar procesos de adsorción y degradación de las drogas presentes en el agua es la extracción de la misma en cartuchos de fase sólida y la conservación de estos cartuchos a -20 °C, con el consecuente ahorro de espacio que ello implica en comparación con el almacenamiento de grandes volúmenes de muestra. Esta misma conclusión fue arrojada por Baker y Kasprzyk-Hordern tras extraer muestras de influente dopado en cartuchos Oasis MCX y conservarlos a -20 °C: realizando la elución al cabo de 6 semanas y analizando los extractos comprobaron que ninguno de los compuestos adicionados había experimentado un nivel de desaparición apreciable [65].

2.2. EXTRACCIÓN EN FASE SÓLIDA *OFF-LINE*

La inmensa mayoría de los trabajos publicados hasta la fecha para la determinación de drogas de abuso en aguas están basados en la modalidad *off-line* de la SPE. En la **Tabla II.13** se recogen las condiciones empleadas en algunos de ellos así como los porcentajes de recuperación de los métodos desarrollados.

Dado el carácter básico y polar de la mayoría de las drogas, los adsorbentes utilizados para su retención son o bien materiales poliméricos de fase reversa y balance hidrofílico-lipofílico (Oasis HLB) [30,31,35,62] o adsorbentes, también poliméricos, en modo mixto fase reversa-intercambiador catiónico fuerte (Oasis MCX [33,66,67], Strata-XC [32,58]). En el primer caso, las muestras, de entre 100 y 1000 mL dependiendo de su naturaleza, se extraen sin llevar a cabo ningún ajuste de pH (en aguas residuales este pH se sitúa habitualmente entre 6 y 7 unidades). Para reducir la coelución de sales y de especies potencialmente interferentes, puede introducirse una etapa de lavado con agua ultrapura previa a la elución de los analitos, que se realiza con disolventes moderadamente polares como el metanol [30,31,62] o la acetona [35].

Con los adsorbentes en modo mixto, el procedimiento más frecuente consiste en acidificar las muestras hasta valores de pH próximos a 2 o 3 y, tras la carga, lavar la fase estacionaria con agua ultrapura acidificada (adicionalmente, el agua de lavado puede contener un pequeño porcentaje de disolvente orgánico para favorecer la elución de interferencias orgánicas) [32,58,66]. Finalmente, los analitos (básicos) se neutralizan y se eluyen haciendo pasar un pequeño volumen de metanol, acetona o acetona:acetato de etilo conteniendo entre un 2 y un 5% de hidróxido amónico.

Como es habitual en los procedimientos de SPE *off-line*, la concentración de los analitos en el eluato se incrementa previamente a su introducción en el sistema cromatográfico mediante la evaporación de parte de (o de todo) el disolvente presente en el extracto. Este proceso puede implicar pérdidas de los compuestos por volatilización o descomposición térmica, aunque generalmente este efecto no ha sido estudiado de forma separada y sólo se ha tenido en cuenta en el valor de recuperación global de todo el procedimiento de extracción. Excepcionalmente, Baker y Kasprzyk-Hordern evaluaron la influencia de la temperatura de evaporación, conjuntamente con la naturaleza del disolvente a evaporar (el disolvente de elución), en la recuperación de diferentes sustancias de abuso [65]. En general, los porcentajes de recuperación más elevados se consiguieron al llevar a cabo la evaporación a 20 °C; un incremento en esta temperatura afectó negativamente a algunos de los compuestos estudiados (e.g. ketamina) y de forma más acusada en los extractos constituidos por metanol basificado (metanol conteniendo un 7% de hidróxido amónico) que en los extractos formados

por metanol puro. Los autores atribuyeron este comportamiento a que, a valores de pH altos, los analitos básicos están en forma neutra y, por tanto, son más apolares y más volátiles que en estado catiónico (a pH inferiores). La morfina se desmarcó de esta tendencia mostrando recuperaciones superiores en metanol basificado. Por otro lado, la deacetilación de la heroína para dar lugar a la 6-acetilmorfina resultó favorecida en medio básico (20% de descenso e incremento, respectivamente, en sus recuperaciones con respecto al metanol puro) y a altas temperaturas de evaporación. En vista de estos resultados, y dada la necesidad de alcanzar un compromiso entre estabilidad y rapidez, los autores recomendaron no superar los 40 °C durante la etapa de evaporación del disolvente, especialmente si éste está combinado con una base.

En todo caso, la adición de patrones marcados isotópicamente permite compensar posibles pérdidas de analito ocurridas durante los procesos de pretratamiento, extracción y/o postratamiento de las muestras. De esta forma, y siempre y cuando se mantengan dentro de unos porcentajes razonables, estas pérdidas no afectan significativamente a la exactitud de las determinaciones obtenidas.

2.3. EXTRACCIÓN EN FASE SÓLIDA *ON-LINE*

Como alternativa a los métodos *off-line*, Postigo et al. [15] desarrollaron un método de extracción en fase sólida *on-line* para la concentración de 17 sustancias de abuso y metabolitos (incluyendo al agente precursor efedrina) en aguas residuales. Utilizando un sistema robótico, se hacen pasar 5 mL de muestra filtrada a través de cartuchos de 10 mm × 2 mm (diámetro interno) Oasis HLB (para la determinación de cannabinoides) o PLRP-S (para los restantes analitos) previamente acondicionados con 1 mL de acetonitrilo y 1 mL de agua. Tras la carga, el adsorbente se lava con 1 mL de agua ultrapura y los analitos se eluyen directamente hacia la columna cromatográfica con la misma fase móvil utilizada para su posterior separación. Todos los pasos están controlados por el procesador automático de muestras, que actúa como un muestreador automático acoplado al cromatógrafo de líquidos, y además es posible preconcentrar una muestra mientras se lleva a cabo el análisis de la anterior, incrementando considerablemente el rendimiento temporal del sistema. Además de una completa automatización, las principales ventajas de esta técnica radican en el bajo consumo de disolventes orgánicos y muestra (5 mL), la mínima manipulación de esta última y la elevada reproducibilidad y sensibilidad alcanzadas (con límites de detección de entre 0.07 y 1.94 ng L⁻¹). Entre sus inconvenientes, los fuertes efectos de matriz combinados con las bajas recuperaciones obtenidas en agua residual (8-59%) hacen imprescindible la utilización de patrones internos marcados isotópicamente para la cuantificación.

Tabla II.13. Determinación de drogas de abuso en aguas mediante extracción en fase sólida *off-line*.

| Análitos | Muestras | Almacenamiento/ pretratamiento | Extracción | Postratamiento | Detección | %R | Ref. |
|---|--|--|---|--|--------------------------|---|------|
| MOR, COD, 6-AM, HER, FEN, EDDP, MET, THC, THCCOOH (+ otros 2 metabolitos) | 200 mL (río, inf.) Puntuales (río) Integr., 24 h (inf.) | A 4 °C (máx 3 días) Filtración | Oasis HLB 200 mg Acond.: 5 mL MeOH, 5 mL H ₂ O Lavado: 3 mL H ₂ O Elución: 8 mL MeOH | Conc. a 0.5 mL (40 °C) 0.25 mL a LC-MS (cannabinoides) 0.25 mL a sequedad Reconstitución en H ₂ O Filtración | UPLC- (ESI)- MS/MS | 44-99 (río) 42-96 (inf.) | [62] |
| COC, BE, AMP, MAMP, MDA, MDMA, MDEA, LSD, KET, PCP, FEN (+ 4 drogas blandas) | 100 mL (río, inf., ef.) Puntuales (río) Integr., 24 h (inf., ef.) | A 4 °C (máx 1 día) Filtración | Oasis HLB 200 mg Acond.: 10 mL MeOH, 10 mL H ₂ O Lavado: 8 mL 5% MeOH/H ₂ O Elución: 6 mL MeOH | Evap. a sequedad Reconstitución en 0.5 mL 5% MeOH/H ₂ O Filtración | UPLC- (ESI)- MS/MS | 75-99 (río) 70-101 (inf.) | [30] |
| MOR, 6-AM, MET, EDDP, COC, BE, COE, AMP, MAMP, MDA, MDMA, MDEA, THCCOOH (+ otros 3 metabolitos) | 50 mL (ef., inf.) Integr., 24 h | A 4 °C (máx 3 días) Filtración A pH 2 | Oasis MCX 60 mg Acond.: 6 mL MeOH, 3 mL H ₂ O, 3 mL H ₂ O pH 2 Elución: 3 mL MeOH, 3 mL 2% NH ₄ OH/MeOH | Evap. a sequedad Reconstitución en 0.2 mL H ₂ O Centrifugación | LC- (ESI)- MS/MS | 61-107 (ef.) 51-112 (inf.) | [33] |
| MOR, COD, MET, BE (+ otras drogas) | 1000 mL (río, agua subterránea, grifo) 200 mL (ef.) 100 mL (inf.) | A 4 °C (máx 1 día) Filtración | Oasis HLB 200 mg Acond.: 2 mL <i>n</i> -heptano, 2 mL acetona, 3 x 3 mL MeOH, 4 x 2 mL H ₂ O pH 7 Elución: 4 x 2 mL acetona | Evap. a 50 µL Dilución a 0.5 mL con 10% MeOH/H ₂ O | LC- (ESI)- MS/MS | 71-92 (sub.) 25-87 (grifo) 70-86 (río) 42-77 (ef.) 29-48 (inf.) | [35] |
| MOR, MET, EDDP, COC, BE, COE, MDMA, LSD, KET (+ 3 drogas) | 500 mL Puntuales (río) Integr., 24 h (ef., inf.) | Filtración A pH 6 SPE en 24 h cartuchos a -20 °C | Strata-XC 200 mg Acond.: 2 x 6 mL MeOH, 2 x 6 mL H ₂ O, Lavado: 50 mL 10% MeOH/H ₂ O 100 mM HCOOH Elución: 10 mL 5% NH ₄ OH/ Acetona: EtOAc 1:1 | Evap. a sequedad Reconstitución en 0.2 mL 30% MeOH/ H ₂ O 5 mM NH ₄ OAc pH 4.5 | LC- (ESI)- MS/MS | 4-65 (río) | [32] |

Tabla II.13 (continuación). Determinación de drogas de abuso en aguas mediante extracción en fase sólida *off-line*.

| Análitos | Muestras | Almacenamiento/ pretratamiento | Extracción | Posttratamiento | Detección | %R | Ref. |
|---|--|--|---|--|--------------------------|---|------|
| MOR, 6-AM, COC, BE, COE, AMP, MAMP, MDMA, MDEA (+ otras drogas y metabolitos) | 200 mL (inf.) Integr., 24 h | Filtración A pH 2 A -20 °C (máx 2 días) | Strata-XC 500 mg Acond.: 10 mL MeOH, 10 mL H ₂ O (pH 2) Lavado: 10 mL H ₂ O (pH 2), 10 mL 2% HCOOH/H ₂ O, 10 mL 5% MeOH/H ₂ O Elución: 10 mL 2% NH ₄ OH/MeOH | Evap. a 0.5 mL Dilución con 1 mL 0.1% HCOOH/H ₂ O | LC- (ESI)- MS/MS | 55-103 (agua desionizada) | [58] |
| COD, COC, BE, AMP (+ PPCPs) | 1000 mL (río) 250 mL (ef., inf.) Puntuales | A pH 2 A 4 °C Ad. 500 mg Na ₂ EDTA Filtración | Oasis MCX 60 mg Acond.: 2 mL MeOH, 2 mL 2% HCOOH/H ₂ O Lavado: 2 mL 2% HCOOH/H ₂ O Elución: 2 mL MeOH, 2 mL 5% NH ₄ OH/MeOH | Evap. a sequedad Reconstitución en 0.5 fase móvil Filtración | UPLC- (ESI)- MS/MS | 70-131 (río) 49-109 (ef.) 43-105 (inf.) | [66] |
| COC, BE, AMP, MAMP, MDMA, MDA | 200 mL (ef.) 100 mL (inf.) Integr., 24 h | A 4 °C (máx 2 días) Filtración A pH 3 | Oasis MCX 150 mg Acond.: 6 mL MeOH, 10 mL H ₂ O (pH 3) Lavado: 3 mL 5% MeOH/H ₂ O Elución: 2 x 3 mL 5% NH ₄ OH/MeOH, 5 mL 5% NH ₄ OH/MeOH | Evap. a sequedad Reconstitución en 0.4 mL MeOH:H ₂ O 1:1 | LC- (ESI)- MS/MS | - | [22] |
| COC, BE, AMP, MAMP, (+ buprenorfina) | 500 mL (ef.) 250 mL (inf.) Integr., 24 h | A 4 °C (máx 1 día) Filtración | Oasis HLB 500 mg Acond.: 2 x 5 mL MeOH, 2 x 5 mL H ₂ O Lavado: 5 mL H ₂ O Elución: 10 mL MeOH | Evap. a sequedad Reconstitución en 0.5 mL MeOH | LC- (ESI)- MS/MS | 74-89 (inf.) | [31] |
| 6-AM, MET, EDDP, COC, BE, AMP, MAMP, MDMA | 50 mL (inf.) Integr., 24 h | A pH 2 A -20 °C Filtración | Oasis MCX 60 mg Acond.: 6 mL MeOH, 4 mL H ₂ O, 4 mL H ₂ O (pH 2) Lavado: 3 mL H ₂ O Elución: 4 mL MeOH, 4 mL 5% NH ₄ OH/MeOH | Evap. a sequedad Reconstitución en 0.1 mL ACN Ad. 0.1 mL ACN:H ₂ O 5 mM NH ₄ OAc (9:1) | LC- (ESI)- MS/MS | 61-103 | [67] |

PPCPs: fármacos y productos de cuidado personal; ef.: efluente; inf.: influente; integr.: integradas; Ad.: adición; Acond.: acondicionamiento; Evap.: evaporación; Conc.: concentración; MeOH: metanol; NH₄OH: hidróxido amónico; HCOOH: ácido fórmico; EtOAc: acetato de etilo; NH₄OAc: acetato amónico; ACN: acetonitrilo.

2.4. INYECCIÓN DIRECTA EN EL SISTEMA LC-MS/MS

Otra alternativa para reducir el tiempo de análisis consiste en prescindir de la etapa de extracción en fase sólida e introducir directamente la muestra en el cromatógrafo de líquidos, (*direct injection*, DI). Generalmente, esta técnica implica la inyección de volúmenes de muestra superiores a los habituales (*large volume injection*, LVI) utilizando columnas compatibles con fases 100% acuosas. Combinada con instrumentación analítica de nueva generación (altamente sensible), permite llevar a cabo la determinación de algunos compuestos a niveles ambientalmente relevantes minimizando el coste, el tiempo y la complejidad del análisis.

Chiaia et al. [59] aplicaron por primera vez la LVI a la determinación de drogas de abuso en aguas centrifugando las muestras de influente e inyectando 1.8 mL del sobrenadante (en dos ciclos de 0.9 mL) en un bucle de acero inoxidable acoplado a la columna analítica (Atlantis T3 C₁₈). Los límites de cuantificación para las 19 drogas de abuso y metabolitos considerados oscilaron entre 2.5 y 10 ng L⁻¹.

En un estudio similar, Bisceglia et al. [58] desarrollaron un método que les permitió detectar 17 sustancias ilícitas y metabolitos en agua residual sin tratar a concentraciones inferiores a 50 ng L⁻¹. Los límites de detección para otros analitos considerados, con menores tiempos de retención, fueron considerablemente más altos (e.g. 670 ng L⁻¹ en el caso de la morfina). El pretratamiento recibido por las muestras consistió en su filtración a través de filtros de 1.2 y 0.2 μm y en su combinación con un 0.07% (v/v) de ácido fórmico, inyectando 5 μL de la disolución resultante directamente en una columna Restek Viva pentafluorofenil propil (PFPP). Para poder extender la determinación a otros compuestos a niveles ambientales reales (inferiores a 50 ng L⁻¹) desarrollaron una versión modificada del método incorporando una etapa de extracción en fase sólida (**Tabla II.13**).

Berset et al. [26] inyectaron 100 μL de muestra, previamente filtrada y acidificada a pH 2, en la columna cromatográfica (Hydro RP C₁₈) y obtuvieron, para la mayoría de las drogas y metabolitos considerados (12 en total), límites de cuantificación de 20 ng L⁻¹ en agua residual y de 0.2 ng L⁻¹ en agua superficial.

Recientemente, Martínez-Bueno et al. [28] desarrollaron un método para la determinación de drogas de abuso y metabolitos en agua fluvial y residual basado en la inyección directa de tan sólo 10 μL de muestra en una columna Zorbax Eclipse XDB C₈. Comprobando que el pH no ejercía una influencia significativa en los resultados obtenidos, analizaron las muestras a pH 3 (habían sido previamente acidificadas para minimizar la degradación de los analitos) y consiguieron límites de cuantificación de entre 10 y 700 ng L⁻¹ en agua residuales y de entre 0.5 y 700 ng L⁻¹ en agua fluvial.

2.5. SISTEMAS DE MUESTREO PASIVO

El empleo de muestreadores pasivos para compuestos orgánicos polares (*polar organic chemical integrative samplers*, POCIS) fue descrito por primera vez para la determinación de drogas de abuso en aguas por Jones-Leep et al. en 2004 [61]. Esta técnica, propiamente una técnica de muestreo y no de preparación de muestra, utiliza un adsorbente de SPE empaquetado entre dos membranas microporosas a través de las cuales difunden los analitos. El dispositivo se expone a la matriz acuosa durante un tiempo controlado y, posteriormente, el adsorbente se transfiere a una columna de vidrio y los compuestos retenidos se eluyen por gravedad con un disolvente orgánico. La principal ventaja de esta metodología es que permite muestrear grandes volúmenes, disminuyendo los límites de detección y ofreciendo valores medios de concentración en el tiempo. Sin embargo, requiere la realización de estudios de calibración para determinar las velocidades de difusión de los analitos, con el consecuente incremento de la complejidad y la duración del análisis.

Jones-Leep et al. [61] utilizaron un prototipo de POCIS con una superficie de exposición de 18 cm² y 100 mg de adsorbente Oasis HLB para la retención de diversos fármacos y dos derivados anfetamínicos (metanfetamina y MDMA) en agua residual tratada. El sistema se expuso a la corriente de efluente durante un período de tiempo comprendido entre 28 y 30 días y la elución de los analitos se llevó a cabo con metanol.

Bartelt-Hunt et al. [60] emplearon esta misma técnica para determinar anfetamina, metanfetamina y varias drogas legales en efluentes de estaciones depuradoras y en aguas fluviales sometidas a las descargas de estos efluentes. En este caso, cada dispositivo presentaba un área superficial de 41 cm² y contenía 200 mg de adsorbente Oasis HLB; el período de exposición se fijó en 7 días (salvo en una de las localizaciones, en donde el muestreo se extendió a un mes) y los analitos se eluyeron con 50 mL de metanol.

Recientemente, Harman et al. [68] monitorizaron durante un año las concentraciones de 11 drogas de abuso y metabolitos en agua residual exponiendo POCIS a una corriente de influente por períodos de dos semanas. Para ello utilizaron un diseño con una superficie de exposición de 18 cm² y 100 mg de adsorbente (Oasis HLB); tras un lavado de esta fase con 2 × 6 mL agua conteniendo un 20% de metanol, los compuestos retenidos se eluyeron con 6 mL de metanol con hidróxido amónico (0.5%) seguidos de 6 mL de metanol con ácido acético (1%).

3. DETERMINACIÓN

La inmensa mayoría de los métodos desarrollados hasta la fecha para la determinación de drogas de abuso y metabolitos en aguas están basados en la cromatografía líquida (LC) acoplada a la espectrometría de masas en tándem como sistema de detección. La cromatografía de gases acoplada a la espectrometría de masas (simple o en tándem), de gran aplicación para estas sustancias en el ámbito clínico y forense, ha sido mucho menos utilizada con fines medioambientales debido a su menor sensibilidad y mayores requisitos de preparación de muestra. El siguiente apartado recoge una breve revisión bibliográfica de las aplicaciones de ambas técnicas a la determinación de drogas de abuso en muestras de agua.

3.1. CROMATOGRAFÍA DE LÍQUIDOS ACOPLADA A ESPECTROMETRÍA DE MASAS

En la **Tabla II.14** se presentan algunos de los estudios existentes en la bibliografía para la determinación de drogas en aguas mediante LC-MS. La mayor parte de ellos han empleado la cromatografía de líquidos de alta eficacia en columnas de fase reversa (de entre 100 y 200 mm de longitud y 2.1-4 mm de diámetro) para lograr una separación satisfactoria [15,22,26,28,32,33,59]. En menor medida, se han utilizado columnas de esta misma fase pero haciendo uso de la cromatografía de líquidos de ultra-alta presión [30,62,66]. Esta técnica proporciona separaciones más eficaces incrementando la sensibilidad de los métodos analíticos y acortando el tiempo de análisis, pero los estrechos picos cromatográficos obtenidos con ella obligan a emplear espectrómetros de masas capaces de operar a velocidades de barrido muy elevadas [69]. Algunos trabajos han demostrado que los compuestos más polares, como la ecgonina metil éster, se retienen mejor empleando columnas de interacción hidrofílica (HILIC, *hydrophilic interaction liquid chromatography*) [64,67].

Entre los disolventes orgánicos empleados como fase móvil, el acetonitrilo y el metanol han sido los más utilizados. La naturaleza de los modificadores adicionados depende del modo de ionización seleccionado para la detección por MS. La mayoría de las drogas se detectan en modo positivo; en consecuencia, se ha considerado la adición de un ácido (acético o fórmico) a la fase acuosa [22,28,33,59] o a ambas fases, acuosa y orgánica [58,66], para favorecer su ionización. Los cannabinoides se pueden ionizar también en modo negativo y, en este caso, algunos autores han utilizado fases móviles básicas como agua con 0.05% de trietilamina [33]. Otros de los modificadores empleados han sido las sales de formiato amónico o acetato amónico a diferentes valores de pH [26,30-32,35,58,62,67].

El acoplamiento LC-MS se ha llevado a cabo, principalmente, utilizando interfases de electrospray. Aunque otras fuentes de ionización, como la ionización química a presión atmosférica (APCI) son menos sensibles a los efectos de matriz (especialmente importantes cuando se analizan matrices complejas como las aguas residuales), no resultan adecuadas para todos los analitos, particularmente para los más polares [70]. Para corregir estos efectos en ESI, la mayoría de los estudios considerados en la **Tabla II.14** han optado por añadir a las muestras, antes de procesarlas, análogos deuterados de los compuestos objetivo para emplearlos como patrones o surrogados internos (nombrados como patrones internos, indistintamente, a lo largo de la presente memoria).

Los espectrómetros más utilizados han sido los analizadores de triple cuadrupolo [26,30,31,33,35,58,59,62,66,67] o los sistemas híbridos cuadrupolo-trampa de iones lineal utilizados como sistemas de triple cuadrupolo [15,22,28]. Operando en MS/MS en modo SRM, estos analizadores proporcionan resultados sensibles y selectivos y, monitorizando dos transiciones por compuesto, cumplen con los requisitos establecidos en la Unión Europea para la identificación y confirmación de determinadas sustancias y sus residuos en los animales vivos y sus productos [71]. Sin embargo, si la fragmentación de los compuestos es muy extensa, la sensibilidad de su cuantificación por MS/MS puede verse drásticamente reducida. Los instrumentos de alta resolución, como el analizador de resonancia de ion ciclotrón (*ion cyclotron resonance*, ICR) o el Orbitrap, se han utilizado para determinar drogas de abuso en aguas en contadas ocasiones [72]. Los analizadores híbridos cuadrupolo-tiempo de vuelo, por su parte, sólo se habían empleado en este campo, hasta la presente tesis doctoral, con fines cualitativos [73].

3.2. CROMATOGRAFÍA DE GASES ACOPLADA A ESPECTROMETRÍA DE MASAS

Comparada con LC-MS, la cromatografía de gases acoplada a la espectrometría de masas es una técnica de menor coste y menos susceptible a los efectos de matriz, tan notorios en el análisis de aguas residuales. Aunque presenta una larga tradición en la determinación de drogas de abuso en el ámbito clínico y forense, sus aplicaciones ambientales son todavía muy limitadas: hasta la fecha, tan sólo se han desarrollado dos métodos de GC-MS (uno de los cuales está incluido en la presente tesis doctoral) para la cuantificación de sustancias ilícitas en muestras de agua.

El primero fue optimizado por Mari et al. [63] para la determinación de morfina, cocaína y heroína, en aguas residuales sin tratar. Tras extraer las muestras mediante SPE, concentraron los extractos a sequedad y derivatizaron los grupos hidroxilo de la morfina con

50 μL de BSTFA conteniendo un 1% de trimetilclorosilano (catalizador). La disolución resultante (1 μL) se inyectó en el cromatógrafo de gases en modo *splitless* y la separación de los compuestos se llevó a cabo en una columna de 5% difenil 95% dimetilpolisiloxano de 12×0.2 mm y $0.33 \mu\text{m}$ de espesor de fase. La detección se llevó a cabo en modo SIM utilizando un espectrómetro de masas de cuadrupolo simple.

El trabajo desarrollado en la presente tesis doctoral fue optimizado para la determinación de diversas drogas de abuso y metabolitos (14 en total) pertenecientes a cuatro clases diferentes. La gran variedad de moléculas presentes obligó a la utilización del MSTFA como agente derivatizante; la separación se realizó en una columna HP-5 MS y la determinación se llevó a cabo en MS/MS mediante el empleo de una trampa de iones.

En el ámbito clínico y forense, la determinación de drogas de abuso mediante cromatografía de gases ha sido abordada, mayoritariamente, incluyendo una reacción previa de sililación o acilación de los grupos polares (alcohol, amina, carboxilo). Entre los agentes sililantes, los más comunes son el BSTFA, el MSTFA y el MTBSTFA. Para la acilación se emplean reactivos como el MBTFA o los anhídridos pentafluoropropiónico (PFPA) y heptafluorobutírico (HFBA) (en el caso de los alcaloides, algunos autores acompañan el anhídrido de hidróxido pentafluoropropiónico, PFPOH, o de hexafluoro-2-propanol, HFIP) [74].

Tabla II.14. Condiciones empleadas para la determinación de drogas de abuso y metabolitos en aguas mediante LC (o UPLC)-MS.

| Análitos | Matriz | Extracción | Separación | Columna | Fase móvil (modificador) | (Ionización) Detección | LOD/LOQ ng L ⁻¹ | Ref. |
|--|--|-----------------------|------------|--|--|--|--|------|
| MOR, COD, 6-AM, HER, FEN, EDDP, MET, THC, THCCOOH (+ otros 2 metabolitos) | río, inf. | SPE | UPLC | Acquity BEH C ₁₈ (100 mm × 2.1 mm; 1.7 µm) | H ₂ O (NH ₄ OCOH/HCOOH 50 mM, pH 3.8) / MeOH | (ESI +) QqQ-MS/MS modo SRM | 0.1-10 (río) 0.3-20 (inf) LOQ | [62] |
| COC, BE, AMP, MAMP, MDA, MDMA, MDEA, LSD, KET, PCP, FEN (+ 4 drogas blandas) | río, ef. inf. | SPE | UPLC | Acquity BEH C ₁₈ (100 mm × 2.1 mm; 1.7 µm) | H ₂ O (NH ₄ OCOH/HCOOH 30 mM, pH 3.5) / ACN (HCOOH 0.1%) | (ESI +) QqQ-MS/MS modo SRM | 0.1-3.1 (río) 0.2-5.0 (inf) LOQ | [30] |
| MOR, 6-AM, MET, EDDP, COC, BE, COE, AMP, MAMP, MDA, MDMA, MDEA, THCCOOH (+ otros 3 metabolitos) | ef., inf. | SPE | LC | XTerra MS C ₁₈ (100 mm × 2.1 mm; 3.5 µm) | H ₂ O (HOAc 0.05%) / ACN (ESI +) H ₂ O (TEA 0.05%) / ACN (ESI -) | (ESI +) (THCCOOH: ESI -) QqQ-MS/MS modo SRM | 0.6-3.2 (ef.) 1.0-8.7 LOQ | [33] |
| MOR, COD, MET, BE (+ otras drogas) | río, ef. inf., agua subterránea, grifo | SPE | LC | Synergi Polar-RP 80 Å (150 mm × 3 mm, 4 µm) | H ₂ O (NH ₄ OCOH 10 mM, HCOOH pH 4) / ACN | (ESI +) QqQ-MS/MS modo SRM | 1-2 (río) 5-10 (ef.) 10-20 (inf.) LOQ | [35] |
| MOR, MET, EDDP, COC, BE, COE, MDMA, LSD, KET (+ 3 drogas) | río, ef., inf. | SPE | LC | Onyx monolithic C ₁₈ (200 mm × 3 mm) | H ₂ O (NH ₄ OAc 5 mM) / MeOH | (ESI) MS/MS modo SRM | 2-856 (río) LOQ | [32] |
| MOR, 6-AM, COC, BE, COE, AMP, MAMP, MDMA, MDEA (+ otras drogas y metabolitos) | inf. | SPE | LC | Restek Viva PFPP (10 mm × 2.1 mm, 5 µm) Restek Ultra IBD (10 mm × 2.1 mm, 3 µm) | H ₂ O (HCOOH 0.1%) / ACN (HCOOH 0.1%) H ₂ O (NH ₄ OAc 10 mM) / ACN | (ESI +) QqQ-MS/MS modo SRM | 0.03-7.7 LOQ | [58] |
| MOR, 6-AM, HER, COC, BE, COE, AMP, MAMP, MDMA, THC, THCCOOH, LSD, O-H-LSD (+ otras drogas y metabolitos) | ef., inf. | SPE <i>on-line</i> | LC | Purospher Star RP-C ₁₈ (125 mm × 2 mm; 5 µm) | H ₂ O / ACN | (ESI +) (THCCOOH, THC: ESI -) QqLIT-MS/MS modo SRM | 0.07-1.94 LOD | [15] |

Tabla II.14 (continuación). Condiciones empleadas para la determinación de drogas de abuso y metabolitos en aguas mediante LC (o UPLC)-MS.

| Análitos | Matriz | Extracción | Separación | Columna | Fase móvil (modificador) | (Ionización) Detección | LOD/LOQ ng L ⁻¹ | Ref. |
|---|-----------|------------|------------|---|--|------------------------------|----------------------------|------|
| COD, COC, BE, AMP (+ PPCPs) | ef., | SPE | UPLC | ACQUITY UPLC BEH C ₁₈ (100 mm × 1 mm; 1.7 µm) | H ₂ O:MeOH (HOAc 0.5%) / MeOH (HOAc 0.5%) | (ESI +) QqQ-MS/MS modo SRM | 0.1-1 (río) | [66] |
| | inf. | | | | | | 1-3 (ef.) | |
| COC, BE, AMP, MAMP, MDMA, MDA | ef., | SPE | LC | Genesis C ₁₈ (150 mm × 3 mm; 4 µm) | H ₂ O (HCOOH 0.1%) / ACN | (ESI +) QqLIT-MS/MS modo SRM | 3-22 | [22] |
| | inf. | | | | | | LOQ | |
| COC, BE, AMP, MAMP, (+ buprenorfina) | ef., | SPE | LC | Xbridge Phenyl (150 mm × 3 mm; 3.5 µm) | H ₂ O (NH ₄ OCO ₂ H 10 mM) / ACN | (ESI +) QqQ-MS/MS modo SRM | 1.2-12.8 | [31] |
| | inf. | | | | | | (inf.) | |
| 6-AM, MET, EDDP, COC, BE, AMP, MAMP, MDMA | inf. | SPE | LC | Luna HILIC (150 mm × 3 mm; 5 µm) | H ₂ O (NH ₄ OAc 5 mM) / ACN | (ESI +) QqQ-MS/MS modo SRM | 1-2 | [67] |
| | | | | | | | LOQ | |
| MET, COC, BE, AMP, MAMP, MDA, MDMA, MDEA, KET, LSD, O-H-LSD, PCP (+ otras drogas y metabolitos) | inf. | - | DI-LVI- LC | Atlantis T3 C ₁₈ (150 mm × 4.6 mm; 5 µm) | H ₂ O:MeOH (HOAc 0.1%) / ACN | (ESI +) QqQ-MS/MS modo SRM | 2.5-10 | [59] |
| | | | | | | | LOQ | |
| MOR, 6-AM, COD, MET, EDDP, COC, BE, AMP, MAMP, MDMA, THC, THCCOOH (+ 6-acetilcodeína) | río, ef., | - | DI-LVI- LC | Synergy Hydro RP (150 mm × 2 mm; 2.5 µm) | H ₂ O (NH ₄ OCO ₂ H 2 mM, HCOOH 0.2%) / ACN | (ESI +) QqQ-MS/MS modo SRM | 0.2 (río) | [26] |
| | inf. | | | | | | 20 (inf.) | |
| MOR, 6-AM, COD, HER, MET, EDDP, COC, BE, AMP, MAMP, MDA, MDMA, MDEA, KET (+ otras drogas) | río, ef., | - | DI-LVI- LC | Zorbax Eclipse XDB-C ₈ (150 mm × 4.6 mm; 5 µm) | H ₂ O (HCOOH 0.1%) / ACN | (ESI +) QqLIT-MS/MS modo SRM | 0.5-700 | [28] |
| | inf. | | | | | | 10-700 | |

PPCPs: fármacos y productos de cuidado personal; ef.: efluente; inf.: influente; Sep.: separación; MeOH: metanol; ACN: acetonitrilo; HOAc: ácido acético; HCOOH: ácido fórmico; NH₄OCO₂H: formiato amónico; NH₄OAc: acetato amónico; TEA: trietilamina; LIT: trampa de iones lineal.

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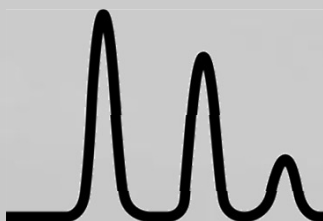
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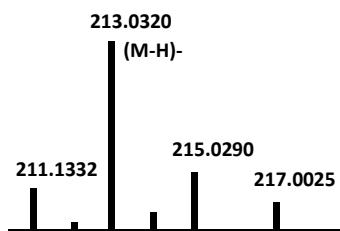
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III. METODOLOGÍA DESARROLLADA



A. AGENTES ANTIMICROBIANOS

INTRODUCCIÓN Y ESQUEMAS DE LOS MÉTODOS DESARROLLADOS

Al igual que muchos otros compuestos presentes en la formulación de productos de cuidado personal, los agentes antimicrobianos considerados en esta tesis doctoral se introducen diariamente en las aguas residuales arrastrados por el agua de baños y duchas. A pesar de que un porcentaje apreciable es eliminado durante los tratamientos de depuración, parte es emitido con los efluentes a las aguas superficiales, donde su impacto eco-toxicológico puede llegar a ser importante.

En este sentido, el primero de los trabajos desarrollados en la presente tesis doctoral propone un método simple y robusto para la determinación simultánea de triclosán, triclocarbán y siete parabenos (incluyendo, por primera vez, la distinción entre los isómeros *iso*- y *n*- del propil y del butil parabén), en aguas de diversa naturaleza. Como técnica de preparación de muestra se seleccionó la SPE debido a su sencillez y a que proporciona elevados factores de preconcentración y eficacias de extracción; como técnica de separación y detección se utilizó la cromatografía líquida acoplada a la espectrometría de masas en tándem; la determinación del triclocarbán mediante cromatografía de gases es especialmente complicada dado que no es estable térmicamente y que su derivatización presenta múltiples dificultades. El método se optimizó y validó utilizando dos equipos LC-QqQ-MS comerciales diferentes, lo que permitió comparar sus características analíticas y su distinta respuesta a los efectos de matriz.

El escaso conocimiento existente en torno a la distribución ambiental de los derivados halogenados de los parabenos (susceptibles de formarse como consecuencia de la cloración o bromación del compuesto correspondiente) llevó a adaptar el método anteriormente descrito a la determinación de los mismos siete parabenos junto con el metil parabén monoclorado, el diclorado y el dibromado, en agua residual. En este caso, se utilizó un cromatógrafo de líquidos acoplado a un espectrómetro de masas provisto de un analizador híbrido cuadrupolo-tiempo de vuelo. Estos sistemas permiten evaluar la presencia de compuestos no preseleccionados originalmente como analitos una vez que el análisis ha finalizado y sin necesidad de utilizar patrones; de esta forma, fue posible extender la determinación (a nivel cualitativo) a otros parabenos halogenados de los que no se disponía de patrón comercial. La aplicación del método a muestras de influente y efluente permitió comparar las concentraciones de los parabenos y de sus derivados halogenados en tres estaciones depuradoras, aportando los primeros datos relativos al comportamiento (degradación) de estos últimos durante los tratamientos de depuración. El estudio se completó mediante dos ensayos de biodegradabilidad aeróbica, el primero siguiendo las directrices de la norma ISO

7827 y el segundo utilizando agua residual tomada a la entrada de una estación depuradora urbana.

Como alternativa a la SPE (que generalmente implica la concentración de grandes volúmenes de muestra, con el consecuente incremento en el consumo de disolventes y la duración del análisis) el tercero de los métodos descritos en esta memoria se basó en su versión miniaturizada, la microextracción con adsorbentes empaquetados, para extraer seis parabenos, triclosán y metil triclosán en agua residual. Al estar basada en los mismos procesos de adsorción, idealmente todas las moléculas de analito quedan retenidas en la fase aceptora, lo que le permite alcanzar mayores eficacias de extracción que otras técnicas de microextracción basadas en procesos de equilibrio. La desorción directa de los analitos en el inyector de un cromatógrafo de gases-espectrómetro de masas permitió combinar las etapas de preparación de muestra, separación y detección en un procedimiento totalmente automático.

Finalmente, teniendo en cuenta que el carácter hidrofóbico del triclosán y de su producto de biometilación (el metil triclosán) favorece su adsorción sobre los sedimentos de aguas contaminadas y sobre los lodos en las estaciones depuradoras, se decidió optimizar un procedimiento para su determinación en estas dos matrices. Conocer los niveles de metil triclosán en lodos es una cuestión de especial importancia para comprender el comportamiento del triclosán durante los tratamientos de depuración, determinar si los microorganismos existentes en el agua son capaces de llevar a cabo procesos de biometilación y evaluar el riesgo de que esta especie alcance los suelos como consecuencia de la reutilización de biosólidos como fertilizantes. Las metodologías descritas para tal fin hasta el momento en el que se decidió abordar este trabajo eran complejas (PLE, MAE, Soxhlet) y requerían, en la mayoría de los casos, una etapa posterior de limpieza del extracto. Alternativamente, el procedimiento propuesto combinó los procesos de extracción, limpieza y elución mediante un sencillo método de dispersión de la matriz en fase sólida. La posterior determinación se llevó a cabo mediante GC-MS.

A continuación, se presentan los esquemas de las metodologías analíticas empleadas en cada uno de estos estudios (**Figuras III.1 a III.4**), así como las publicaciones científicas derivadas de los mismos.

Figura III.1. Esquema para la determinación de siete parabenos, triclosán y triclocarbán en aguas fluviales y residuales mediante SPE-LC-(ESI)-MS/MS (QqQ).

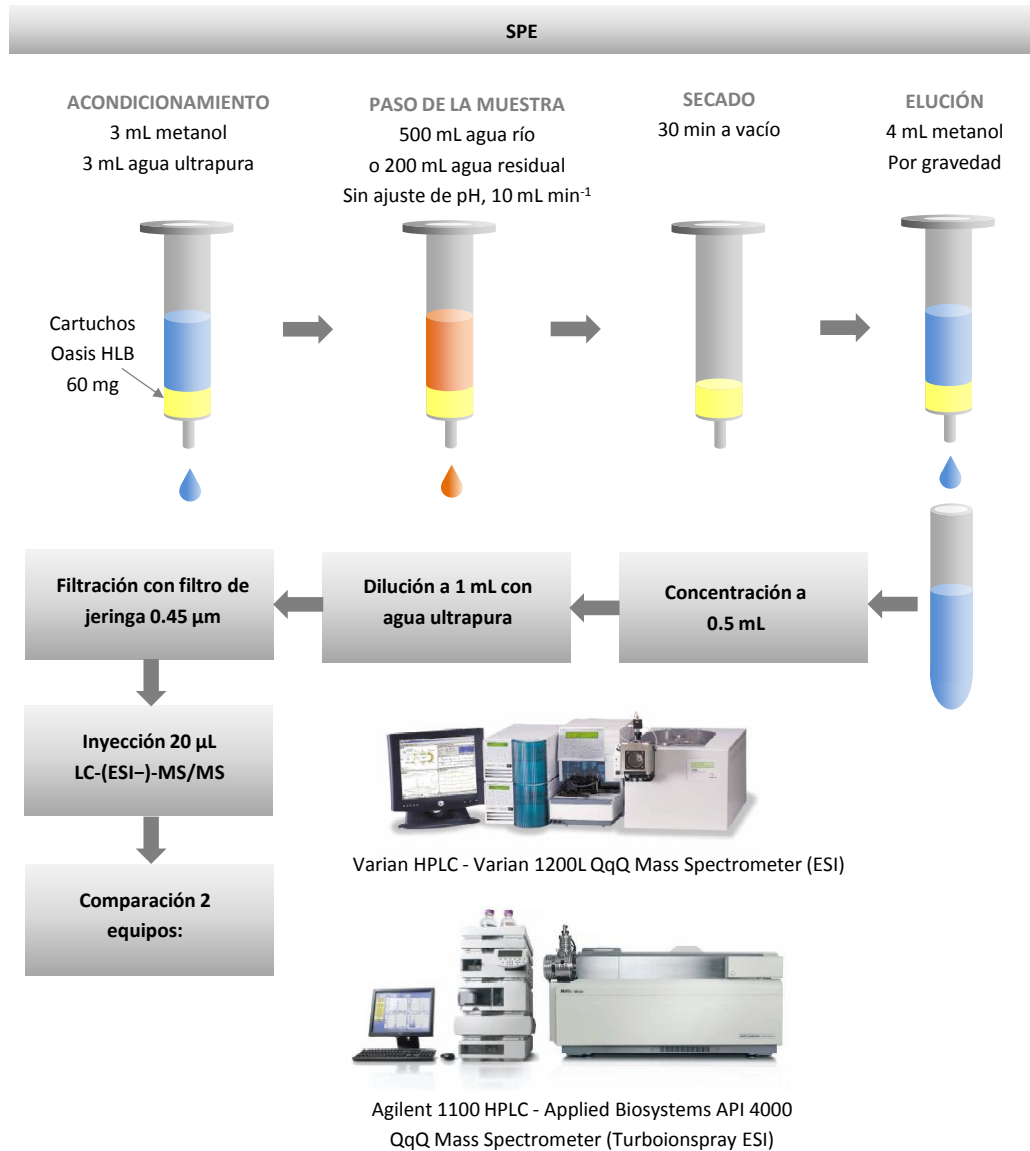


Figura III.2. Esquema para la determinación de siete parabenos y tres derivados halogenados del metil parabén en aguas residuales mediante SPE-LC-(ESI)-MS (MS/MS para confirmación) (QTOF).

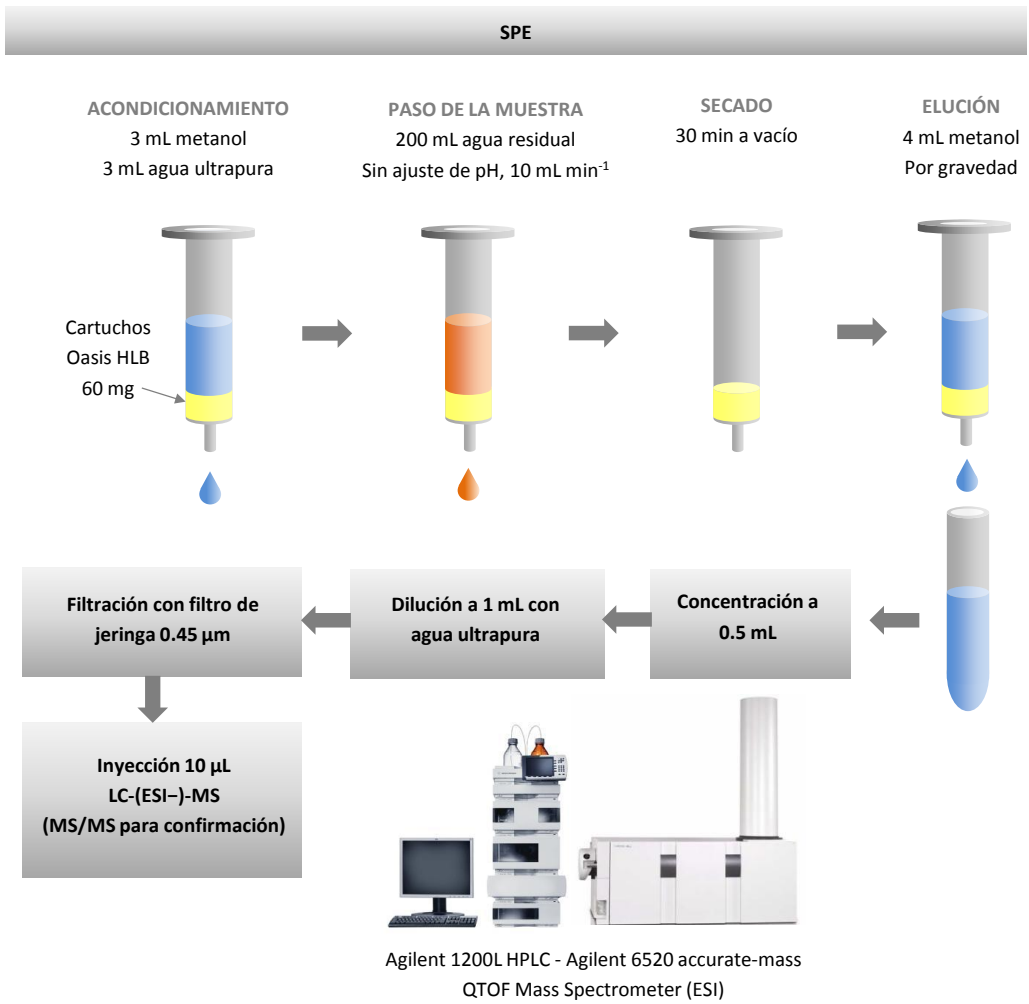


Figura III.3. Esquema para la determinación de seis parabenes, triclosán y metil triclosán en aguas residuales mediante MEPS-GC-(EI)-MS (Q).

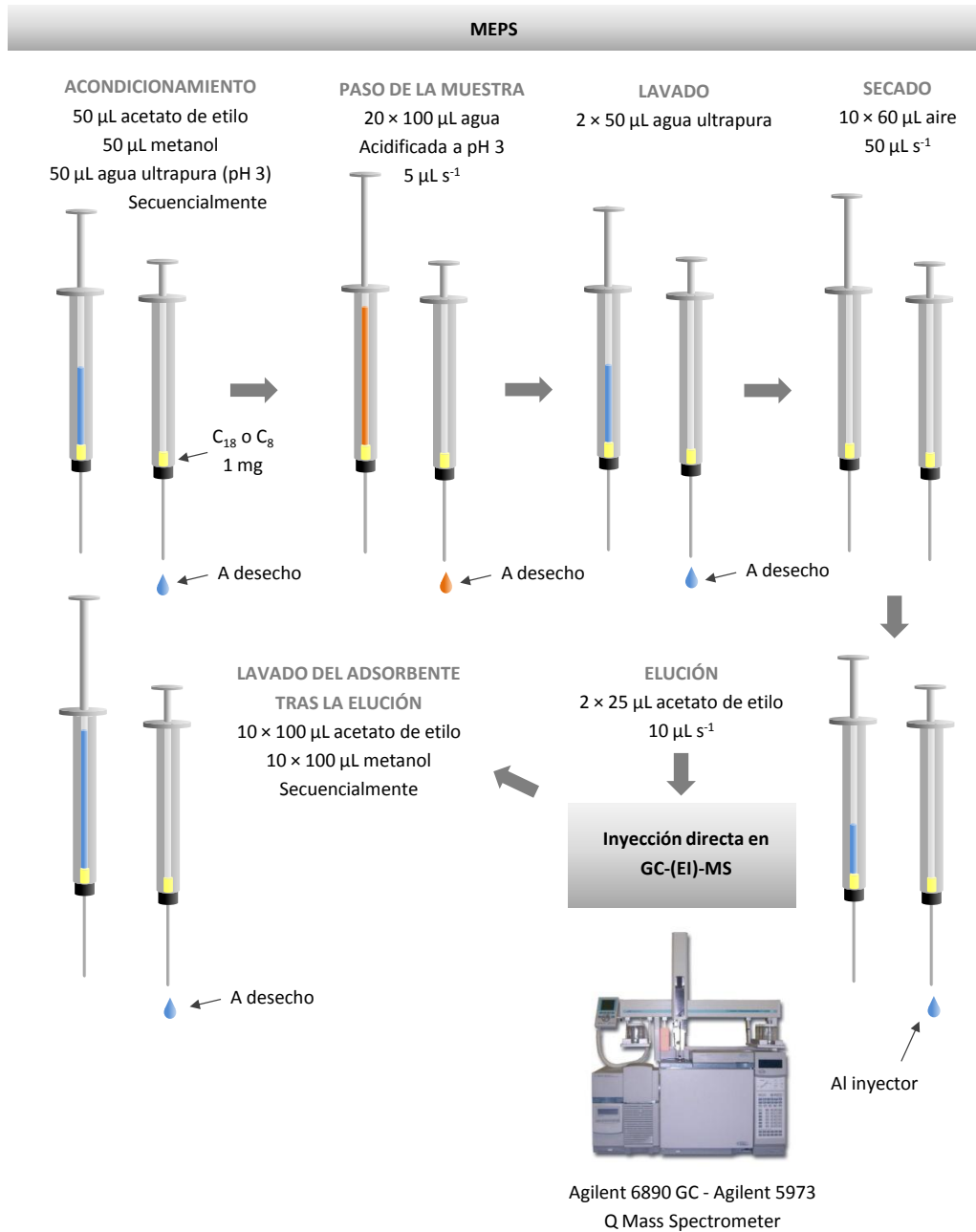
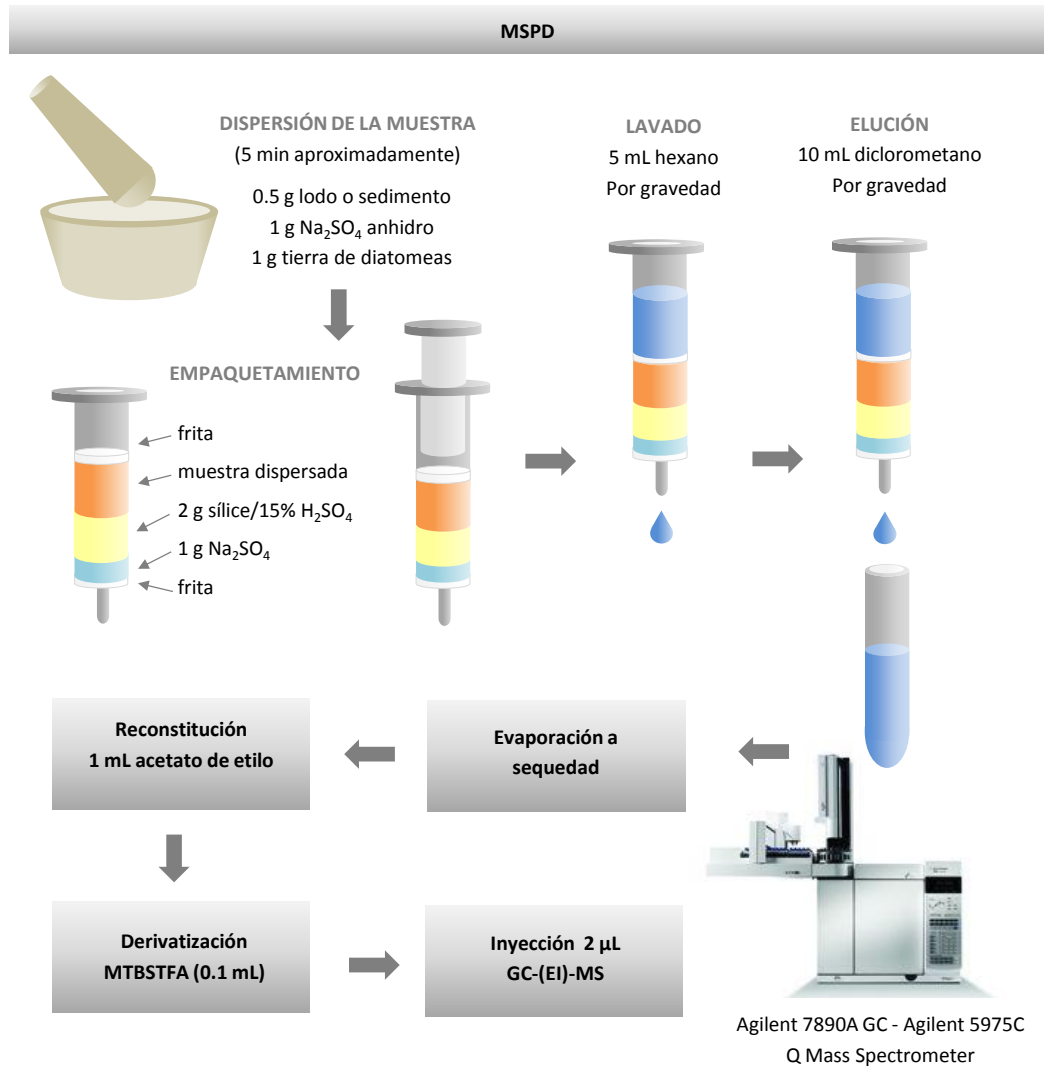


Figura III.4. Esquema para la determinación de triclosán y metil triclosán en lodos y sedimentos mediante MSPD-GC-(EI)-MS (Q).



PUBLICACIÓN III.1

SIMULTANEOUS DETERMINATION OF PARABENS, TRICLOSAN AND TRICLOCARBAN IN WATER BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONISATION-TANDEM MASS SPECTROMETRY

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SIMULTANEOUS DETERMINATION OF PARABENS, TRICLOSAN AND TRICLOCARBAN IN WATER BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONISATION-TANDEM MASS SPECTROMETRY

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Abstract

A method for the determination of several household biocides in water by liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) is presented. It permits the simultaneous determination of triclosan (TCS), triclocarban (TCC) and seven parabens, including the distinction between branched and linear isomers of propyl (*i*-PrP and *n*-PrP) and butyl parabens (*i*-BuP and *n*-BuP). Prior to LC-MS/MS, analytes are preconcentrated by solid-phase extraction (SPE) on Oasis HLB (60 mg) cartridges at natural sample pH and subsequently eluted with 4 mL of methanol. This simple SPE procedure provides extraction recoveries above 85% except for raw wastewater, where it falls to 67% for TCC. The performance of the method was tested with two triple quadrupole LC-MS instruments from a low-mid and mid-high market range: a Varian 1200L and an API-4000. The latter system provided between 3 and 80 times lower limits of quantification (LOQs) than the first one, in the 0.008-0.44 ng L⁻¹ range for surface water. Moreover, a comparison of matrix effects on both instruments showed a very different behaviour, particularly in the case of parabens. For these compounds signal suppression was observed in the 1200L instrument and signal enhancement with the API-4000. As a result, different calibration approaches were chosen for them and this pointed to the need of matrix effect re-evaluation in method transfer between different LC-MS systems. The application of the method to real samples showed the ubiquity of methyl paraben (MeP) and *n*-PrP (at the 1-6 µg L⁻¹ in raw wastewater) and the coexistence of *i*-BuP and *n*-BuP at similar levels (ca. 100-200 ng L⁻¹ in raw wastewater).

Keywords: biocides; bactericides; solid-phase extraction (SPE); liquid chromatography-mass spectrometry (LC-MS); electrospray ionisation (ESI); matrix effects; signal suppression; water analysis.

1. INTRODUCTION

Esters of 4-hydroxybenzoic acid (parabens), 5-chloro-2-(2,4-dichloro-phenoxy)-phenol (triclosan, TCS) and *N*-(4-chlorophenyl)-*N'*-(3,4-dichlorophenyl)-urea (triclocarban, TCC) are widely employed as bactericides and preservatives in personal care products such as tooth pastes, deodorants, beauty creams, bath gels and shampoos. In addition, parabens are added as preservatives to canned foods and beverages, and TCS is incorporated as a biocide in footwear, carpets, plastic toys and kitchenware [1-4].

The extensive inclusion of these chemicals in everyday consumption products has raised concerns about their potential long-term effects on human health and on wildlife. Parabens show oestrogenic activity which, although relatively weak in comparison with that of 17 β -oestradiol [5], cannot be considered negligible as parabens occur in the environment at much higher concentration than the latter compound. In addition, a recent study has suggested a possible relationship between them and breast cancer, involving the use of paraben-containing deodorants [6]. This hypothesis has not been fully proved and additional studies are needed to confirm their carcinogenicity.

TCS and TCC are known to be endocrine disruptors [7,8], but the main concern about them is that they can turn into more toxic and persistent species such as chlorinated phenols [9,10], polychlorinated diphenyl ethers [11], polychlorinated dibenzodioxins [12] and mono and dichlorinated anilines [13].

These bactericides are continuously released in the aquatic media through urban wastewater, their main entry route into the environment. They have been detected in water samples at concentrations ranging from ng L⁻¹ to μ g L⁻¹, depending on the compound and the nature of the sample. Parabens and TCS are effectively removed during conventional sewage treatments, so their levels in wastewater are usually much higher than in surface water, where they can still be found at the ng L⁻¹ level [14-20]. Removal of TCC during wastewater treatment has seldom been studied and the level is rather variable, between 0 and 98% in measurements from wastewater treatment plants (WWTPs) in the USA and Canada [13,18,21,22].

Analytical methods for the determination of these compounds in water samples are based on a preconcentration step, usually solid-phase extraction (SPE) [7,18,23,24] or solid-phase microextraction (SPME) [15,25], followed by a subsequent determination by gas chromatography-mass spectrometry (GC-MS) [15,19,26] or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [14,17,18,23]. Currently, this latter technique is preferred because of its high sensitivity and because derivatisation of analytes – required with

GC-MS analysis – is not needed. Actually, the derivatisation procedure for TCC is particularly difficult, as it is also the case for the structurally related phenylurea herbicides [27].

The goal of this work was to develop an analytical procedure for determining seven parabens, TCS and TCC simultaneously in water samples. SPE was selected as the sample preparation technique because of its simplicity and compatibility with chromatographic analysis. Separation and determination of the target compounds was carried out by LC-MS/MS with electrospray ionisation (ESI) in the negative ion mode. Experimental parameters were optimised to achieve the maximum efficiency and, finally, the method was tested with two different LC-triple quadrupole instruments (from different vendors and market range) with real samples, in order to compare their different matrix effects response and analytical performance.

2. EXPERIMENTAL

2.1. Chemicals and stock solutions

Methyl (MeP), ethyl (EtP), *n*-propyl (*n*-PrP), *n*-butyl (*n*-BuP) and benzyl (BzP) esters of 4-hydroxybenzoic acid, as well as triclosan (TCS) and triclocarban (TCC), were purchased from Aldrich (Milwaukee, WI, USA). *Iso*-propyl paraben (*i*-PrP) and *iso*-butyl paraben (*i*-BuP) were obtained from TCI Europe (Zwijndrecht, Belgium). Stock solutions of each compound ($1000 \mu\text{g mL}^{-1}$) and mixtures of all of them ($10 \mu\text{g mL}^{-1}$) were prepared in methanol and stored at -20°C until use. As internal standards (ISs), methyl 4-hydroxybenzoate-2,3,5,6- d_4 (MeP- d_4) was from CDN Isotopes (Quebec, Canada), and solutions of $^{13}\text{C}_{12}$ -triclosan ($^{13}\text{C}_{12}$ -TCS; $100 \mu\text{g mL}^{-1}$ in nonane, 99%) and $^{13}\text{C}_6$ -triclocarban ($^{13}\text{C}_6$ -TCC; $100 \mu\text{g mL}^{-1}$ in acetonitrile, 99%) were from Cambridge Isotope Laboratories (Andover, MA, USA). Solutions containing these three labelled compounds ($2 \mu\text{g mL}^{-1}$) were prepared in methanol:acetone (95:5).

Calibration standards with increasing concentrations of analytes and 100 ng mL^{-1} of ISs were prepared in methanol:water (1:1). HPLC grade methanol, acetic acid, formic acid and ammonia were supplied by Merck (Darmstadt, Germany). Acetone was from Prolabo (Cerdanyola del Vallès, Spain).

2.2. Samples

Four surface water samples: Sar River (two sampling points), Sarela River and “Dos Pasos” Creek, a WWTP influent and an effluent were collected. They were taken in amber

glass bottles previously rinsed with ultrapure water and methanol and stored in the dark at 4 °C until analysis. Particulate matter was filtered just before extraction through a combination of glass fibre prefilters and 0.45 µm nitrocellulose filters (Millipore, Bedford, MA, USA). Filtered samples were spiked with labelled standards (100 ng) and, in the case of recovery studies, also with analytes.

Surface water samples were taken in May and October in the area of Santiago de Compostela (Galicia, NW Spain). Raw and treated wastewater samples were collected in the same months at an urban WWTP equipped with primary and secondary treatments that receives the combined, urban and industrial, wastewater from a population of 125000 inhabitants.

2.3. Solid-phase extraction

A solid-phase extraction procedure was developed to isolate and preconcentrate the target compounds from water samples.

Under the final conditions, Oasis HLB cartridges (3 mL, 60 mg), obtained from Waters (Milford, MA, USA), were sequentially conditioned with 3 mL methanol and 3 mL water before use. Samples (500 mL for river water, 200 mL for sewage) spiked with surrogate ISs (100 ng each) were passed through the cartridges at a flow rate of $\approx 10 \text{ mL min}^{-1}$. Cartridges were then dried under vacuum for 30 min and analytes were eluted with 4 mL methanol. Finally, extracts were concentrated with a gentle stream of nitrogen (99.999%) in a Turbovap II concentrator (Zymark, Hopkinton, MA, USA) down to ca. 0.5 mL and diluted to a final volume of 1 mL with ultrapure water.

2.4. Instrumentation

For comparative purposes, samples were analysed with two different triple quadrupole LC-MS/MS instruments: one low-mid end (A) and another of mid-high end (B) systems, in terms of equipment cost and expected performance.

Instrument A consisted of two ProStar 210 high-pressure mixing pumps (Varian, Walnut Creek, CA, USA), a vacuum membrane degasser (Metachem Technologies, Bath, UK), and an autosampler and a thermostated LC column compartment ProStar 410 module (Varian). The LC system was interfaced to a triple quadrupole 1200L mass spectrometer equipped with an ESI interface (Varian). Nitrogen, used as nebulising and drying gas, was provided by a nitrogen

generator (Domnick Hunter, Durham, UK). Argon (99.999%) was used as the collision gas. Instrument control and data acquisition were controlled with the Varian MS Workstation software.

Instrument B comprised an Agilent Technologies (San Jose, CA, USA) 1100 Series HPLC system consisting of a binary pump, vacuum degasser, autosampler and column thermostated compartment. This HPLC system was interfaced to an API-4000 triple quadrupole mass spectrometer equipped with a Turboionspray ESI interface (Applied Biosystems, Foster City, CA, USA). Nitrogen was generated with a generator (Peak Scientific, Bedford, MA, USA) and employed as ESI and collision gas. This instrument was controlled with the Analyst software (Applied Biosystems).

2.5. LC-MS/MS determination

System A was operated with a needle potential of 4.5 kV, a source temperature of 50 °C, a desolvation temperature of 200 °C, a nebulising gas pressure of 50 psi (345 kPa) and a drying gas pressure of 20 psi (138 kPa). The argon pressure in the collision cell was kept at 1.5 mTorr for MS/MS measurements.

For the second LC-MS system, the ion spray voltage was 4.0 kV, the source temperature 500 °C, the curtain gas pressure 45 psi (310 kPa), and the ion source gas 1 and 2 pressures, both 40 psi (276 kPa). The nitrogen pressure in the collision cell was 6 psi (41 kPa).

Selection of the most intense MS/MS transitions in both LC-MS instruments was achieved by infusion of the individual compounds at a concentration level of ca. 2 µg mL⁻¹ in MeOH:water (1:1) in both ESI positive and negative mode. A much higher signal was obtained in the negative mode, where only the [M-H]⁻ ion was produced. The signal intensity of this ion was optimised by varying the capillary voltage (system A) / declustering potential (system B). Subsequently, the [M-H]⁻ ions were subjected to collision induced dissociation (CID) product ion experiments and the most abundant product ions were obtained by varying the collision energy with the aid of the automated routines included within the software packages of both instruments. Collision gas pressures were kept at the recommended instrument values as mentioned above.

Table 1. Experimental LC-ESI-MS/MS parameters used for the determination of parabens, triclosan and triclocarban by SRM in the negative ion mode.

| | Empirical formula | Monoisotopic molecular weight | Transitions (m/z) | Proposed product ion | Instrument A | | Instrument B | |
|------------------------------------|---|-------------------------------|------------------------|---|--------------------------------|---|--------------------------------|---|
| | | | | | CV / CE / DT ^a | I ₁ / I ₂ ± tol. ^b | DP / CE / DT ^c | I ₁ / I ₂ ± tol. ^b |
| MeP | C ₈ H ₈ O ₃ | 152.05 | 151 → 92 151 → 136 | [M-H-CH ₃ -CO ₂] ⁻ [M-H-CH ₃] ⁻ | 45 / 20 / 300 45 / 13 / 300 | 1.92 ± 0.38 - | 49 / 29 / 100 49 / 19 / 100 | 2.17 ± 0.54 - |
| MeP-d ₄ | C ₈ H ₈ O ₃ | 156.07 | 155 → 96 | [M-H-CH ₃ -CO ₂] ⁻ | 45 / 20 / 300 | - | 49 / 29 / 100 | - |
| EtP | C ₉ H ₁₀ O ₃ | 166.06 | 165 → 92 165 → 136 | [M-H-CH ₂ -CH ₃ -CO ₂] ⁻ [M-H-CH ₂ -CH ₃] ⁻ | 40 / 22 / 300 40 / 13 / 300 | 1.40 ± 0.28 - | 48 / 29 / 100 50 / 22 / 100 | 2.95 ± 0.74 - |
| <i>i</i> -PrP | C ₁₀ H ₁₂ O ₃ | 180.08 | 179 → 92 179 → 136 | [M-H-CH ₂ (CH ₃) ₂ -CO ₂] ⁻ [M-H-CH ₂ (CH ₃) ₂] ⁻ | 48 / 23 / 300 48 / 15 / 300 | 1.14 ± 0.23 - | 60 / 36 / 100 60 / 20 / 100 | 2.49 ± 0.62 - |
| <i>n</i> -PrP | C ₁₀ H ₁₂ O ₃ | 180.08 | 179 → 92 179 → 136 | [M-H-CH ₂ -CH ₂ -CH ₃ -CO ₂] ⁻ [M-H-CH ₂ -CH ₂ -CH ₃] ⁻ | 48 / 23 / 300 48 / 15 / 300 | 1.59 ± 0.32 - | 60 / 36 / 100 60 / 20 / 100 | 2.12 ± 0.53 - |
| <i>i</i> -BuP | C ₁₁ H ₁₄ O ₃ | 194.09 | 193 → 92 193 → 136 | [M-H-CH ₂ -CH ₂ (CH ₃) ₂ -CO ₂] ⁻ [M-H-CH ₂ -CH ₂ (CH ₃) ₂] ⁻ | 48 / 22 / 150 48 / 16 / 150 | 1.73 ± 0.35 - | 50 / 33 / 100 50 / 22 / 100 | 2.65 ± 0.66 - |
| <i>n</i> -BuP | C ₁₁ H ₁₄ O ₃ | 194.09 | 193 → 92 193 → 136 | [M-H-CH ₂ -CH ₂ -CH ₂ -CO ₂] ⁻ [M-H-CH ₂ -CH ₂ -CH ₂ -CO ₂] ⁻ | 48 / 22 / 150 48 / 16 / 150 | 1.56 ± 0.31 - | 50 / 33 / 100 50 / 22 / 100 | 2.79 ± 0.70 - |
| BzP | C ₁₄ H ₁₂ O ₃ | 228.08 | 227 → 92 227 → 136 | [M-H-CH ₂ -CH ₂ -Ph-CO ₂] ⁻ [M-H-CH ₂ -Ph] ⁻ | 48 / 22 / 150 48 / 14 / 150 | 1.27 ± 0.25 - | 65 / 34 / 100 55 / 20 / 100 | 1.20 ± 0.24 - |
| TCC | C ₁₃ H ₉ Cl ₃ N ₂ O | 313.98 | 313 → 160 315 → 162 | [M-H-Ph(NCO)Cl] ⁻ [M-H-Ph(NCO)Cl] ⁻ | 55 / 11 / 100 55 / 11 / 100 | 5.13 ± 1.54 - | 45 / 18 / 100 50 / 18 / 100 | 3.37 ± 0.84 - |
| ¹³ C ₆ -TCC | C ₁₃ H ₉ Cl ₃ N ₂ O | 320.00 | 319 → 160 | [M-H-Ph(NCO)Cl] ⁻ | 55 / 11 / 100 | - | 50 / 18 / 100 | - |
| TCS | C ₁₂ H ₇ Cl ₃ O ₂ | 287.95 | 287 → 35 289 → 35 | [Cl] ⁻ [Cl] ⁻ | 55 / 5 / 100 55 / 5 / 100 | 1.92 ± 0.38 - | 50 / 29 / 100 50 / 29 / 100 | 1.34 ± 0.27 - |
| ¹³ C ₁₂ -TCS | C ₁₂ H ₇ Cl ₃ O ₂ | 299.99 | 299 → 35 | [Cl] ⁻ | 55 / 5 / 100 | - | 50 / 29 / 100 | - |

^a Capillary voltage (V) / collision energy (eV) / dwell time (ms).^b Intensity ratio transition 1 / transition 2 ± maximum tolerance according to 2002/657/EC Decision [32].^c Declustering potential (V) / collision energy (eV) / dwell time (ms).

In both systems, quantification of all compounds was carried out by recording two transitions – from the $[M-H]^-$ precursor ion to the selected product ions – for each analyte and one for each IS in the selective reaction monitoring (SRM) mode. In instrument A, these transitions were grouped into different segments, so that a maximum of six transitions were recorded simultaneously in order to maximise dwell times (100-300 ms; see **Table 1**) and, thus, sensitivity. In instrument B, equipped with enhanced collision cell and electronics, all transitions were recorded simultaneously in a single segment (dwell time 100 ms per transition). Individual CID (MS/MS) parameters for each compound and each LC-MS instrument are shown in **Table 1**.

Several mobile phase additives were considered in order to improve compound separation and sensitivity; viz. ammonium acetate (NH_4OAc), formic acid and acetic acid. The influence of additives on the sensitivity was studied by flow-injection analysis (FIA), injecting 5 μL of a standard solution ($10 \mu g mL^{-1}$ for every analyte) into a stream of methanol:water (1:1) containing variable concentrations of each modifier (0-20 mM, 0-0.2% and 0-2%, respectively) and registering responses in SRM mode.

LC separation was carried out on a 100×2.1 mm Halo C_{18} ($2.7 \mu m$) porous shell column, which consists of a $1.7 \mu m$ inert core coated with a $0.5 \mu m$ C_{18} layer (Advanced Materials Technology, Nes-Ziona, Israel). The column was protected with a 4×2 mm C_{18} guard cartridge provided by Phenomenex (Torrance, CA, USA) and the temperature was set at $45 \text{ }^\circ C$. A dual eluent system of water (A) and methanol (B), both with 5 mM NH_4OAc , was used. The flow rate was $0.2 mL \text{ min}^{-1}$ and the gradient was as follows: 0 min (40% B), 5 min (55% B), 11 min (55% B), 13 min (100% B), 20 min (100% B), 21 min (40% B) and 29 min (40% B). A volume of 20 μL of sample was injected into the HPLC system and analytes were detected by ESI-MS/MS in the negative ion mode.

MeP- d_4 , $^{13}C_{12}$ -TCS and $^{13}C_6$ -TCC were selected as surrogated ISs for MeP, TCS and TCC, respectively. The remaining parabens were quantified by either external calibration (system A) or by the internal standard method (system B) with MeP- d_4 as IS, as discussed in detail in the Results and Discussion section.

Distinction between SPE recoveries, matrix effects during the LC-MS/MS run and overall method recoveries was carried out by spiking samples either before or after SPE with the same amount of analytes. Thus, four aliquots of samples (surface water, raw and treated wastewater) were spiked with the analytes and ISs before SPE and the other four were spiked after SPE (i.e. over the extract). In addition, two samples were measured without analytes spike. Response factors of all the spiked samples (after non-spiked sample signal subtraction) were then compared with the response factor of a calibration curve prepared in MeOH:H₂O. In

this way, three response factors are obtained: one from the pure standard (R_1), a second one from the spiked samples before SPE (R_2) and a third one from the spike over the extract (R_3). Therefore, matrix effects percentage (%ME) is calculated as $100 \times R_3/R_1$; % recovery accounting exclusively the sample preparation step as $100 \times R_2/R_3$; and, finally, overall method recovery percentage as $100 \times R_2/R_1$. Further details on this methodology have already been described in the literature [28-30].

3. RESULTS AND DISCUSSION

3.1. Mass spectrometry

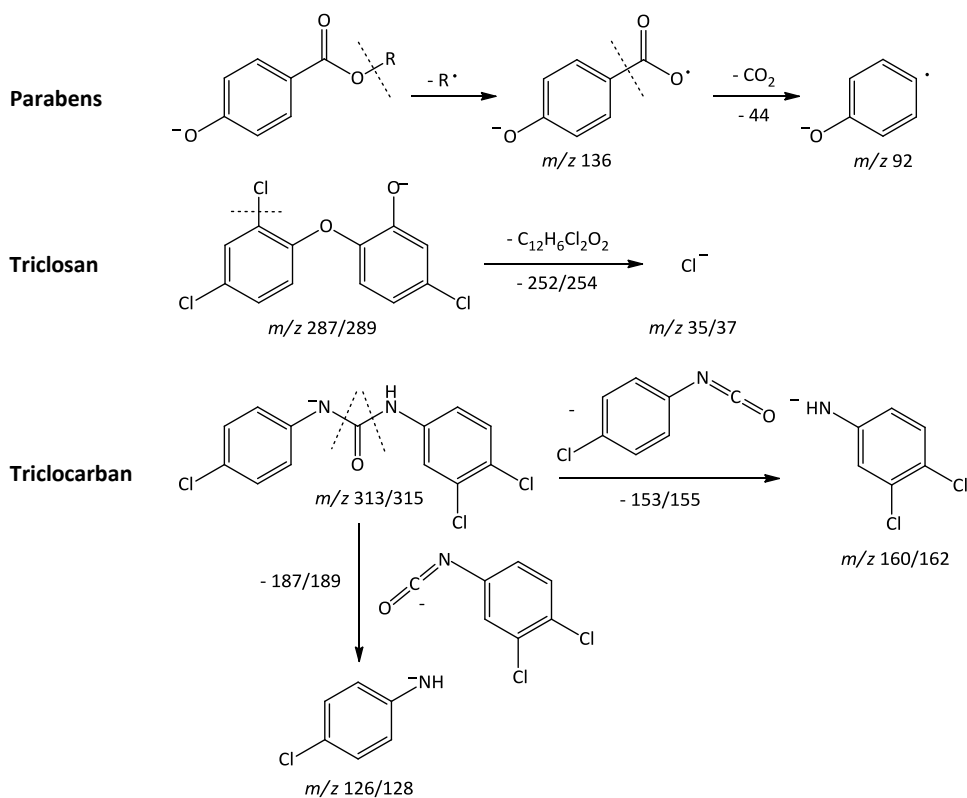
Figure 1 shows the main CID fragmentation pathways of the deprotonated target compounds. For parabens, this pathway consists of the loss of the alkyl chain bonded to the ester group (resulting in an ion at m/z 136) followed by the loss of CO_2 (m/z 92) as observed in previous studies [17,18,31]. This characteristic fragmentation pattern may prove to be very useful in screening for other parabens by working in the Precursor Ion Scanning mode.

On the other hand, CID of the TCS anion only leads to the formation of chloride ions (m/z 35 and 37), without any other products being observed, as already reported by Quintana and Reemtsma [30] and Hua et al. [14], who also used a triple quadrupole mass spectrometer for the quantification of this compound.

Finally, the dominant fragmentation pattern observed for the deprotonated TCC consists of cleavage of the nitrogen-carbon bond closest to the aromatic ring carrying two chlorine atoms, as reported by Sapkota et al. [24], yielding a product ion at m/z 160 + 162 (**Figure 1**). The monochlorinated aniline is also produced but to a minor extent (m/z 126 + 128).

According to the 2002/657/EC decision [32], two different MS/MS transitions are required to confirm the identity of target analytes. The most intense of the above discussed transitions was selected for quantification in the SRM mode and the second one was used for confirmation. The ratio between the signal intensities of the two transitions (transitions ratio) must be monitored and be within the ranges shown in **Table 1** in order to fulfil that regulation [32]. For ISs, just one transition was employed, as the concentration of these species added to the samples is high enough so that their identification does not raise any doubt.

Figure 1. Negative ion mode ESI-MS/MS fragmentation pathways of the deprotonated analytes.

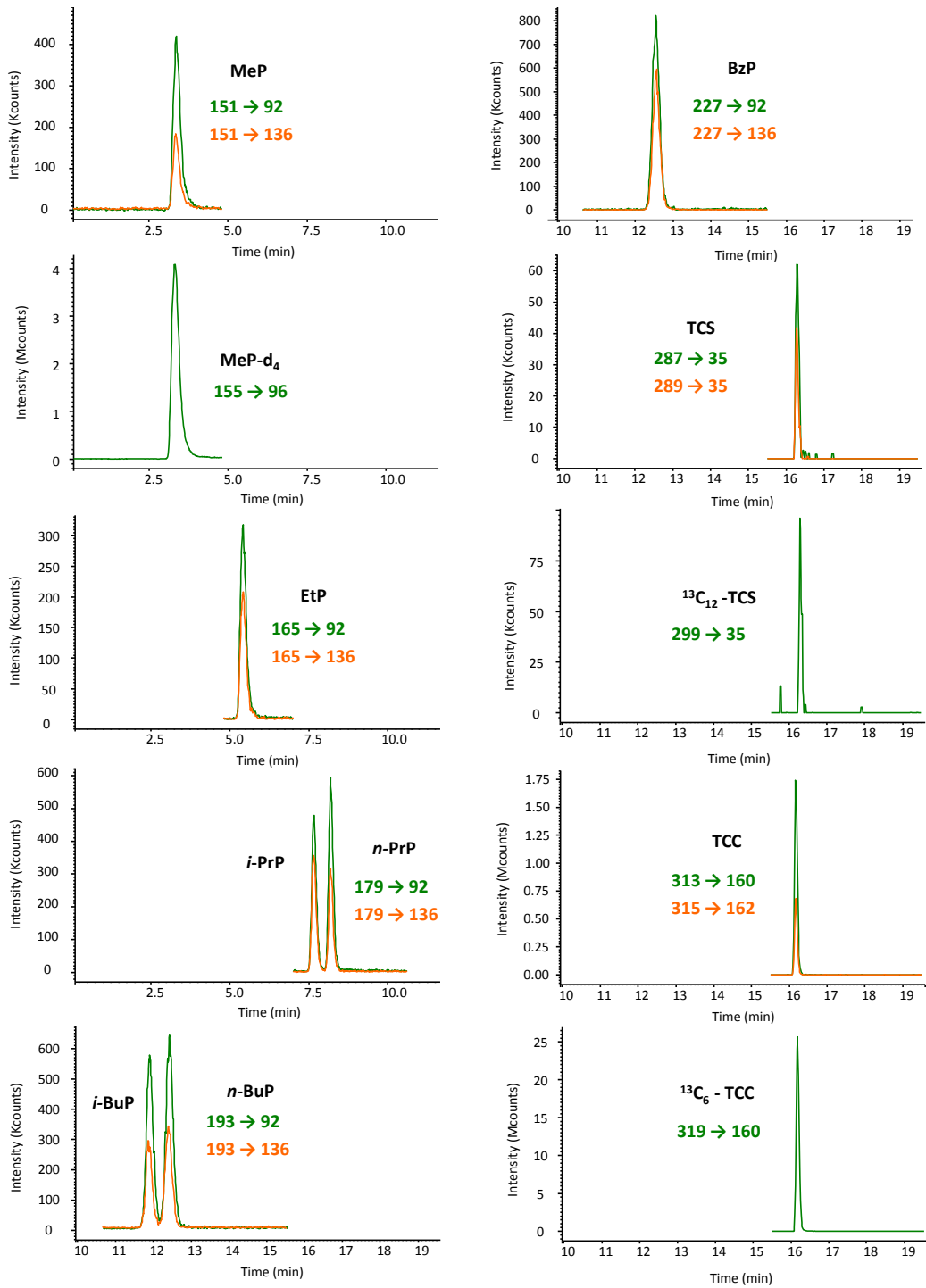


3.2. Mobile-Phase additives

The effect of adding an organic modifier to the mobile phase was studied. Three different modifiers at different concentrations were considered: ammonium acetate (0-20 mM), formic acid (0-0.2%) and acetic acid (0-2%). For each modifier and concentration level, 5 μL of a 10 $\mu\text{g mL}^{-1}$ mixture standard was injected by FIA and responses were registered in SRM mode.

A reduction in the signals of analytes as NH_4OAc concentration in the mobile phase is increased was observed. This effect has been observed for several other analytes classes [33,34] and can be explained because addition of NH_4OAc increases the concentration of anions in the aerosol, competing with the target compounds for drop surface. However, it contrasts with the observations made by Choi et al. [35], who found that addition of ammonium acetate or formate at a concentration above 5 mM caused a slight enhancement in parabens LC-MS/MS (ESI-) response.

Figure 2. Chromatogram of a standard (8 ng mL⁻¹ except TCS and internal standards: 80 and 100 ng mL⁻¹, respectively) injected in instrument A (Varian 1200L).



The presence of organic acids led to a much stronger signal suppression for parabens and TCS, particularly with the most acidic modifier, formic acid. This was expected, as these acids turn parabens ($pK_a \approx 8.3$ [36]) and TCS ($pK_a 7.8$ [36]) towards their neutral form, decreasing their negative ESI-MS/MS responses. Thus, this effect was not observed for TCC, a very weak acid ($pK_a 12.8$ [36]), which is already protonated at neutral pH with the unmodified mobile phase. Again, the above pattern differs from that reported by Choi et al. for parabens and TCS [35], although it matches the observations of same authors for other acidic endocrine-disrupting chemicals like oestradiol or bisphenol A.

In view of these results, avoiding the use of eluent modifiers should lead to the best sensitivity. However, 5 mM NH_4OAc was finally chosen since it is expected to provide a good buffering capacity, keeping retention times stable and leading to a loss in sensitivity lower than 35%. Under final working conditions, compounds were separated in 20 min and the chromatographic programme was completed in 29 min. As it can be seen in **Figure 2**, good separation was achieved between lineal and branched parabens, which is essential in order to determine this type of isomers in real water samples, since they have the same SRM transitions.

3.3. LC-ESI-MS/MS performance

Table 2 summarises some data related to the performance of both LC-MS/MS systems considered in this work.

Linearity was investigated by injection of standard solutions at seven different concentrations up to 800 ng mL^{-1} . The representation of peak area versus analyte concentration fitted a linear model with determination coefficients (R^2) from 0.990 to 0.999. Instrumental precision studies were performed by six injections of the same standard (80 ng mL^{-1} level) carried out over a 48 h period. Obtained relative standard deviations (RSDs) were below 5% in both systems, except for TCS on system A (11%, **Table 2**). For limits of quantification (LOQs), defined as a signal to noise ratio (S/N) of 10, noticeable differences were observed between the two instruments and, as expected, system B was more sensitive than A, providing 3- to 14-fold lower LOQs for parabens and TCC. In the case of TCS, this was markedly important, as system A provided an 80-fold higher LOQ. This improvement is attributed to a poor transmission efficiency for low masses, $m/z 35$ for TCS, in LC-MS system A, which also resulted in lower repeatability for this particular chemical compared with parabens and TCC (**Table 2**). A typical chromatogram of a standard is presented in **Figure 2**. In general, LOQs provided by the newest generation of LC-MS/MS instruments (system B) are better than those obtained by Kasprzyk-Hordern et al. [17] for parabens (they reported LC-MS LOQs of 0.2

and 0.4 ng mL⁻¹ for methyl, ethyl, *n*-propyl and *n*-butyl paraben) and by Agüera et al. [23] and Quintana and Reemstma [30] for TCS (1 and 0.7 ng mL⁻¹, respectively).

Table 2. Performance of both LC-MS/MS instruments (A and B).

| | R ² ^a | | LOQ (ng mL ⁻¹) ^b | | %RSD (n = 4) ^c | |
|---------------|-----------------------------|--------|---|-------|---------------------------|-----|
| | A | B | A | B | A | B |
| MeP | 0.9999 | 0.9999 | 0.7 | 0.2 | 2.5 | 0.6 |
| EtP | 0.9960 | 0.9993 | 0.6 | 0.07 | 4.9 | 4.8 |
| <i>i</i> -PrP | 0.9954 | 0.9984 | 0.6 | 0.08 | 4.2 | 2.8 |
| <i>n</i> -PrP | 0.9945 | 0.9997 | 0.6 | 0.06 | 4.3 | 4.0 |
| <i>i</i> -BuP | 0.9955 | 0.9996 | 0.6 | 0.02 | 3.4 | 0.8 |
| <i>n</i> -BuP | 0.9900 | 0.9994 | 0.6 | 0.04 | 4.7 | 1.3 |
| BzP | 0.9922 | 0.9997 | 0.2 | 0.04 | 2.7 | 1.1 |
| TCC | 0.9991 | 0.9926 | 0.01 | 0.004 | 3.6 | 1.5 |
| TCS | 0.9926 | 0.9995 | 10 | 0.12 | 11.0 | 1.8 |

^a Seven-point calibration LOQ – 800 ng mL⁻¹.

^b S/N = 10, methanol:water (1:1) standards.

^c Injection of a standard at the 80 ng mL⁻¹ level during a 48 h period (n=6).

3.4. SPE-LC-ESI-MS/MS performance

Solid-phase extraction conditions were optimised with 100 mL samples of ultrapure water spiked with the target compounds at 15 ng mL⁻¹ and using methanol as elution solvent. In the preliminary experiments, 10 mL of this solvent were used. Working under these conditions, Oasis HLB cartridges (60 mg) provided between 10 and 15% higher recoveries than the mixed-mode ones, Oasis MAX (60 mg), for the concentration of acidified (pH 2) ultrapure water samples. With basified samples (pH 12) parabens could not be eluted from the Oasis MAX sorbent with pure methanol due to anion-exchange interactions (data not given). Although addition of an organic acid could overcome this problem, it would then require the complete blowing down of the extract to eliminate the acid, which could result in losses of analytes. Thus, the first sorbent was used in further experiments.

Subsequently, the effect of the pH on the retention of analytes using Oasis HLB cartridges was investigated with 100 mL volume samples, adjusted at five different pH values: 2.0, 5.8, 7.1, 8.3 and 12.1. For MeP and EtP, which are the most polar of the tested compounds (log K_{ow}: 2.0 and 2.5, respectively [36]), the efficiency of the extraction underwent a dramatic reduction for samples adjusted at pH 12 and remained unchanged in the range between pH 2 and 8.5 (data not shown). The yield of the SPE for the rest of compounds was

not affected by the pH of the water. This effect can be easily understood taking into account the pK_a values of parabens and TCS (≈ 8.3 and 7.8 [36]). At pH 12, these analytes become more hydrophilic because they are transformed in their anionic form, and thus retention of the most polar analytes (MeP and EtP) fails. On the basis of this behaviour, samples were processed as received without any pH adjustment (natural and wastewater samples are expected to have a pH value in the 5.5-8.5 range) simplifying the sample preparation process and avoiding retention of humic acids in the cartridge due to sample acidification.

Finally, using two cartridges connected in series, it was verified that the breakthrough volume of the 60 mg cartridges was higher than 1 L for all compounds in spiked ultrapure water and that 4 mL methanol sufficed their quantitative elution from the sorbent; moreover, further concentration of this extract to a volume of ca. 0.5 mL, using a gentle stream of nitrogen at room temperature, did not lead to significant losses of analytes. Therefore, 500 and 200 mL were selected as sample volumes for river and wastewater, respectively; extracts were concentrated to ca. 0.5 mL and made up to 1 mL using ultrapure water. An aliquot of this final extract (20 μ L) was injected in the LC-MS/MS system.

Table 3 shows the recoveries obtained for spiked samples of ultrapure water (400 ng L^{-1}), river water (150 ng L^{-1}) and raw and treated wastewater (1500 ng L^{-1}). After blank correction, the concentration of each compound in the SPE extracts was established by standard addition over the extract [28-30], to compensate for potential matrix effects, and compared with that added to the sample (see Experimental section). In general, for ultrapure water, river water and treated wastewater, recoveries greater than 85% were obtained; slightly lower values, particularly for TCC, the most lipophilic of the compounds, were attained for raw wastewater, probably due to adsorption to particulate matter (**Table 3**). When extracting 500 mL of surface water, LOQs ranged from 0.02 to 20 ng L^{-1} with the LC-MS/MS system A and from 0.008 to 0.44 ng L^{-1} with the system B. For raw and treated wastewater (200 mL sample volume), they varied between 0.05 and 50 ng L^{-1} (A) and between 0.02 and 1.1 ng L^{-1} (B) (**Table 3**). Obviously, LOQ values obtained with the latter instrument are better than those obtained with the first one, but they are also lower than those reported by other authors like Benijts et al. [31] (1-2 ng L^{-1} for parabens in river water), Kasprzyk-Hordern et al. [17] (0.3-0.5 ng L^{-1} for parabens in the same matrix) and Hua et al. [14] (4 ng L^{-1} in river water and 10 ng L^{-1} in treated wastewater, both for TCS).

Table 3. SPE recoveries and LOQs after sample SPE with both instruments (A and B).

| | % SPE Recovery (%RSD) | | | | LOQ (ng L ⁻¹) for SPE-LC-MS/MS ^d | | | | |
|---------------|------------------------------|-------------|--------------------------|---------------------------------|---|-------------|------|------------|--|
| | Ultrapure water ^a | | River water ^b | Treated wastewater ^c | Raw wastewater ^c | River water | | Wastewater | |
| | A | B | | | A | B | A | B | |
| MeP | 97.8 (2.0) | 99.2 (14.0) | 96.1 (4.5) | 95.5 (9.8) | 1.4 | 0.44 | 3.5 | 1.11 | |
| EtP | 88.9 (12.9) | 87.9 (18.9) | 101.1 (4.0) | 79.4 (7.7) | 1.2 | 0.13 | 3.0 | 0.34 | |
| <i>i</i> -PrP | 88.1 (10.6) | 91.7 (15.1) | 102.8 (5.4) | 81.3 (8.4) | 1.2 | 0.15 | 3.0 | 0.38 | |
| <i>n</i> -PrP | 89.3 (12.8) | 88.9 (17.4) | 102.5 (4.5) | 73.2 (8.4) | 1.2 | 0.11 | 3.0 | 0.28 | |
| <i>i</i> -BuP | 90.7 (10.7) | 93.2 (14.0) | 109.3 (4.8) | 86.6 (20.6) | 1.2 | 0.05 | 3.0 | 0.12 | |
| <i>n</i> -BuP | 91.8 (8.0) | 92.9 (15.5) | 103.9 (4.2) | 89.7 (20.7) | 1.2 | 0.08 | 3.0 | 0.21 | |
| BzP | 92.6 (5.7) | 94.8 (14.5) | 103.5 (5.1) | 93.9 (26.1) | 0.4 | 0.08 | 1.0 | 0.2 | |
| TCC | 89.2 (1.0) | 92.5 (7.5) | 86.1 (3.6) | 66.8 (6.0) | 0.02 | 0.008 | 0.05 | 0.02 | |
| TCS | 80.6 (8.4) | 93.8 (24.0) | 90.9 (20.4) | 102.9 (25.1) | 20 | 0.23 | 50 | 0.57 | |

^a SPE of 500 mL samples spiked at the 400 ng L⁻¹ level (n = 4).

^b SPE of 500 mL samples spiked at the 150 ng L⁻¹ level (n = 4).

^c SPE of 200 mL samples spiked at the 1500 ng L⁻¹ level (n = 4).

^d S/N = 10.

3.5. Matrix effects

The main drawback of LC-MS with atmospheric pressure interfaces, and in particular with ESI, is signal suppression or enhancement during ionisation because of co-extracted matrix compounds competing with analytes or changing the physicochemical characteristics of the droplet [37,38].

Thus, matrix effects were studied in river, raw and treated wastewater, processing every sample with the optimised method and spiking the extracts with all analytes at 80 ng mL⁻¹. Matrix effects (%ME), calculated by comparing the spiked extract response with that of standards in methanol:water (1:1) [28-30], are presented in **Figure 3** for both instruments.

For instrument A, the matrix produces signal suppression for parabens, with the suppression being reduced as the retention time increases. This phenomenon has often been observed [30] and it is attributed to salts and polar components of the sample overloading the column capacity and eluting early in the chromatogram [35]. Surprisingly, for instrument B, matrix effects lead to signal enhancement (%ME higher than 100), that, again, is less important as retention time increases. Finally, TCS and TCC show similar effects with both instruments: signal suppression, which is particularly noticeable for raw wastewater due to hydrophobic matrix components, and which is stronger in system B.

The use of internal standards (MeP-d₄, ¹³C₁₂-TCS and ¹³C₆-TCC) effectively compensates matrix effects for their analogous native analytes (MeP, TCS and TCC) with both LC-MS/MS systems (**Figure 3**). However, for the remaining parabens, for which there are no isotopically labelled standards available, there is a signal overcorrection when using MeP-d₄ as internal standard with instrument A, while all parabens can be measured by this quantification approach with instrument B (**Figure 3**). As compiled in **Table 4**, acceptable overall method recoveries (see Experimental section) are obtained with both instruments.

Although the study of matrix effects on different instruments is not commonly carried out, a previous work of Mei et al. [39] confirms our finding that matrix effects may vary greatly between different systems, both with ESI and atmospheric-pressure chemical ionisation (APCI) sources. The different behaviour can only be attributed to the different design of the ESI source among the different LC-MS manufacturers, which incorporate different approaches to assist the electrospray desolvation process. These results clearly show that translation of a method to a different LC-MS instrument requires a matrix effect re-evaluation in order to define the appropriate quantification approach.

Figure 3. Matrix effects (%ME) by external standard calibration (ES) and by internal standard calibration (IS) for river water, treated and raw wastewater; (a) Instrument A; (b) Instrument B.

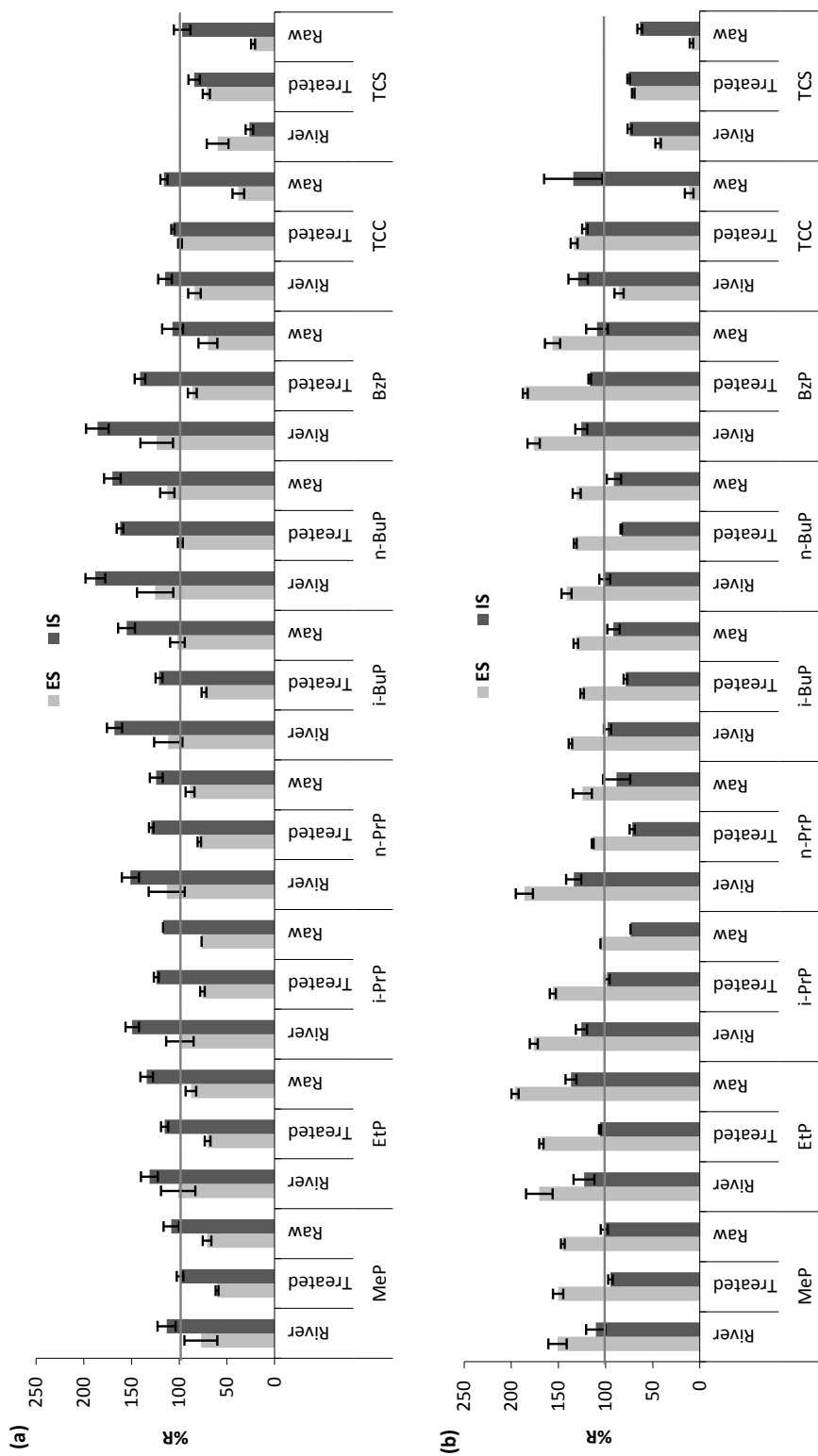


Table 4. Overall method recoveries for spiked samples with both instruments (n = 4).

| | %Recovery (%RSD) | | | | | | | | | | | | | | | |
|---------------|------------------------------|----------------|----------------|----------------|--------------------------|----------------|----------------|----------------|---------------------------------|----------------|----------------|----------------|-----------------------------|----------------|-------------|--|
| | Ultrapure water ^a | | | | River water ^b | | | | Treated wastewater ^c | | | | Raw wastewater ^c | | | |
| | A ^d | B ^e | A ^d | B ^e | A ^d | B ^e | A ^d | B ^e | A ^d | B ^e | A ^d | B ^e | A ^d | B ^e | | |
| MeP | 102.6 (2.0) | 107.1 (1.4) | 112.3 (11.3) | 108.7 (5.2) | 95.3 (2.8) | 90.9 (2.9) | 103.4 (6.2) | 94.7 (6.7) | 94.2 (12.9) | 109.2 (3.6) | 88.9 (6.5) | 107.1 (5.2) | 71.0 (2.8) | 105.1 (4.2) | 136.9 (4.5) | |
| EtP | 98.5 (10.6) | 125.0 (3.0) | 91.1 (4.4) | 113.7 (7.2) | 77.7 (4.9) | 100.1 (5.0) | 62.1 (5.9) | 73.4 (16.1) | 98.4 (12.8) | 131.3 (3.9) | 100.5 (4.8) | 118.9 (9.6) | 80.9 (4.1) | 73.3 (7.0) | 88.7 (20.0) | |
| <i>n</i> -PrP | 99.6 (10.8) | 125.4 (4.0) | 103.9 (4.5) | 89.9 (8.6) | 80.8 (4.1) | 78.5 (3.7) | 88.3 (19.1) | 90.0 (10.4) | 104.7 (8.0) | 129.5 (3.4) | 116.4 (2.8) | 93.5 (7.7) | 102.8 (3.3) | 83.6 (4.1) | 88.5 (12.1) | |
| <i>i</i> -BuP | 110.2 (5.7) | 112.6 (6.0) | 117.2 (4.5) | 113.7 (7.6) | 89.1 (2.3) | 113.4 (4.5) | 65.8 (24.2) | 99.0 (14.5) | 103.7 (1.0) | 134.4 (1.9) | 106.2 (4.1) | 131.0 (5.9) | 91.8 (3.3) | 123.8 (1.5) | 136.2 (2.7) | |
| BzP | 82.4 (8.4) | 113.8 (14.1) | 89.9 (18.9) | 68.9 (6.9) | 76.7 (19.2) | 69.0 (4.9) | 99.8 (41.9) | 72.0 (12.3) | TCC | | | | | | | |
| TCC | | | | | | | | | TCS | | | | | | | |
| TCS | | | | | | | | | | | | | | | | |

^a SPE of 500 mL samples spiked at the 400 ng L⁻¹ level (n = 4).

^b SPE of 500 mL samples spiked at the 150 ng L⁻¹ level (n = 4).

^c SPE of 200 mL samples spiked at the 1500 ng L⁻¹ level (n = 4).

^d MeP, TCS and TCC were measured by the internal standard method, with MeP-d₄, ¹³C₁₂-TCS and ¹³C₆-TCC being the ISS, respectively.

All other analytes were quantified by the external standard methodology (see text for details).

^e All analytes were quantified by the internal standard method: MeP-d₄ was used as IS for parabens, ¹³C₁₂-TCS for TCS and ¹³C₆-TCC for TCC (see text for details).

3.6. Application to real samples

The proposed method was applied to determine the levels of the target bactericides in grab samples from different rivers and sewage waters from the area of Santiago de Compostela (**Table 5**). A chromatogram of a river sample is presented in **Figure 4**.

The most often found species were MeP and *n*-PrP, in agreement with their ubiquitous presence in cosmetic formulations. These two compounds occurred in almost all analysed samples and only in some WWTP effluent and river samples their levels were below the LOQs of the method. Although they reached a level of 5.1 and 1.3 $\mu\text{g L}^{-1}$ (MeP and *n*-PrP, respectively) in raw wastewater, their elimination at the WWTP was higher than 99.9% (**Table 5**), in agreement with previous published results [14-20] and previous measurements from the same WWTP [15]. Thus, the fact that they were then detected in river waters at up to 69 ng L^{-1} (*n*-PrP) points out to small discharges of untreated wastewater or leaks from the sewage plumbing system. Regarding the other parabens, EtP, *n*-BuP and *i*-BuP were also present in all the WWTP influents and in some of the surface water samples and the WWTP effluents (**Table 5**); on the other hand, *i*-PrP was only detected at very low concentrations (4.6 ng L^{-1}) in a WWTP influent and BzP was below the LOQ in all the analysed samples. It is noteworthy that *i*-BuP was detected in the same concentration range (ca. 100-200 ng L^{-1}) as its linear analogue (*n*-BuP) in raw sewage, while PrP was found in the samples almost exclusively as the linear isomer (*n*-PrP). Although the few WWTP samples analysed point to a similar elimination rate of both isomers of BuP, under certain circumstances the branched isomer is expected to be less biodegradable than the linear one. However, to our knowledge, this is the first study that has considered both isomers.

Another of the widely used biocides, TCS, appeared in one of the raw sewage samples at the 0.9 $\mu\text{g L}^{-1}$ level and at 59 ng L^{-1} in a treated WWTP sample. However, it could not be detected above the LOQ in surface waters because of the low sensitivity of system A for this particular compound, as explained before (see **Tables 2** and **3**).

Finally, TCC was only detected in one of the WWTP influent samples at a very low level (4.7 ng L^{-1}), which may be a consequence of the limited market for this compound in Europe compared with the USA, where it occurs at the $\mu\text{g L}^{-1}$ level in raw wastewater [13,24]. Nevertheless, this assumption should be confirmed by the analysis of more samples from different locations in Europe.

Table 5. Concentration of analytes (n = 4) found in surface water and municipal wastewater samples from the surrounding area of Santiago de Compostela (Galicia, NW Spain).

| Concentration in ng L ⁻¹ (%RSD); May samples | | | | | | |
|---|------------|-----------|------------|------------|-------------|-------------|
| | Sarela | Dos Pasos | Sar 1 | Sar 2 | Treated | Raw |
| MeP | 6.8 (15.3) | 8.2 (6.8) | 3.4 (9.5) | 3.4 (32.7) | <LOQ | 5138 (3.9) |
| EtP | <LOQ | <LOQ | <LOQ | 3.0 (15.2) | <LOQ | 549 (3.7) |
| <i>i</i> -PrP | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| <i>n</i> -PrP | 1.0 (11.8) | 4.3 (7.2) | 5.9 (17.5) | 69 (3.4) | <LOQ | 1147 (4.5) |
| <i>i</i> -BuP | <LOQ | <LOQ | <LOQ | 4.6 (3.7) | <LOQ | 83.6 (11.0) |
| <i>n</i> -BuP | <LOQ | <LOQ | 1.0 (14.8) | 7.0 (8.3) | 3.6 (31.0) | 150 (6.8) |
| BzP | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| TCC | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| TCS | <LOQ | <LOQ | <LOQ | <LOQ | 59.0 (19.3) | <LOQ |

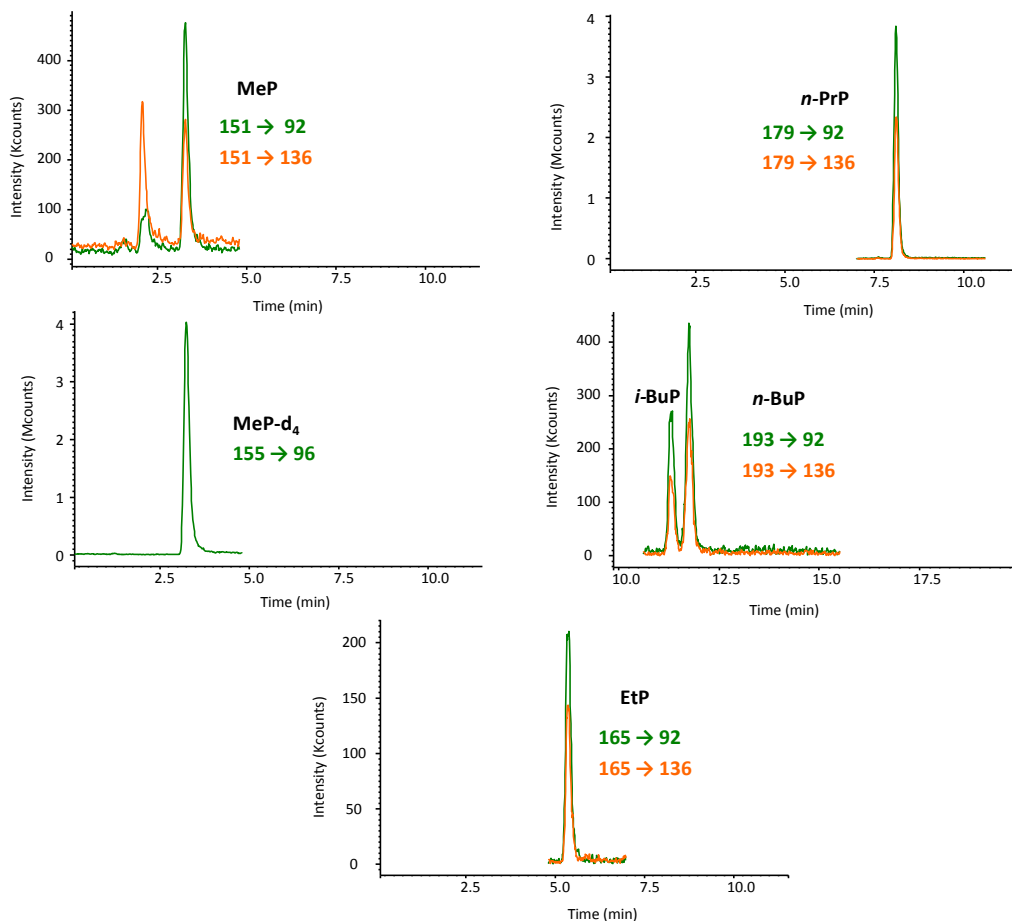
| Concentration in ng L ⁻¹ (%RSD); October samples | | | | | | |
|---|------------|-----------|------------|------------|------------|------------|
| | Sarela | Dos Pasos | Sar 1 | Sar 2 | Treated | Raw |
| MeP | 3.4 (5.4) | 9.0 (1.2) | 17.3 (0.8) | 1.8 (15.4) | 1.5 (14.9) | 1926 (5.4) |
| EtP | <LOQ | 1.2 (3.0) | 2.7 (28.4) | <LOQ | <LOQ | 452 (3.0) |
| <i>i</i> -PrP | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 4.6 (7.1) |
| <i>n</i> -PrP | 1.5 (10.8) | 5.9 (5.1) | <LOQ | 25.5 (6.7) | <LOQ | 1302 (2.2) |
| <i>i</i> -BuP | <LOQ | <LOQ | <LOQ | 1.3 (7.7) | <LOQ | 89.1 (1.7) |
| <i>n</i> -BuP | <LOQ | 1.0 (0.3) | <LOQ | 1.2 (7.4) | <LOQ | 181 (3.5) |
| BzP | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| TCC | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 4.7 (21.3) |
| TCS | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 936 (20.3) |

4. CONCLUSIONS

A method for the simultaneous determination of seven parabens, TCS and TCC in water samples by means of SPE followed by LC-ESI-MS/MS determination has been developed and validated with two different triple quadrupole LC-MS systems: a low-mid (A: Varian 1200L) and mid-high (B: API-4000) market range instrument.

SPE with 60 mg Oasis HLB cartridges produced efficient retention of the analytes at the natural pH of the samples, without breakthrough even when 1 L of water was concentrated, and easy desorption with only 4 mL of MeOH. SPE recoveries obtained were normally higher than 85% except for some analytes in raw wastewater, where they were still higher than 65%. The combination of this SPE method with LC-ESI-MS/MS provided LOQs at the sub-ng L⁻¹ level with system B and slightly higher with system A, with the exception of TCS, that produced higher LOQs in the Varian instrument (50 ng L⁻¹ for wastewater).

Figure 4. SRM traces of the analytes detected in the Sar River 2 sample (May); injected into instrument A (Varian 1200L).



LC-ESI-MS/MS matrix effects were also evaluated in both systems with surface and wastewater samples after SPE. These experiments showed a very different behaviour of the two instruments; thus, for parabens, signal suppression occurred in system A, while signal enhancement was noticed in B. As a result, it is evident that the calibration strategies need to be re-evaluated whenever the instrument is changed.

Finally, the proposed method was applied to the analysis of real samples. From these analyses it is worth mentioning the high levels of MeP and *n*-PrP in raw wastewater (1.1-5.1 $\mu\text{g L}^{-1}$) and the co-occurrence of both isomers of BuP (*i*-BuP and *n*-BuP) at similar concentrations (ca. 100-200 ng L^{-1}), which cannot be distinguished in other reported methods.

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PUBLICACIÓN III.2

EVALUATION OF THE OCCURRENCE AND BIODEGRADATION OF PARABENS AND HALOGENATED BY-PRODUCTS IN WASTEWATER BY ACCURATE-MASS LIQUID CHROMATOGRAPHY-QUADRUPOLE-TIME-OF-FLIGHT-MASS SPECTROMETRY (LC-QTOF-MS)

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Abstract

An assessment of the sewage occurrence and biodegradability of seven parabens and three halogenated derivatives of methyl paraben (MeP) is presented. Several wastewater samples were collected at three different wastewater treatment plants (WWTPs) during April and May 2010, concentrated by solid-phase extraction (SPE) and analysed by liquid chromatography-electrospray-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS). The performance of the QTOF system proved to be comparable to triple quadrupole instruments in terms of quantitative capabilities, with good linearity ($R^2 > 0.99$ in the 5-500 ng mL⁻¹ range), repeatability (RSD < 5.6%) and LODs (0.3-4.0 ng L⁻¹ after SPE). MeP and *n*-propyl paraben (*n*-PrP) were the most frequently detected and the most abundant analytes in raw wastewater (0.3-10 µg L⁻¹), in accordance with the data displayed in the bibliography and reflecting their wider use in cosmetic formulations. Samples were also evaluated in search for potential halogenated by-products of parabens, formed as a result of their reaction with residual chlorine contained in tap water. Monochloro- and dichloro-methyl paraben (ClMeP and Cl₂MeP) were found and quantified in raw wastewater at levels between 0.01 and 0.1 µg L⁻¹. Halogenated derivatives of *n*-PrP could not be quantified due to the lack of standards; nevertheless, the monochlorinated species (ClPrP) was identified in several samples from its accurate precursor and product ion mass/charge ratios (*m/z*). Removal efficiencies of parabens and MeP chlorinated by-products in WWTPs exceeded 90%, with the lowest percentages corresponding to the latter species. This trend was confirmed by an activated sludge biodegradation batch test, where non-halogenated parabens had half-lives lower than 4 days, whereas halogenated derivatives of MeP turned out to be more persistent, with up to 10 days of half-life in the case of the dihalogenated derivatives. A further stability test performed with raw wastewater also showed that parabens degrade rapidly in real sewage,

with half-lives lower than 10 h for *n*-butyl-paraben, while dihalogenated species turned out to be more stable, with half-lives longer than a week.

Keywords: parabens; halogenated by-products; biodegradation; water samples; accurate-mass mass spectrometry; wastewater.

1. INTRODUCTION

Parabens, esters of 4-hydroxybenzoic acid, are extensively employed as preservatives not only in a wide range of personal care products (PCPs) such as tooth pastes, deodorants, beauty creams, bath gels and shampoos, but also in canned foods, beverages and pharmaceuticals [1,2]. This extensive use has awakened the concern about their potential long-term effects on human health and, in fact, recent studies have suggested a possible relationship between them and breast cancer, presumably favoured by prolonged dermal expositions to paraben-containing deodorants [3]. Even though this hypothesis has not been fully proved and additional studies are needed to confirm their carcinogenicity, a new generation of paraben-free PCPs has emerged in the market recently.

As in the case of many other personal care chemicals, these preservatives are continuously released in urban wastewater at relatively high levels [4-7] and, despite being considerably removed during conventional sewage treatments [6,8-10], they have still been detected in river water samples at low ng L⁻¹ [5,7,11,12]. The main concern once they reach the environment is that they have proved to show oestrogenic activity [13-15], relatively weak compared to that of 17 β -oestradiol but not negligible, as they occur at much higher concentrations than the latter compound. Besides this, they can easily react with free chlorine when mixed with chlorinated tap water [16], yielding mostly mono and dichlorinated/brominated derivatives that have already been detected in raw wastewater. Although halogenation masks the apparent oestrogenic activity of the parent compounds [17], the resulting chlorinated by-products show higher acute toxicity responses in the *Daphnia magna* test [18], a fact that should be taken into account in case they reach the aquatic medium. However, the occurrence of such derivatives in the environment has not been investigated yet and, to the best of our knowledge, the biodegradability of parabens and their by-products during wastewater treatments still remains unknown.

Therefore, the aim of this work was the evaluation of the occurrence and biodegradability of parabens and their chlorination by-products in raw and treated wastewater. To this end, samples were preconcentrated by solid-phase extraction (SPE) followed by liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-

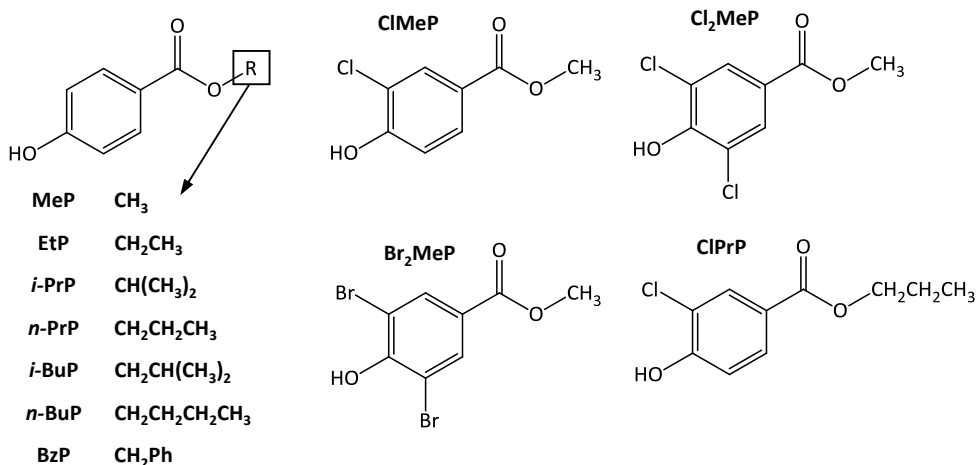
MS) determination. The use of LC-QTOF-MS has emerged in the last years as a valuable technique for the identification of by-products from emerging pollutants [19] due to the unique combination of high selectivity and structural information derived from accurate mass MS and MS/MS spectra, as well as novel software implementations, which allow the comparison with empirical formulae databases [20]. Thus, the potential of a modern LC-QTOF-MS was evaluated in terms of both qualitative and quantitative capabilities.

2. MATERIALS AND METHODS

2.1. Chemicals

Figure 1 shows the structure of the analytes included in this study. Methyl (MeP), ethyl (EtP), *n*-propyl (*n*-PrP), *n*-butyl (*n*-BuP) and benzyl (BzP) esters of 4-hydroxybenzoic acid were purchased from Aldrich (Milwaukee, WI, USA); *iso*-propyl paraben (*i*-PrP) and *iso*-butyl paraben (*i*-BuP) were from TCI Europe (Zwijndrecht, Belgium). Halogenated derivatives of MeP, 3-chloro-, 3,5-dichloro- and 3,5-dibromo-methyl paraben (ClMeP, Cl₂MeP and Br₂MeP) were obtained from ABCR GmbH&Co (Karlsruhe, Germany). As internal standards (ISs), methyl 4-hydroxybenzoate-2,3,5,6-d₄ (MeP-d₄) and *n*-propyl 4-hydroxybenzoate-2,3,5,6-d₄ (*n*-PrP-d₄) were from CDN Isotopes (Quebec, Canada). Stock solutions of each compound (1000 µg mL⁻¹) and mixtures of all of them or their deuterated analogues (10 µg mL⁻¹) were prepared in methanol and stored at -20°C until use. Calibration standards with increasing concentrations of the analytes and 100 ng mL⁻¹ of ISs were prepared in methanol:water (1:1).

Figure 1. Structures of parabens and halogenated derivatives included in the study.



HPLC grade methanol and ammonium acetate, employed as mobile phase additive, were supplied by Merck (Darmstadt, Germany). Salts used in the preparation of the assay medium during the activated sludge biodegradation study (ammonium chloride, potassium dihydrogen phosphate anhydrous, dipotassium monohydrogen phosphate anhydrous and disodium monohydrogen phosphate dihydrate) were also purchased from Merck. Magnesium sulphate heptahydrated was from Aldrich (Milwaukee, WI, USA), and calcium chloride and iron (III) chloride hexahydrated were supplied by Riedel de Haën (Seelze, Germany).

2.2. Samples and sample extraction

Raw and treated sewage samples were collected in different days during April and May 2010, at three different urban WWTPs: codes A (3 days study), B (3 days study) and C (5 days study). These plants receive the discharges from small and medium size cities (18000, 15000 and 125000 inhabitants, respectively) located in the same metropolitan area of the northwest of Spain. All of them comprise a primary and a secondary (activated sludge) treatment. Samples were collected in amber glass bottles previously rinsed with methanol and ultrapure water and extracted (SPE) in less than 6 h after sampling. Prior to extraction, particulate matter was removed using a combination of glass fibre prefilters and 0.45 μm nitrocellulose filters (Millipore, Bedford, USA).

Extraction was performed as detailed elsewhere [5]. In brief, Oasis HLB cartridges (60 mg, 3 mL), obtained from Waters (Milford, MA, USA), were sequentially conditioned with 3 mL of methanol and 3 mL of ultrapure water. Subsequently, 200 mL filtered samples, spiked with isotopically labelled standards and, in the case of recovery studies, also with analytes, were passed through them at a flow rate of approximately 10 mL min^{-1} . The sorbent was dried under vacuum for 30 min and the analytes were eluted with 4 mL of methanol. Extracts were concentrated down to ca. 0.5 mL with a gentle stream of nitrogen (99.999%) in a Turbovap II concentrator (Zymark, Hopkinton, MA, USA), diluted to a final volume of 1 mL with ultrapure water and injected (10 μL) into the LC-MS system.

2.3. LC-QTOF-MS

LC separations were carried out on a 100 \times 2.1 mm Halo C₁₈ (2.7 μm) porous shell column, consisting of a 1.7 μm inert core coated with a 0.5 μm C₁₈ layer (Advanced Materials Technology, Nes-Ziona, Israel). The column was protected with a 4 \times 2 mm C₁₈ guard cartridge provided by Phenomenex (Torrance, CA, USA) and thermostated at 45 °C. A dual eluent system

of water (A) and methanol (B), both containing 5 mM of ammonium acetate and adjusted to pH 4.5 with acetic acid, was used. The flow rate was maintained at 0.2 mL min⁻¹ and the gradient was as follows: 0 min (5% B), 10 min (40% B), 15 min (55% B), 20 min (55% B), 28 min (100% B), 30 min (100% B), 32 min (5% B) and 42 min (5% B).

Analyses were performed by LC-QTOF-MS using an Agilent 1200 Series liquid chromatograph (comprising a membrane degasser, a binary high-pressure gradient pump, a thermostated LC column compartment and an autosampler) interfaced to a quadrupole-time-of-flight mass spectrometer (Agilent 6520 accurate mass) equipped with a Dual electrospray ion source. Nitrogen, used as nebulising and drying gas, was provided by a nitrogen generator (Erre Due srl, Livorno, Italy). High purity nitrogen (99.9995%, Carburos Metálicos, A Coruña, Spain) was used as collision gas for MS/MS measurements.

The voltage of the ESI needle was set at 4 kV in the negative ionisation mode. The gas temperature of the source was 350 °C, the drying gas flow 11 L min⁻¹ and the nebulising gas pressure 45 psig. In the collision cell, nitrogen was kept at 18 mTorr. Analytes were quantified in single-MS mode from the accurate-mass extracted chromatograms (10 ppm mass window). Moreover, MS/MS spectra were simultaneously recorded for confirmation purposes (2 spectra per second, time window of 1.5 min centred in the retention time of each analyte). The fragmentor voltage was set at 160 V and the collision energy at 20 V. The instrument was operated in the 2 GHz (extended-dynamic range) mode and tuned at the beginning of each analyses series (ca. every 1-2 days) with a tuning solution containing different m/z values in the 100-1700 m/z range, according to the manufacturer instruction (Agilent Technologies). During each chromatographic run, the mass-axis was constantly recalibrated. To this end, the second sprayer was continuously infused with a reference solution provided by Agilent Technologies, for which in the negative mode (ESI-), the reference masses were 68.995758, 112.985587 and 980.016375 m/z (FWHM resolution: ca. 4700 at m/z 113 and ca. 11000 at m/z 980). The m/z values of the MS quantification ions, also used as precursors for MS/MS, and confirmation fragment ions, as well as acquisition times and internal standards used for each analyte, are compiled in **Table 1**.

Instrument control, data acquisition and evaluation were performed with the MassHunter software (Agilent Technologies). A database containing the empirical formulae of all the mono and dihalogenated possible parabens, with the halogen being Cl or Br, was created in order to identify those by-products for which standards were not available.

Table 1. Accurate m/z ratios of the $[M-H]^-$ precursor ions, confirmation fragment ions and internal standards used in each case.

| | Retention time (min) | IS | $[M-H]^-$ (m/z) | Confirmation fragment ions (m/z) | (m/z) |
|---------------------|-------------------------|-----------------|------------------------|---|-----------|
| MeP | 15.9 | MeP- d_4 | 151.0401 | 136.0166 | 92.0268 |
| EtP | 19.3 | n -PrP- d_4 | 165.0557 | 136.0166 | 92.0268 |
| ClMeP | 19.8 | n -PrP- d_4 | 185.0011 | 169.9776 | 125.9878 |
| Cl ₂ MeP | 20.3 | n -PrP- d_4 | 218.9621 | 159.9488 | 131.9539 |
| Br ₂ MeP | 21.1 | n -PrP- d_4 | 308.8591 | 249.8458 | 78.9189 |
| i -PrP | 21.6 | n -PrP- d_4 | 179.0714 | 136.0166 | 92.0268 |
| n -PrP | 22.2 | n -PrP- d_4 | 179.0714 | 136.0166 | 92.0268 |
| i -BuP | 25.6 | n -PrP- d_4 | 193.0870 | 136.0166 | 92.0268 |
| n -BuP | 26.1 | n -PrP- d_4 | 193.0870 | 136.0166 | 92.0268 |
| BzP | 26.3 | n -PrP- d_4 | 227.0714 | 136.0166 | 92.0268 |
| ClPrP | 26.8 | n -PrP- d_4 | 213.0324 | 170.9854 | 125.9878 |

2.4. Aerobic biodegradability and stability in raw wastewater

The biological degradation of parabens was evaluated through two different series of assays.

First of all, a batch of aerobic biodegradation tests was carried out based on the ISO 7827:1994 international standard [21]. Thus, 1.2 L glass bottles were filled with 1 L of ultrapure water containing a phosphate buffer, a pool of inorganic salts [21] and 5 mg L⁻¹ of one of the considered parabens (MeP, EtP, n -PrP, i -BuP, n -BuP, ClMeP, Cl₂MeP or Br₂MeP). Then, 20 mg of activated sludge from WWTP-C were added to each solution as inoculum. Control tests, without and with poisoned (HgCl₂) sludge, and with aniline as a control substrate, were also run in parallel in order to check for abiotic degradation, sorption processes and sludge activity. All solutions were kept in the dark at 20 ± 2 °C under continuous stirring. Samples (ca. 40 mL) were taken every few days, immediately filtered through 0.45 µm membrane filters (cellulose acetate; Sartorius, Goettingen, Germany) and stored frozen until being analysed by LC-QTOF-MS

A second series of assays was performed in order to evaluate the stability of parabens in real wastewater. A raw wastewater sample, characterized by a pH of 7.1, a total suspended solids content of 100 mg L⁻¹ and a chemical oxygen demand of 240 mg L⁻¹, collected from WWTP-C, was used for this purpose. Non-filtered aliquots (10 mL) were poured in 16 mL amber vials, spiked with one of the considered parabens (50 ng mL⁻¹) and the closed vials were

kept in the dark at room temperature (20 ± 2 °C). Control poisoned (HgCl_2) tests were carried out in parallel. Fractions of ca. 1 mL were taken at different times, from a few hours up to 6 days, passed through $0.20 \mu\text{m}$ membrane filters (cellulose acetate; Sartorius, Goettingen, Germany) and stored frozen until analysis. In this case, $50 \mu\text{L}$ were injected in the LC-MS system, so that the achieved LODs stayed below 0.2 ng mL^{-1} for all species. Thus, it was possible to follow their degradation up to a percentage higher than 99%.

All degradation data were fitted to a logistic model with the software Graphpad Prism 5. This type of mathematic model, very common in enzymatic reactions, can account for the initial lag phase and the subsequent first-order degradation. A simplified model [22] was used:

$$\frac{C}{C_0} = \frac{K}{1 + c \cdot e^{rt}}$$

Where K , c and r are the fitting parameters. K represents the inhibiting factor and r the rate constant. In the case that K and c become very large, then the logistic function will approach a first-order exponential decay.

Thus, the half-lives ($t_{1/2}$) can be calculated as follows [22]:

$$t_{1/2} = \ln\left(\frac{K - 0.5}{c \cdot 0.5}\right) \cdot \left(\frac{1}{r}\right)$$

Or, in general, the time when $C/C_0 = x$, t_x , is calculated from the general expression:

$$t_x = \ln\left(\frac{K - x}{c \cdot x}\right) \cdot \left(\frac{1}{r}\right)$$

3. RESULTS AND DISCUSSION

3.1. Method performance

The SPE-LC-QTOF method was based on a previously published work [5]. However, since the instrument was changed from a triple-quadrupole to a QTOF system and several halogenated by-products were included among the target analytes, SPE and LC-MS procedures required slight adaptations and were therefore revalidated. The information on the performance of the method is compiled in **Table 2**.

In the first instance, the original composition of the LC eluents, which were originally buffered with 5 mM ammonium acetate (pH ca. 7) [5], needed to be acidified to pH 4.5. Otherwise, the halogenated parabens showed broad peaks and were poorly retained into the

reversed-phase C₁₈ column, due to their more acidic character (calculated pK_a = 5.3-6.8) versus the original parabens (calculated pK_a values in the 8-8.5 range) [23]. Then, the performance of the LC-QTOF-MS method was tested in terms of repeatability and, particularly, LODs and linearity, since a short dynamic range has been reported as one of the main drawbacks of (Q)TOF instruments in quantitative analysis [24,25]. However, new implementations in modern instruments can overcome this problem. In the case of the system used in this work, the Analog to digital converter (ADC) can either work in the 4 GHz mode, which grants a higher mass resolution, and in the 2 GHz mode (resolution ca. half of 4 GHz), where data are acquired at two gain levels, expanding the linear range. Thus, this latter mode was employed, and the obtained linearity was satisfactory from 5 to 500 ng mL⁻¹ (IS concentration of 100 ng mL⁻¹) with R² values varying from 0.9986 to 0.9996 (Table 2).

Table 2. Performance parameters of the method.

| | LC-TOF-MS | | | | | SPE-LC-TOF-MS | | |
|---------------------|-----------------------------|-------------------------------|------------------------|-------------------------|--|-------------------------|------------------------------|-------------------------|
| | R ² ^a | LOD (ng mL ⁻¹) | %RSD ^b | | Mass accuracy relative error (ppm) ^c | | LOD (ng L ⁻¹) | %R ^d ± SD |
| | | | 20 ng mL ⁻¹ | 200 ng mL ⁻¹ | 10 ng mL ⁻¹ | 100 ng mL ⁻¹ | | |
| MeP | 0.9996 | 0.8 | 3.9 | 3.4 | 2.7 | 1.7 | 4.0 | 99 ± 4 |
| EtP | 0.9990 | 0.3 | 2.0 | 2.9 | 2.1 | 0.9 | 1.4 | 104 ± 4 |
| ClMeP | 0.9995 | 0.05 | 3.2 | 4.0 | 6.5 | 1.4 | 0.3 | 99 ± 2 |
| Cl ₂ MeP | 0.9986 | 0.2 | 5.4 | 5.6 | 2.1 | 1.4 | 0.9 | 119 ± 2 |
| Br ₂ MeP | 0.9986 | 0.09 | 3.1 | 5.6 | 1.5 | 1.0 | 0.5 | 125 ± 5 |
| <i>i</i> -PrP | 0.9992 | 0.3 | 3.2 | 3.0 | 2.0 | 0.8 | 1.6 | 92 ± 2 |
| <i>n</i> -PrP | 0.9991 | 0.3 | 2.1 | 4.7 | 1.4 | 0.6 | 1.3 | 103 ± 5 |
| <i>i</i> -BuP | 0.9987 | 0.4 | 1.6 | 4.8 | 1.6 | 0.8 | 2.2 | 108 ± 8 |
| <i>n</i> -BuP | 0.9993 | 0.4 | 2.1 | 2.2 | 1.6 | 0.5 | 2.2 | 103 ± 7 |
| BzP | 0.9994 | 0.4 | 3.4 | 2.9 | 1.8 | 1.1 | 1.9 | 102 ± 8 |

^a Calibration range 5-500 ng mL⁻¹ (IS 100 ng mL⁻¹).

^b n = 7 replicates.

^c Mean value from two replicates.

^d Percentages of recovery for 200 mL ultrapure water samples spiked with 1.25 ng mL⁻¹ of each analyte and 0.5 ng mL⁻¹ of each IS.

Instrumental precision studies were carried out at two different levels (20 and 200 ng mL⁻¹) by seven injections of the same standard over a 24 h period; obtained relative standard deviations (RSDs) ranged between 1.6 and 5.4% in the first case and between 2.2 and 5.6% in the second one. Instrumental LODs of the LC-MS method were defined for a peak-to-peak signal-to-noise (S/N) ratio of 3 (mean of three replicates), measuring the noise in the ca. 2 min

time region before and after the retention time of each analyte. Achieved LODs ranged from 0.05 ng mL⁻¹ (ClMeP) to 0.80 ng mL⁻¹ (MeP), considering an injection volume of 10 µL. Thus, the performance of the LC-QTOF system in the single-MS mode turned out to be comparable with that of triple-quadrupole instruments [5]. Furthermore, valuable qualitative information was provided and accurate-mass MS/MS spectra could be recorded simultaneously for the target analytes, minimising the risk of false positives. A further tested parameter was the relative error of mass assignments to [M-H]⁻ ions, which was maintained below 3 ppm, except for ClMeP (6.5 ppm), for a concentration level of 10 ng mL⁻¹, and below 2 ppm for all compounds at 100 ng mL⁻¹ concentrations (**Table 2**).

Finally, recoveries of the SPE protocol were also re-evaluated using 200 mL aliquots of ultrapure water spiked with 1.25 ng mL⁻¹ of analytes and 0.5 ng mL⁻¹ of ISs; obtained values ranged from 92.0% (*i*-PrP) to 125.4% (Br₂MeP).

3.2. Screening of halogenated parabens

As it has been proved by Canosa et al. [16], halogenated derivatives of parabens can be formed by reaction of the parent bactericides with residual chlorine in tap water during showering and bathing. Thereafter, these by-products might enter the aquatic environment through sewage water. Occurrence of such by-products was screened by LC-QTOF-MS, as pure standards were only available for some of the MeP derivatives.

First, a database containing all the potential mono and dichlorinated/brominated derivatives of the target parabens was created with the Personal Compound Database Library software, included within the Mass Hunter package. For each possible halogenated paraben, this database comprises its name and empirical formula; other information such as structure, CAS No. and Chemspider No. (directly linking to PubChem and Chemspider public internet databases with more information on each particular chemical) can be also added. Then, the Mass Hunter software provides a “Find by Formula” function that automatically generates the accurate *m/z* values of the ionised compounds according to the ESI-MS polarity (negative in this case) and the parameters considered, i.e. deprotonation, formation of ammonia adducts, etc. In this case, neither adducts nor dimers/trimers were expected to occur, so they were not contemplated. Subsequently, the software searches for peaks with these accurate masses (within an *m/z* window of ±5 ppm), accurate mass extracted chromatograms are generated and their peak spectra compared with the calculated one in terms of three parameters: mass accuracy, isotopic match and spacing between the different molecular ions observed in each cluster of signals, determined by the natural abundance of elemental isotopes in each ion.

These three parameters are combined into an overall score, where the mass accuracy contributes a 48%, the isotopic distribution a 28% and the spacing between ions a 24%. Hence, an overall score of 100 would represent a perfect match. More details on the Mass Hunter algorithm have been previously reported by Gómez et al. elsewhere [20]. Other (Q)TOF manufacturers use similar algorithms [26].

Applying this methodology, ClMeP and Cl₂MeP were found in all the influents and in a few effluents and ClPrP could be also detected in all the influents. Indeed, in the case of the chlorinated derivatives of MeP, this screening approach was unnecessary as standards were commercially available and unequivocal identification and quantification could be performed. Nevertheless, Cl₂MeP illustrates a good example of QTOF potential due to the finding of two possible peaks with the same empirical formula and different retention times in a sample of WWTP-B. As shown in **Figure 2a**, the MS spectrum of the first eluting compound matched very well (overall score 99.56) with the theoretical spectrum of the deprotonated Cl₂MeP (ion formula: C₈H₅Cl₂O₃). This fact indicates that this species has the same empirical formula than Cl₂MeP, but, obviously, this is not enough for a positive identification, as a search in e.g. SciFinder Scholar database [23] revealed 169 known chemicals whose formula is C₈H₆Cl₂O₃. Yet, when both compounds were submitted to reinjection and CID fragmentation, their high resolution MS/MS spectra turned out to be completely different, proving that the first eluting compound was actually a different one and not Cl₂MeP itself. In fact, its fragmentation was dominated by a decarboxylation and a further, or simultaneous, loss of HCl, indicating that it is a carboxylic acid (**Figure 2b**). On the other hand, Cl₂MeP fragmented through the typical parabens pattern, i.e. loss of the carboxylic group together, or sequentially, with the side chain (nominal *m/z* 160).

On the other hand, the monochlorinated derivative of PrP, ClPrP, could not be quantified in any of the samples since no standard was available in the laboratory. However, it was identified in all of the raw sewage samples at low concentrations from its accurate-mass MS and MS/MS spectra (**Figure 3a** and **3b**, respectively); the latter one shows the typical paraben fragmentation pattern, i.e. loss of the side chain from the ester (nominal *m/z* 171) and production of the chloro-phenolate anion by elimination of the carboxylic ester group (nominal *m/z* 126), confirming the identity of ClPrP. Obviously, once a by-product is identified for the first time it needs to be re-injected in order to obtain the MS/MS spectrum. However, when the retention time is known, its precursor ion can be introduced in the targeted MS/MS list of the method in order to automatically obtain the MS/MS confirmatory spectrum; therefore, a single injection suffices a confident identification in the remaining set of samples.

Hence, the findings from the screening study corroborated the data described by Canosa et al., who also detected the presence of dichlorinated forms of methyl and propyl paraben in raw wastewater by GC-MS with an ion-trap instrument after analytes' derivatisation [16]. The non-detection of brominated derivatives can be attributed to the low levels of bromide in the geographical area of investigation. Also, it is logical to detect only MeP and *n*-PrP derivatives, as these two precursor parabens are the ones found at higher concentrations in wastewater (see section 3.3).

Figure 2. Distinction between Cl₂MeP and an unknown compound with the same empirical formula and same precursor *m/z*: (a) extracted accurate-mass MS chromatogram and MS spectrum of the unknown compound and empirical formula match; (b) MS/MS spectrum of Cl₂MeP facing MS/MS spectrum of the unknown species.

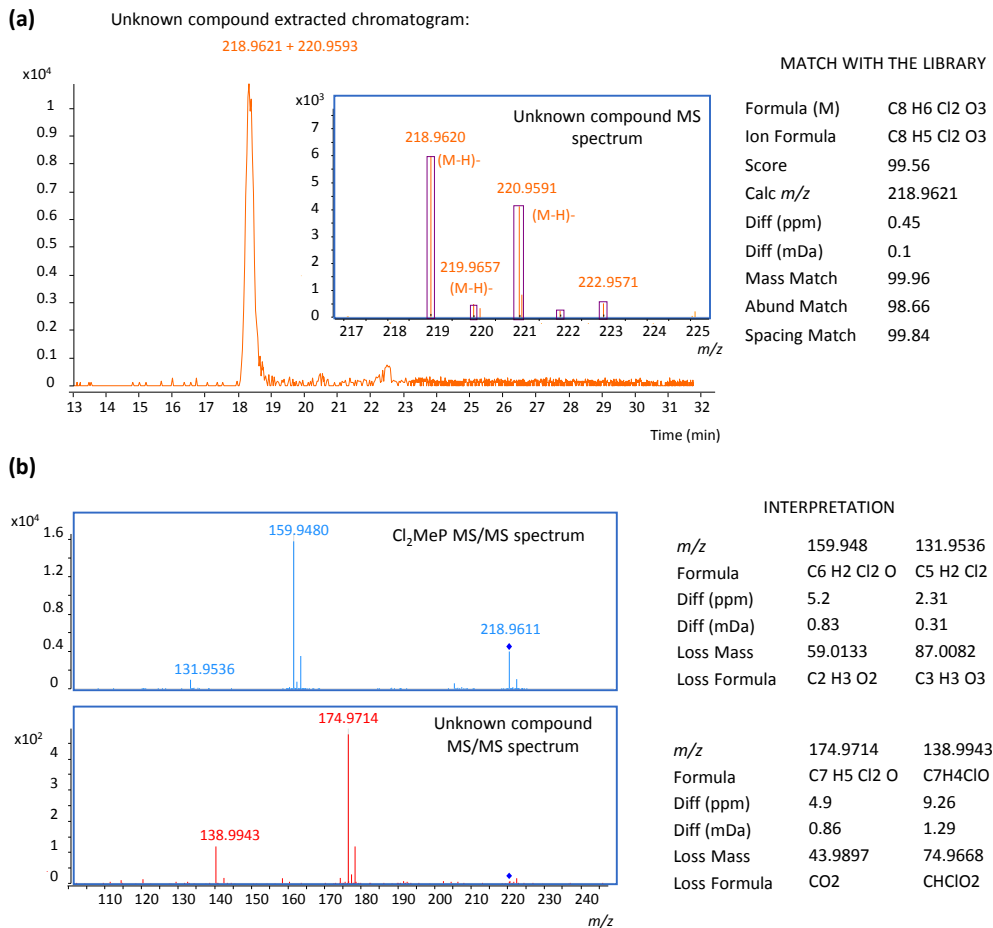
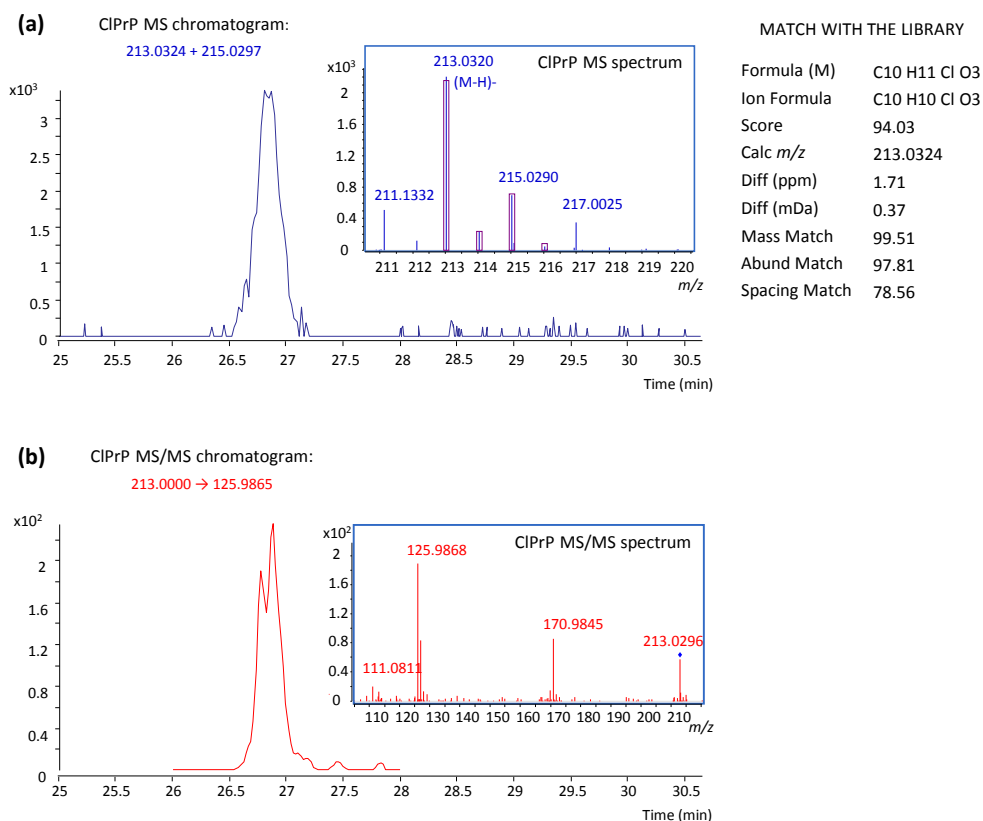


Figure 3. Identification of ClPrP from its accurate-mass precursor and product ion mass spectra: (a) extracted MS chromatogram and MS spectrum and empirical formula match; (b) MS/MS chromatogram and spectrum and empirical formula match.



3.3. Occurrence of target parabens and halogenated by-products in urban wastewaters

After the screening procedure, samples were also investigated in order to quantitate those compounds with commercially available standards.

Table 3 compiles the overall concentrations found in all the WWTPs influents and effluents, the frequency of detection and the removal efficiency. Although these data were obtained from grab samples, they arise from 11 pairs of samples from three different WWTPs, so they provide a reliable estimation of the occurrence of the target analytes. In fact, due to the instability of parabens in influent (raw) wastewater, 24-h composite samples could even lead to a severe underestimation of their concentrations (see section 3.4). For the calculation of average and median values, samples below the LOD were treated as if their concentrations were half of the LOD.

MeP was the most frequently detected compound, in 100% of the samples, and also the most abundant (average concentrations of 4200 ng L⁻¹ and 25 ng L⁻¹ in raw and treated wastewater, respectively). This fact is a reflection of its ubiquitous presence in cosmetic formulations. Following this trend, *n*-PrP and EtP, the next two most frequently used parabens, also occurred in all the influents (average values of 1400 and 880 ng L⁻¹, respectively) and in some of the analysed effluents (maximum concentration of 21 ng L⁻¹). *n*-BuP and *i*-BuP were also present in all the raw wastewater samples, although at lower concentrations (average values: 140 and 57 ng L⁻¹, respectively) but they were not detected in any of the treated wastewater samples. Finally, *i*-PrP was only found in some of the influents of plants B and C at marginal levels (lower than 6 ng L⁻¹) and BzP was not detected in any sample. These concentration ranges are in good agreement with the literature, showing the prevalence of MeP followed by either EtP or *n*-PrP and then *n*-BuP, whereas detection of BzP has only been reported at very low levels [6,8,10].

It is also interesting to notice that the *n*-BuP/*i*-BuP influent concentration ratio was quite constant (2.4 ± 0.3 , average \pm standard deviation) and very similar to the value found in two different grab samples in the only published data concerning the levels of both compounds in raw wastewater (1.9 ± 0.1 and 2.0 ± 0.1 , respectively) [5]. This may simply be due to the fact that they are produced as a technical mix of both isomers.

Regarding halogenated parabens, Br₂MeP was not detected in any sample, whereas ClMeP and Cl₂MeP were found in all the influents at similar levels (average values of 40 and 46 ng L⁻¹, respectively) and could still be found in some of the effluents at lower concentrations (up to 12 ng L⁻¹). To our knowledge, these are the first quantitative data on the occurrence of halogenated parabens in wastewater.

Taking into account the highest oestrogenic values published for these compounds [17], the sum of average paraben concentrations in influents and effluents would be equivalent to ca. 1.6 ng L⁻¹ and 0.01 ng L⁻¹ of 17 β -oestradiol, respectively. Therefore, they do not seem to represent a hazard in terms of oestrogenicity, as concentrations of natural and synthetic oestrogens are much higher, ca. 10-100 ng L⁻¹ of 17 β -oestradiol equivalents in raw and treated wastewater [27]. However, more investigations are required in the case of halogenated derivatives, which are more toxic than their precursors [18] and may appear at higher concentrations in other areas where higher chlorine doses are applied and/or tap water contains significant amounts of bromide.

Finally, an estimate of the removal efficiency was calculated from the average concentration values measured in influents and effluents. As it can be seen in **Table 3**, removal percentages were higher than 90% in all cases. Yet, it is noteworthy that they were higher than

99% for all the non-halogenated parabens that could be detected in some of the effluents. The removal of *i*-PrP was not calculated as its influent concentration was already marginal. Also, the average removals of the butylated parabens could only be assured to be higher than 96%, as they were not detected in effluents and the calculation of their removal is limited by their LODs.

Regarding the two chlorinated by-products, their removals were slightly lower than that of their precursor compound, MeP, ranging from 94% (Cl₂MeP) to 97% (ClMeP). Although this does not represent a statistically relevant difference, it may suggest a slightly higher persistence of the halogenated derivatives compared to their parent compounds, which was studied in detail within a laboratory biodegradation test (section 3.4). Removal values of non-halogenated parabens are in good agreement with previous findings for some of these chemicals in Europe and America [6,8-10], and also recent investigations in gray water showed a good removal (>90%) on laboratory-scale bioreactors [28].

Table 3. Concentrations (ng L⁻¹), percentage of samples above the LOD in both raw and treated wastewater and mean removal values considering the three WWTPs (n = 11 samples); BzP and Br₂MeP were not detected in any sample; n.d.: not detected (<LOD).

| | Sample | Average | Median | SD | Min | Max | % samples > LOD | Mean removal (%) |
|---------------------|----------|---------|--------|------|------|-------|-----------------|------------------|
| MeP | Influent | 4200 | 2500 | 3200 | 290 | 10000 | 100 | 99.4 |
| | Effluent | 25 | 19 | 14 | 6.1 | 50 | 100 | |
| EtP | Influent | 880 | 760 | 520 | 250 | 1600 | 100 | 99.5 |
| | Effluent | 4.0 | 2.5 | 3.3 | n.d. | 9.8 | 73 | |
| ClMeP | Influent | 40 | 39 | 18 | 12 | 61 | 100 | 96.9 |
| | Effluent | 1.2 | n.d. | 2.0 | n.d. | 6.9 | 18 | |
| Cl ₂ MeP | Influent | 46 | 40 | 29 | 8.0 | 90 | 100 | 94.3 |
| | Effluent | 2.6 | n.d. | 4.3 | n.d. | 12 | 45 | |
| <i>i</i> -PrP | Influent | 2.1 | n.d. | 1.8 | n.d. | 5.6 | 45 | - |
| | Effluent | n.d. | n.d. | n.d. | n.d. | n.d. | 0 | |
| <i>n</i> -PrP | Influent | 1400 | 1400 | 670 | 520 | 2800 | 100 | 99.7 |
| | Effluent | 4.8 | n.d. | 6.4 | n.d. | 21 | 36 | |
| <i>i</i> -BuP | Influent | 57 | 65 | 28 | 13 | 110 | 100 | >96.2 |
| | Effluent | n.d. | n.d. | n.d. | n.d. | n.d. | 0 | |
| <i>n</i> -BuP | Influent | 140 | 130 | 68 | 39 | 270 | 100 | >98.4 |
| | Effluent | n.d. | n.d. | n.d. | n.d. | n.d. | 0 | |

3.4. Biodegradability and wastewater stability evaluation

Taking into account the previous results (section 3.3), only the biodegradation of those parabens which occurred in raw wastewater at significant levels was investigated. Furthermore, it was also assayed for the three halogenated derivatives of MeP, for which there were commercially available standards: ClMeP, Cl₂MeP and Br₂MeP. Although the latter by-product was not detected in any of the analysed samples, it may occur in other locations with higher concentrations of bromide in natural waters [16]. The assay was carried out following the ISO 7827 standard [21] in terms of sludge inoculum preparation and tested concentrations, as detailed in section 2.4.

No concentration changes were observed for any compound neither in the control nor in the inhibited media in the course of the whole study, proving that neither adsorption nor degradation due to abiotic processes occurred and that any loss in the test solutions had to be attributed to biological routes (data not shown).

Biodegradation fitted profiles and experimental data points are displayed in **Figure 4**. **Table 4** compiles the fitting model parameters, as well as estimated half-lives ($t_{1/2}$) and time required to reach a degradation level of 99% for each compound, i.e. $C/C_0 = 0.01$ ($t_{0.01}$). The obtained R^2 values, higher than 0.97, demonstrate that the logistic model fitted the experimental data quite well. This parameter could not be calculated in the case of MeP and EtP, since their degradation was too fast to obtain enough data points.

As it can be seen, all considered non-halogenated parabens were readily biodegraded (**Figure 4a**), presenting half-lives lower than 3 days and reaching a 99% degradation level in less than 5 days (**Table 4**). However, their persistence was observed to be increased slightly with the length of the hydrocarbonated chain. Hence, propylated and butylated parabens required from 3.7 to 4.5 days to reach a 99% of degradation, while MeP and EtP required only 2.1 days. On the other hand, halogenated derivatives of MeP showed slower biodegradation kinetics than their parent compound (**Figure 4b**), with half-lives of 3.3 days for the monochlorinated species, 8.6 days for the dichlorinated and 9.7 days for dibrominated one (**Table 4**). This fact corroborates the findings on the WWTP removal, where efficiency decreases slightly as chlorination degree increases, in agreement with relative stabilities of mono and dihalogenated paraben by-products in presence of free chlorine [16].

No peaks of possible transformation products were detected by the LC-QTOF-MS system, indicating that the tested compounds were completely metabolised and incorporated into the sludge biomass or, at least, that the transformation products were not easily ionised by ESI.

Table 4. Parameters from the logistic kinetic fitting, half-lives ($t_{1/2}$) and time required for 99% degradation ($C/C_0 = 0.01$; $t_{0.01}$) from the activated sludge and real wastewater batch tests. In the last case, C/C_0 after 12 h is also presented (C/C_0 -12h).

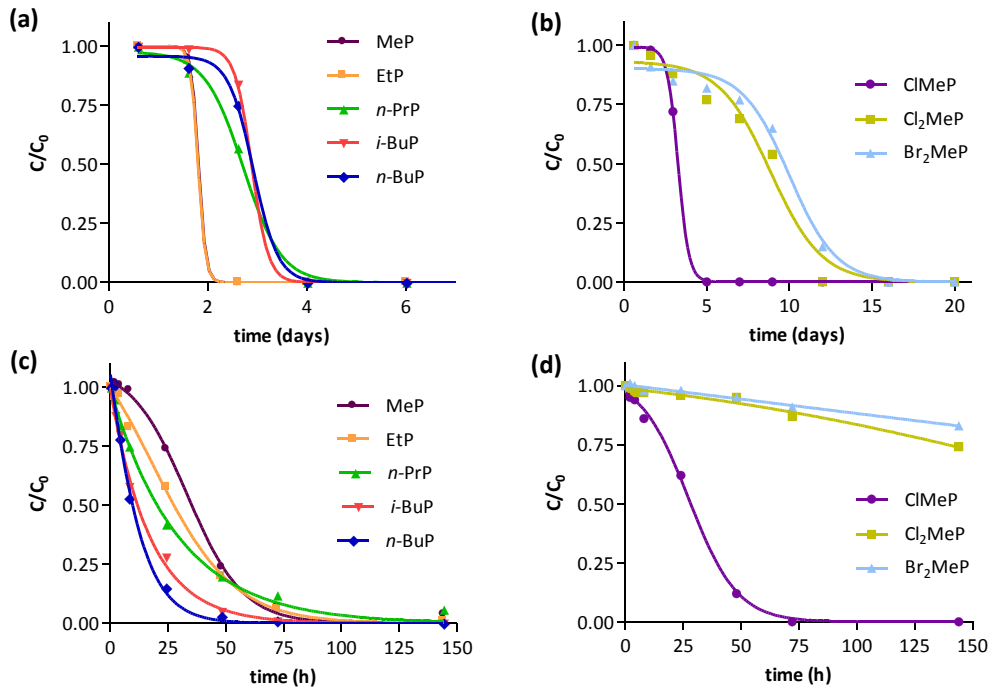
| Activated sludge batch test | | | | | | |
|-----------------------------|------|---------|------------------------|----------------|-------------------------|--------------------------|
| | K | c | r (day ⁻¹) | R ² | t _{1/2} (days) | t _{0.01} (days) |
| MeP | 1.00 | 1.4E-11 | 13.79 | * | 1.8 | 2.1 |
| EtP | 1.00 | 3.0E-11 | 13.43 | * | 1.8 | 2.1 |
| <i>n</i> -PrP | 0.98 | 8.5E-04 | 2.60 | 0.9978 | 2.7 | 4.5 |
| <i>i</i> -BuP | 1.00 | 3.4E-08 | 5.97 | 1.0000 | 2.9 | 3.7 |
| <i>n</i> -BuP | 0.96 | 8.1E-06 | 4.02 | 0.9976 | 2.9 | 4.0 |
| ClMeP | 0.99 | 5.0E-05 | 3.04 | 0.9999 | 3.3 | 4.8 |
| Cl ₂ MeP | 0.93 | 3.3E-03 | 0.65 | 0.9748 | 8.6 | 15.9 |
| Br ₂ MeP | 0.90 | 8.8E-04 | 0.70 | 0.9855 | 9.7 | 16.4 |

| Real wastewater test | | | | | | |
|----------------------|---------|---------|----------------------|----------------|----------------------|-----------------------|
| | K | c | r (h ⁻¹) | R ² | t _{1/2} (h) | C/C ₀ -12h |
| MeP | 1.09 | 6.6E-02 | 0.082 | 0.9974 | 35.2 | 0.93 |
| EtP | 1.35 | 3.5E-01 | 0.057 | 0.9976 | 27.5 | 0.79 |
| <i>n</i> -PrP | 6.3E+11 | 6.4E+11 | 0.034 | 0.9947 | 20.3 | 0.66 |
| <i>i</i> -BuP | 9.3E+11 | 9.4E+11 | 0.058 | 0.9972 | 11.8 | 0.49 |
| <i>n</i> -BuP | 3.01 | 1.9 | 0.103 | 0.9896 | 9.6 | 0.41 |
| ClMeP | 1.04 | 7.4E-02 | 0.095 | 0.9978 | 28.2 | 0.84 |
| Cl ₂ MeP | 1.12 | 1.3E-01 | 0.009 | 0.9753 | 237.1 | 0.97 |
| Br ₂ MeP | 1.95 | 9.4E-01 | 0.003 | 0.9855 | 449.5 | 0.99 |

* R² values not calculated owing to a very high degradation rate

A second series of assays was performed with raw wastewater spiked with parabens at the 50 ng mL⁻¹ level in order to test the stability of these compounds in a real influent for a week (**Figure 4c and d**). A poisoned control sample was run in parallel, without any degradation being observed. As presented in **Table 4**, again degradation kinetics fitted the logistic model quite well. Half-lives of native parabens ranged between 9.6 and 35 h, but, in this case, the species with longer hydrocarbonated chain underwent a faster degradation, in contrast with the activated sludge batch test. This may account for the different bacteriological and enzymatic composition of sewage as compared to activated sludge. On the other hand, stability of methyl paraben derivatives showed the same trend than the previous study, increasing with the number of halogen substituents, as $t_{1/2}$ of ClMeP was 28 h, whereas for Cl₂MeP and Br₂MeP it was longer than 1 week (**Table 4**).

Figure 4. Biodegradation profiles in the activated sludge batch test (a) and (b); and in the raw sewage batch test (c) and (d).



Another important datum shown in **Table 4** for this study is the C/C_0 -12 h. This value represents the concentration of compound remaining in the sample after 12 h, which is the average time that a real sample would stand if the commonly used 24 h composite sampling was performed. Hence, it can be appreciated that, after 12 h, more than 50% of the butylated parabens would have been degraded, while for the rest of native parabens degradation would range from 17 to 34%. Degradation of halogenated parabens during sampling would be also an issue for ClMeP (16% loss) but not for the dihalogenated species (less than 3% of degradation). In view of these results, 24-h composite samples would clearly lead to underestimated concentrations, particularly for the long-length chain parabens. Therefore, it was decided to perform grab sampling through this work, with a total of 11 samples being used to estimate average concentrations and removal percentages. Indeed, this would result in a random error, but it is expected to be much less important than the bias introduced by composite sampling for these personal care compounds. In fact, Ort et al. have recently evaluated the error derived from grab sampling versus different composite sampling approaches [29]. They observed that using just 4 grab samples to derive mean concentrations resulted in less than 30% error for the pharmaceuticals used in a higher extent as compared to the best sampling system, provided that analytes are stable (continuous flow-proportional composite sampling).

In our case, with 11 samples being taken, this error is expected to be reduced to less than 20%, owing also to the wide usage level of parabens; as compared to 24-h composite samples that would lead up to 60% biased results.

4. CONCLUSIONS

- It has been proved that the new generation of QTOF instruments provides adequate limits of detection and good linearity, comparable to triple quadrupole instruments for quantitative purposes. Moreover, their accurate mass determinations, in the full scan and MS/MS acquisition modes, permit the screening of transformation by-products without pure standards, which could otherwise not be detected with triple-quadrupole instruments. This is the case of ClPrP, which could not be quantified due to the lack of standard, but was detected in all the analysed influents.
- Parabens and the halogenated ClMeP and Cl₂MeP were quantified in all the raw wastewater samples, where MeP and *n*-PrP were the prevalent analytes. Their removal in the WWTPs was, however, high in all cases (>94%).
- Laboratory degradation tests, both with activated sludge and raw wastewater demonstrated that the dihalogenated derivatives of MeP have significantly higher half-lives than MeP itself.

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PUBLICACIÓN III.3

FULLY AUTOMATED DETERMINATION OF PARABENS, TRICLOSAN AND METHYL TRICLOSAN IN WASTEWATER BY MICROEXTRACTION BY PACKED SORBENTS AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Analytica Chimica Acta 684 (2011) 59

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Abstract

A fully automated method for the determination of triclosan (TCS), its derivative methyl triclosan (MTCS) and six parabens (esters of 4-hydroxybenzoic acid) including branched and linear isomers of propyl (*i*-PrP and *n*-PrP) and butyl paraben (*i*-BuP and *n*-BuP) in sewage water samples is presented. The procedure includes analytes enrichment by microextraction by packed sorbent (MEPS) coupled at-line to large volume injection-gas chromatography-mass spectrometry (LVI-GC-MS). Under optimised conditions, compounds were extracted from 2 mL samples, adjusted at pH 3, using a C₁₈ MEPS-sorbent. Adsorbed analytes were eluted directly into the Programmed Temperature Vaporizer (PTV) injector of the chromatograph with 2 × 25 µL of ethyl acetate. They were quantified using standard solutions in ultrapure water submitted to the same sample enrichment process as real sewage water samples. After signal normalisation using isotopic labelled species as internal surrogates, no differences were noticed between the extraction efficiency for sewage and ultrapure water; moreover, the proposed method reported lineal calibration curves from 0.1 to 10 ng mL⁻¹, relative standard deviations (%RSD) between 2.0 and 7.1% and limits of detection (LODs) varying from 0.001 to 0.015 ng mL⁻¹ in ultrapure water and from 0.02 to 0.59 ng mL⁻¹ in the most complex sample (raw wastewater).

Keywords: biocides; extraction techniques; microextraction; automation; gas chromatography-mass spectrometry (GC-MS); water analysis.

1. INTRODUCTION

Triclosan (5-chloro-2-(2,4-dichloro-phenoxy)-phenol; TCS) and parabens (alkyl and aryl esters of 4-hydroxybenzoic acid) are extensively employed as bactericides and preservatives in a large variety of personal care products (PCPs) such as shampoos, creams, deodorants and toothpastes. TCS is also included in sportive clothes, footwear, carpets, plastic toys and kitchenware and parabens in pharmaceuticals, processed food and beverages [1,2].

This extensive usage has awakened the concern about their potential long-term effects on human health and wildlife and, in fact, they are known to be weak endocrine disruptors [3,4]. Recent studies have suggested a possible relationship between breast cancer and prolonged dermal expositions to paraben-containing deodorants [5] and, as a result, a new generation of paraben-free PCPs has emerged in the market lately. Regarding TCS, laboratory studies have shown their transformation, under certain conditions, into more toxic and persistent compounds such as chlorinated phenols, polychlorinated biphenyl ethers and polychlorinated dibenzodioxins [6-9]. During wastewater treatment processes it can also turn into methyl triclosan (MTCS), a more lipophilic and bio-accumulative species that has not any commercial application but has already been detected in different environmental compartments [10,11].

As in the case of many other PCP chemicals, parabens and TCS are continuously released into the environment through urban wastewater and, although most of them (particularly parabens) are completely removed during conventional sewage treatments [12-14], they have already been detected in surface water at the ng L^{-1} level [15-18].

Analytical methods for their determination in water samples are based on a pre-concentration step followed by the subsequent separation and detection by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Common sample preparation strategies such as solid-phase extraction (SPE) or liquid-liquid extraction (LLE) require the concentration of very large sample volumes, which is time-, solvent- and labour-consuming and shows a limited automation grade particularly in combination with GC. In an attempt to overcome these problems, microextraction techniques have come up like a promising approach and, in fact, some of them have been applied to the determination of parabens and/or TCS in water: solid phase microextraction (SPME) [12,13,19], stir bar sorptive extraction (SBSE) [20,21], hollow-fibre liquid phase microextraction (HF-LPME) [22], single-drop microextraction (SDME) [23], dispersive liquid-liquid microextraction (DLLME) [24], ultrasound-assisted emulsification-microextraction (USAEME) [25] and membrane-assisted liquid-liquid extraction (MALLE) [26]. Although most of the above approaches lead to sensitive methods, with limits of detection in

the low ng L⁻¹ level, in some cases they are difficult to automate (e.g. DLLME, USAEME and SDME), show slow extraction kinetics (except DLLME and USAEME) and/or provide non-quantitative extraction yields.

Microextraction by packed sorbents (MEPS), first described by Abdel-Rehim [27], presents some interesting features that allow to alleviate some of the above reported drawbacks. MEPS follows the SPE principles but applied in a miniaturised scale, where a small amount of sorbent is packed between the body and the metallic needle of a chromatographic syringe. So, it does not require hardware modifications and it is supposed to show better enrichment factors than the aforementioned strategies. It also allows the direct injection of the complete elution volume into the liquid or gas chromatograph, becoming a promising at-line sample preparation approach applied successfully to the determination of different organic compounds in plasma [28-32], urine [33-37] and even water [38,39] and wine [40]. Most of these studies combine the MEPS with LC-MS/MS analysis, although a few applications with GC-MS have also been published [27,34,38,41-43]. In this line, the goal of this work was the development of an at-line MEPS-GC-MS method for the simultaneous determination of 6 parabens, TCS and MTCS in sewage water. Experimental parameters were optimised to achieve the maximum efficiency during analytes extraction and elution. After validation, the procedure was applied to several wastewater samples collected in the northeast of Germany. Although derivatisation is normally performed in order to improve the GC analytical determination and/or extractability of the analytes (except MTCS) [11-13,26], it was avoided in this work in order to simplify the analytical method.

2. EXPERIMENTAL

2.1. Chemicals and samples

Methyl (MeP), ethyl (EtP), *n*-propyl (*n*-PrP) and *n*-butyl (*n*-BuP) esters of 4-hydroxybenzoic acid, as well as triclosan and methyl triclosan, were purchased from Aldrich (Milwaukee, WI, USA). The branched isomers of propyl paraben (*i*-PrP) and butyl paraben (*i*-BuP) were obtained from TCI Europe (Zwijndrecht, Belgium). As internal standards (ISs), methyl 4-hydroxybenzoate-2,3,5,6-d₄ (MeP-d₄) and *n*-propyl 4-hydroxybenzoate-2,3,5,6-d₄ (*n*-PrP-d₄) were from CDN Isotopes (Quebec, Canada), while a solution of ¹³C₁₂-triclosan (¹³C₁₂-TCS; 100 µg mL⁻¹ in nonane, 99%) was from Cambridge Isotope Laboratories (Andover, MA, USA).

Individual stock solutions of each analyte (1000 µg mL⁻¹) were dissolved in methanol. Mixtures of all of them or their deuterated analogues were prepared in methanol or acetone, respectively, when used to fortify water samples, and in ethyl acetate, when considered to

evaluate the performance of the GC-MS system. The above solutions were stored in the dark at -20 °C.

HPLC grade methanol, ethyl acetate, acetone, *n*-hexane and cyclohexane were purchased from Merck (Darmstadt, Germany). Solutions of hydrochloric acid (25%, w/w) and ammonia (25%, w/w) were also from this company.

Influent and effluent wastewater samples were collected in July 2010 at five different sewage treatment plants (STPs) located in Leipzig (Germany) and receiving the discharges from about 10000 inhabitants. Effluent samples were characterized by total organic carbon (TOC) contents between 35.3 and 56.4 mg L⁻¹, and the influent by a TOC of 101.9 mg L⁻¹, values determined using a “HighTOC II” analyser (Elementar Analysensysteme, Hanau, Germany).

2.2. MEPS conditions

Microextraction was performed using the commercial version of MEPS (SGE, Melbourne, Australia) consisting of a 100 µL gas-tight syringe body and a small barrel incorporated into the conical shaped needle (“barrel insert and needle”, BIN). The needle barrel accommodates 1 mg of a material commonly used in reversed-phase SPE [44]. Two different, commercially-available, silica gel based sorbents (mean particle size 45 µm, pore size 60 Å) were considered in this study: one modified with C₈ and the other one with C₁₈. The MEPS syringe was installed in a GC-MS instrument furnished with a large volume injector type KAS 4 (Gerstel, Mühlheim an der Ruhr, Germany) and the samples were processed by a Multi Purpose Sampler MPS 2 controlled by the software MAESTRO (Gerstel).

Under optimised conditions, the sorbent was sequentially conditioned with 50 µL of ethyl acetate, 50 µL of methanol and 50 µL of ultrapure water at pH 3. The sample (also adjusted at pH 3) was then extracted by aspirating and discarding 20 cycles of 100 µL at a withdrawing flow rate of 5 µL s⁻¹. In order to remove possible inorganic salts, the sorbent was washed twice with 50 µL of ultrapure water (without pH adjustment) and dried partially by pumping 10 × 60 µL of air at 50 µL s⁻¹. Elution was carried out with two portions of 25 µL of ethyl acetate, pumped up through the sorbent and down directly into the large volume injector of the gas chromatograph, both at 10 µL s⁻¹. After each extraction process, the sorbent was washed first with 10 cycles of 100 µL of ethyl acetate and then with another 10 cycles of 100 µL of fresh methanol, avoiding by this way the carryover problems usually associated to MEPS extraction [38,41]. Globally, the above sample preparation process (sampling, enrichment and elution) required around 15 min and it could be carried out simultaneously

with the chromatographic separation of the previous injection, leading to a very fast automated procedure.

2.3. Gas chromatography-mass spectrometry

Analyses were performed using an Agilent 6890 series gas chromatograph (Agilent Technologies, San José, CA, U.S.A.) equipped with a Programmed Temperature Vaporizer (PTV) injector (KAS 4, Gerstel) and connected to an Agilent 5973 Mass Selective Detector (MSD). Injections ($2 \times 25 \mu\text{L}$) were made in the solvent vent mode using a $100 \mu\text{L}$ syringe and an injection speed of $10 \mu\text{L s}^{-1}$. The injection port consisted of a septumless head and a temperature programmable injector equipped with an empty baffled deactivated glass liner. The inlet temperature was set at $45 \text{ }^\circ\text{C}$ (supported by carbon dioxide) for 0.6 min and then it was increased at 720 K min^{-1} to $300 \text{ }^\circ\text{C}$ (held for 5 min). Initially (0.6 min), in order to purge out most of the solvent, the flow rate through the split vent was set at 100 mL min^{-1} ; after this time, the split valve was closed (switched to the splitless mode) for 2.5 min and opened again with a purge flow of 100 mL min^{-1} .

Table 1. Physicochemical data and target ions for the SIM mode determination (m/z) of the studied compounds.

| | Empirical formula | pK_a^a | $\log K_{ow}^b$ | Nominal MW | Target ions for SIM mode (m/z) ^c | IS |
|------------------------------------|---|-----------------|-----------------|------------|---|------------------------------------|
| MeP-d ₄ | C ₈ H ₄ D ₄ O ₃ | – | – | 156 | <u>125</u> , 156, 97 | – |
| MeP | C ₈ H ₈ O ₃ | 8.30 ± 0.13 | 1.96 | 152 | <u>121</u> , 152, 93 | MeP-d ₄ |
| EtP | C ₉ H ₁₀ O ₃ | 8.30 ± 0.13 | 2.47 | 166 | <u>121</u> , 138, 166 | <i>n</i> -PrP-d ₄ |
| <i>i</i> -PrP | C ₁₀ H ₁₂ O ₃ | 8.40 ± 0.15 | 2.91 | 180 | <u>121</u> , 138, 180 | <i>n</i> -PrP-d ₄ |
| <i>n</i> -PrP-d ₄ | C ₁₀ H ₈ D ₄ O ₃ | – | – | 184 | <u>125</u> , 142, 184 | – |
| <i>n</i> -PrP | C ₁₀ H ₁₂ O ₃ | 8.23 ± 0.15 | 3.04 | 180 | <u>121</u> , 138, 180 | <i>n</i> -PrP-d ₄ |
| <i>i</i> -BuP | C ₁₁ H ₁₄ O ₃ | 8.17 ± 0.15 | 3.40 | 194 | <u>121</u> , 138, 93 | <i>n</i> -PrP-d ₄ |
| <i>n</i> -BuP | C ₁₁ H ₁₄ O ₃ | 8.22 ± 0.15 | 3.57 | 194 | <u>121</u> , 138, 93 | <i>n</i> -PrP-d ₄ |
| ¹³ C ₁₂ -TCS | ¹³ C ₁₂ H ₇ Cl ₃ O ₂ | – | – | 300 | <u>300</u> , 302, 230 | – |
| TCS | C ₁₂ H ₇ Cl ₃ O ₂ | 7.80 ± 0.35 | 4.76 | 288 | <u>288</u> , 290, 218 | ¹³ C ₁₂ -TCS |
| MTCS | C ₁₃ H ₉ Cl ₃ O ₂ | – | 5.27^a | 302 | <u>302</u> , 304, 252 | ¹³ C ₁₂ -TCS |

^a Software estimated values obtained from SciFinder Scholar 2007 database.

^b Experimental values provided by PhysProp database (Syracuse Research Corporation).

^c Most intense ions, with the quantifier being underlined.

Separation was carried out in an HP-5MS type capillary column (30 m × 0.25 mm i.d., d_f 0.25 μm) supplied by Agilent. Helium was used as carrier gas at a constant flow of 1 mL min^{-1} . The GC oven was programmed from an initial hold time of 3 min at 50 °C; then, it was increased to 100 °C at 30 K min^{-1} , to 240 °C at 5 K min^{-1} and, finally, to 280 °C at 30 K min^{-1} (held for 1 min). The total run time was 35 min and the solvent delay 6 min. The transfer line was set at 280 °C, the quadrupole at 150 °C and the source at 230 °C. The mass spectrometer was operated in the electron impact ionisation mode (70 eV), in full scan for identification (50-500 m/z , 1.08 cycles s^{-1}) and in selected ion monitoring (SIM) for quantification (dwell time of 100 ms per ion). The target m/z ratios of the analytes and the isotopically labelled internal standards are listed in **Table 1**.

3. RESULTS AND DISCUSSION

3.1. Extraction regime and carryover

In MEPS, there are two different forms of sampling: in a similar way to a conventional SPE, the sample may be pumped up only once and then discarded into waste (carrying out this operation with one or several aliquots of fresh sample) or it may be pumped up and down several times from the same vial in the multiple draw-eject cycles procedure, analogously to in-tube SPME [38]. This latter mode usually requires a lot of draw-eject cycles to reach high recoveries, since the concentration of the analytes in the sample decreases after each pumping cycle. This induces a high mechanical stress of the syringe plunger and, as a result, a short life time of the MEPS syringe [38]. On the other hand, a lower number of cycles is needed in the SPE mode, and, as the sample volume required is larger but still quite small (≤ 2 mL), it was the extraction regime selected in this study. During the first optimisation studies, seven 100 μL volume aliquots of fresh sample were aspirated and discarded. Later, the influence of the sample volume on the extraction efficiency was studied in detail, taking into account that this parameter is limited by the depth of the syringe into the vial and that the autosampler incorporates positions for 2 mL standard vials (tray "VT 98 cooler") and for 10 mL vessels (tray "VT 32-10"). With the first ones, the maximum volume that can be extracted is 700 μL ; with the second ones, it is 2.0 mL.

Compared to a conventional SPE process with disposable cartridges or membranes, reusing of the MEPS sorbent requires a detailed evaluation of potential carryover phenomena [38,41] and, if necessary, the inclusion of a thorough clean-up step after each extraction process. For this reason, 10 cycles of 100 μL of ethyl acetate followed by 10 cycles of 100 μL of methanol were incorporated into the method as a cleaning procedure. Analyte carryover was

checked afterwards and it was lower than 1.0% when using a C₁₈ MEPS sorbent and lower than 1.3% when using a C₈ one, for all compounds, even at the highest sample concentration of 10 ng mL⁻¹ (data not shown).

3.2. Elution solvent

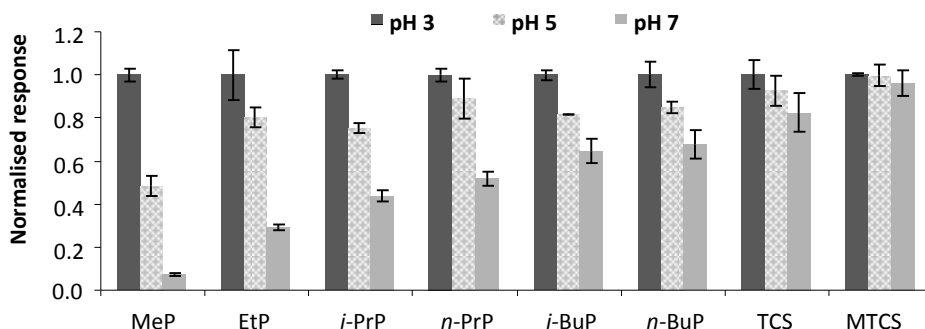
Since the amount of sorbent used in MEPS is very small, desorption may be performed with a relatively small volume of solvent that can be totally transferred into the LVI-GC instrument. Obviously, a GC-compatible, non-polar and volatile solvent is required. Moreover, as retention in silica gel modified with C₈ or C₁₈ is based on hydrophobic interactions, apolar solvents would be enough to disrupt the forces between the analytes and the sorbent. Four different ones (*n*-hexane, cyclohexane, acetone and ethyl acetate) were tried in a preliminary study. Cyclohexane was not able to elute any compound quantitatively, whereas *n*-hexane only eluted efficiently MTCS and TCS. On the other hand, acetone and ethyl acetate showed better and similar responses, hence their efficiency was evaluated in detail: 700 µL of ultrapure water spiked with 6.25 ng mL⁻¹ of every analyte were extracted with a C₁₈ MEPS sorbent and eluted with 3 × 25 µL of each solvent. Fractions were injected separately, the corresponding peak areas were added and the resulting values compared to the responses obtained after a direct injection (25 µL) of a standard solution of 175 ng mL⁻¹ of each analyte. In the case of acetone as elution solvent, recoveries varied from 60 to 112%, while for ethyl acetate they ranged between 73 and 101% (data not shown). On the basis of these data, ethyl acetate was finally chosen as elution solvent for overall analyses.

3.3. Sample pH

The effect of the sample pH on the retention of the analytes was investigated by extracting 7 × 100 µL of spiked ultrapure water (6.25 ng mL⁻¹) in a C₁₈ BIN-sorbent and eluting the compounds with 2 × 25 µL of ethyl acetate. Since their pK_a values are around 8, weak acidic conditions might be the best for their reverse-phase extraction, despite causing the retention of humic acids when working with real samples. In this way, three different pH values were assayed: 3, 5 and 7; highest normalised responses were achieved for pH 3 (**Figure 1**), especially for the most polar analytes (MeP, EtP and *i*-PrP), whereas TCS and particularly MTCS were scarcely affected by the pH of the water sample. The reason of such an increase at low pHs for the above parabens is not clearly understood considering only their pK_a values. However, this effect may be partially attributed to their complete protonation at pH values

lower than 6 and the protonation of the free silanol groups on the C₁₈ BIN, leading to an H-bonding retention of the aforementioned chemicals.

Figure 1. Influence of the sample pH on the extraction efficiency using a C₁₈ BIN-sorbent (n = 3).

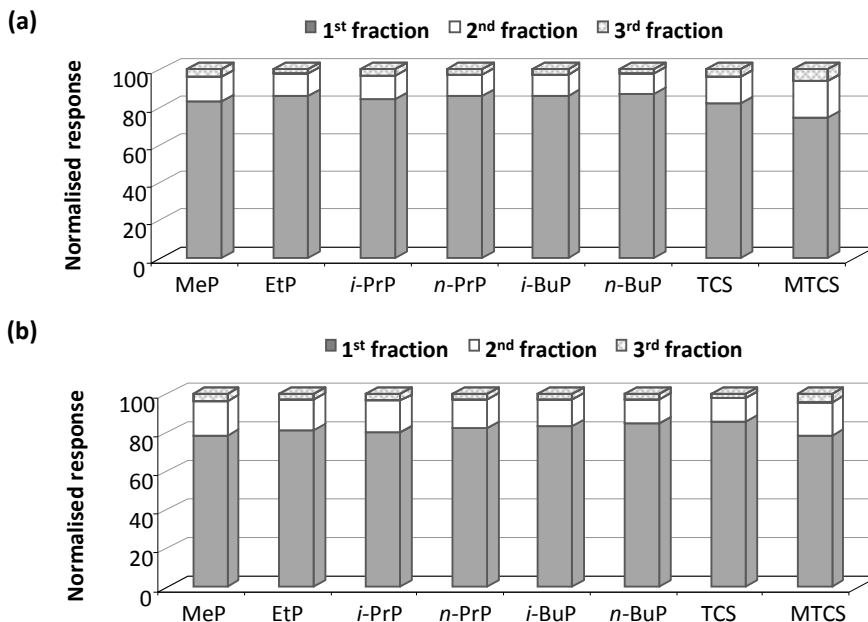


3.4. Comparison between C₈ and C₁₈-MEPS in terms of elution solvent volume, sample volume and extraction efficiency

Two different sorbents commonly used for solid-phase extraction of organic compounds were tested in this study: C₈ and C₁₈ modified silica gel. They were compared in terms of (1) the minimum volume of ethyl acetate required to get a quantitative elution, (2) the maximum volume of sample that could be concentrated without losses and, finally and in view of the previous results, in terms of (3) the overall extraction efficiency.

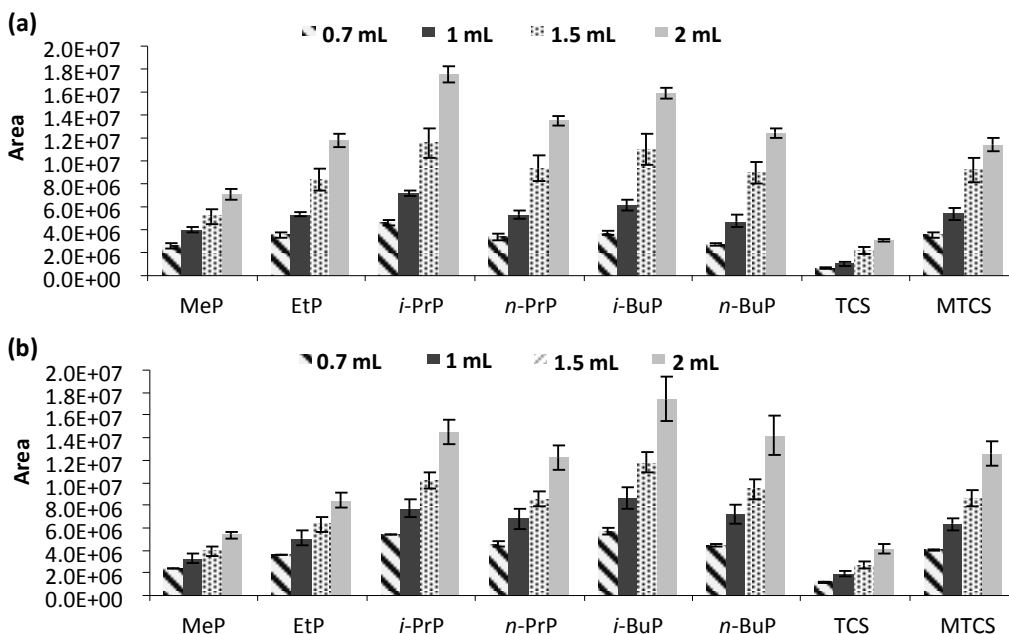
For the first objective, 7 × 100 µL of spiked ultrapure water (3.75 ng mL⁻¹, pH 3) were extracted in the SPE-like regime and analytes were eluted with ethyl acetate in 3 individual portions of 25 µL. Analysing each portion separately, it was verified that 50 µL of ethyl acetate was enough to achieve elution efficiencies higher than 93% from the C₁₈ sorbent and higher than 95% from the C₈ one (**Figure 2**); in both cases, the most retained analyte was the most lipophilic one, MTCS (log K_{ow} 5.27). Taking into account these results, the elution volume was fixed in 50 µL; the best results were obtained when the elution volume of 50 µL was divided in two fractions of 25 µL injected consecutively in the PTV.

Figure 2. Volume of solvent (ethyl acetate) needed for a quantitative elution of the analytes: (a) with a C₁₈ BIN-sorbent (n = 3); (b) with a C₈ BIN-sorbent (n = 3); fractions of 25 μ L.



For sample volume comparison, another autosampler (tray “VT32-10”), which allows the use of 10 mL sample vessels and, consequently, the extraction of 2.0 mL of sample, was tried. Processing increasing volumes of spiked ultrapure water (2 ng mL⁻¹), provided a proportional increase in the response of overall analytes (**Figure 3**); thus, the extracted amount of all analytes increased with the volume of sample. Assuming that the use of larger sample quantities could improve the LODs, 2 mL was selected as the sample volume to extract and both sorbents were finally contrasted in relation to their extraction efficiency. Absolute recoveries were determined by comparison of the responses (peak areas) obtained by MEPS of 2 mL of ultrapure water, spiked at 1 ng mL⁻¹, vs. the responses obtained for a standard in ethyl acetate with an equivalent concentration (40 ng mL⁻¹) injected directly (2 × 25 μ L) in the GC-MS system using a normal injection syringe. With the C₁₈ BIN, extraction efficiency values ranged from 45 to 102% (Uncorrected Extraction Efficiency, **Table 2**) whereas with the C₈ BIN, they varied between 30 and 115% (data not shown); in both cases, the lowest recoveries corresponded to the analytes with the lower log K_{ow} values (MeP and EtP). Having proved that the behaviour of both materials is quite similar, although slightly better recoveries are obtained for the most hydrophilic parabens with C₁₈, further experiments and application to real samples were carried out with this sorbent.

Figure 3. Analytes responses obtained by extracting increasing sample volumes: (a) with a C₁₈ BIN-sorbent (n = 3); (b) with a C₈ BIN-sorbent (n = 3).



3.5. Method performance

As it was shown in the previous section, the most polar target analytes were not extracted quantitatively even with the C₁₈-BIN material, since their extraction efficiency values stayed below 80% when compared to a standard directly prepared in ethyl acetate. The use of internal standards could not correct the extraction efficiency, which still remained slightly under 80% for EtP and MTCS (Corrected Extraction Efficiency, **Table 2**). Taking into account this point and the fact that calibration with standards requires the presence of an operator to replace the MEPS syringe by a normal one (with the same capacity but without the packed bed of sorbent), calibration by extracting spiked ultrapure water samples was proposed as quantification technique, as it is commonly done in microextraction techniques, such as SPME, or in on-line SPE methodologies. Moreover, it did not involve an extra time analysis, as extraction takes place while the preceding chromatogram is running. Calibration curves calculated were linear between 0.1 and 10 ng mL⁻¹, with determination coefficients (R²) ranging from 0.9970 to 0.9999. Repeatability studies were carried out by 8 consecutive extractions of ultrapure water spiked with the analytes at 0.25 ng mL⁻¹ level and with the ISs at 0.5 ng mL⁻¹; %RSD varied between 2.0 and 7.1% (**Table 2**). In the case of wastewater, RSD values were also excellent, in the 0.1-4.1% range (**Table 2**).

In order to evaluate the influence of the matrix on the performance of the whole method, raw and treated wastewater were fortified with 1 ng mL^{-1} of all the target analytes and extracted under optimised conditions. Non-spiked samples were also analysed, peak areas subtracted from those corresponding to the spiked ones and obtained results divided by the responses got with spiked pure water (1 ng mL^{-1}) and multiplied by 100. Relative recoveries calculated by this way (Uncorrected Relative Recovery, **Table 2**) showed a noticeable signal enhancement, probably due to the influence of the matrix components during the injection/evaporation on the PTV injector. Likely, these compounds block potential active points in the liner and/or the head of the GC column, improving the mass transfer efficiency of target compounds from the injector port to the capillary column. The use of internal standards (MeP-d₄ for MeP, *n*-PrP-d₄ for the remaining parabens and ¹³C₁₂-TCS for TCS and MTCS) effectively compensated these effects, keeping the trueness of the method in the 86-120% range for both wastewater matrices as well as improving the precision (Corrected Relative Recovery, **Table 2**).

Finally, estimated LODs of the whole method (calculated for a signal to noise ratio of 3) ranged from 0.001 to 0.015 ng mL^{-1} in ultrapure water, from 0.01 to 0.24 ng mL^{-1} in treated wastewater and from 0.02 to 0.59 ng mL^{-1} in raw wastewater (**Table 2**). Limits of quantification (LOQs) are not presented in **Table 2**, but can be easily calculated as 3.3 times the LODs. These LODs are higher than those published in the literature by other extraction methods (**Table 3**), particularly in influent wastewater, but still suffice the determination of the analytes in this type of samples. Moreover, as compared to other published methods (**Table 3**), it requires a very small volume of sample, does not require derivatisation and affords very good precision in a simple and fully automated method.

3.6. Application to real samples

The developed method was applied to determine the levels of the selected bactericides in treated wastewater sampled from 5 different STPs located in Leipzig (Germany) and in the common raw wastewater feeding all of them. Concerning parabens, three of them were quantified in the raw water sample at ng mL^{-1} level: MeP ($5.81 \pm 0.34 \text{ ng mL}^{-1}$), EtP ($1.13 \pm 0.02 \text{ ng mL}^{-1}$) and *n*-PrP ($2.06 \pm 0.24 \text{ ng mL}^{-1}$), in agreement with their frequent use in cosmetic formulations and with the data described in the literature [18,26]. *n*-BuP was also determined at $0.29 \pm 0.01 \text{ ng mL}^{-1}$, whereas the levels of remaining parabens were below their LODs.

Table 2. Performance parameters of the automated MEPS-LVI-GC-MS method for 2 mL extracted sample.

| R ² | Repeatability %RSD | Extraction efficiency in ultrapure water (%) ^b | | Uncorrected Relative Recovery (%) ^c | | Corrected Relative Recovery (%) ^d | | LOD (ng mL ⁻¹) | | |
|----------------|-----------------------|--|-------------|---|-------------------|---|-------------------|----------------------------|-----------------------|-------------------|
| | | Uncorrected | Corrected | Treated wastewater | Raw wastewater | Treated wastewater | Raw wastewater | Ultrapure water | Treated wastewater | Raw wastewater |
| MeP | 0.9983 | 44.8 (17.2) | 95.3 (4.1) | 239.1 (17.3) | 261.6 (24.4) | 118.1 (3.1) | 110.6 (4.1) | 0.012 | 0.22 | 0.59 |
| EtP | 0.9985 | 74.9 (18.7) | 78.7 (4.1) | 176.3 (13.9) | 179.6 (18.1) | 86.8 (1.0) | 87.3 (2.0) | 0.006 | 0.24 | 0.35 |
| <i>i</i> -PrP | 0.9983 | 90.9 (15.4) | 94.4 (6.0) | 195.7 (10.4) | 211.8 (17.1) | 105.7 (0.4) | 104.1 (1.6) | 0.004 | 0.07 | 0.13 |
| <i>n</i> -PrP | 0.9999 | 90.0 (22.0) | 92.1 (1.9) | 214.7 (16.6) | 251.5 (30.5) | 110.6 (1.9) | 120.5 (3.3) | 0.002 | 0.11 | 0.14 |
| <i>i</i> -BuP | 0.9986 | 102.0 (22.3) | 103.9 (2.2) | 206.5 (11.3) | 189.5 (22.5) | 108.1 (0.6) | 90.4 (2.3) | 0.006 | 0.09 | 0.13 |
| <i>n</i> -BuP | 0.9978 | 97.7 (24.2) | 97.5 (2.7) | 215.2 (15.3) | 218.6 (23.4) | 113.0 (1.2) | 104.4 (1.1) | 0.015 | 0.13 | 0.19 |
| TCS | 0.9995 | 94.1 (30.3) | 85.4 (0.3) | 217.0 (15.1) | 177.9 (29.9) | 111.7 (0.5) | 111.9 (0.4) | 0.003 | 0.01 | 0.02 |
| MTCS | 0.9970 | 87.8 (17.0) | 79.9 (10.5) | 168.1 (9.1) | 126.7 (11.7) | 97.9 (0.1) | 85.7 (0.2) | 0.001 | 0.01 | 0.02 |

^a R² of calibration curve 0.1-10 ng mL⁻¹.

^b Extraction efficiencies in spiked pure water (1 ng mL⁻¹), referred to the direct injection of a standard in ethyl acetate (40 ng mL⁻¹). Expressed as "Mean (%RSD)" for n = 4 replicates.

^c Relative recoveries, referred to a extraction of spiked pure water (1 ng mL⁻¹). Expressed as "Mean (%RSD)" for n = 4 replicates.

^d Relative recoveries, referred to a extraction of spiked pure water (1 ng mL⁻¹) and corrected by the use of internal standard. Expressed as "Mean (%RSD)" for n = 4 replicates.

Table 3. Comparison of the developed method to other published extraction techniques based on GC-MS(/MS) detection.

| | SPE | SPME | DLLME/USAEME | MALLE | MEPS |
|----------------------|-----------------------|---------------------------|-------------------------|----------------------------|--------------------------|
| Sample volume | 1000 mL | 10-22 mL | 10 mL | 18 mL | 2 mL |
| Solvent volume | 20 mL | Solvent-free | 0.1-1 mL | 0.4 mL | 2.2 mL |
| LODs | 10 ng L ⁻¹ | 0.3-17 ng L ⁻¹ | 1-16 ng L ⁻¹ | 0.3-1.4 ng L ⁻¹ | 1-590 ng L ⁻¹ |
| Repeatability (%RSD) | ≤ 6% | ≤ 17% | ≤ 13% | ≤ 17% | ≤ 7% |
| Derivatisation | Yes | Yes | Yes | Yes | No |
| Fully automatable | No | Yes | No | No | Yes |
| Reference | [14] | [12,13,19] | [24,25] | [26] | This work |

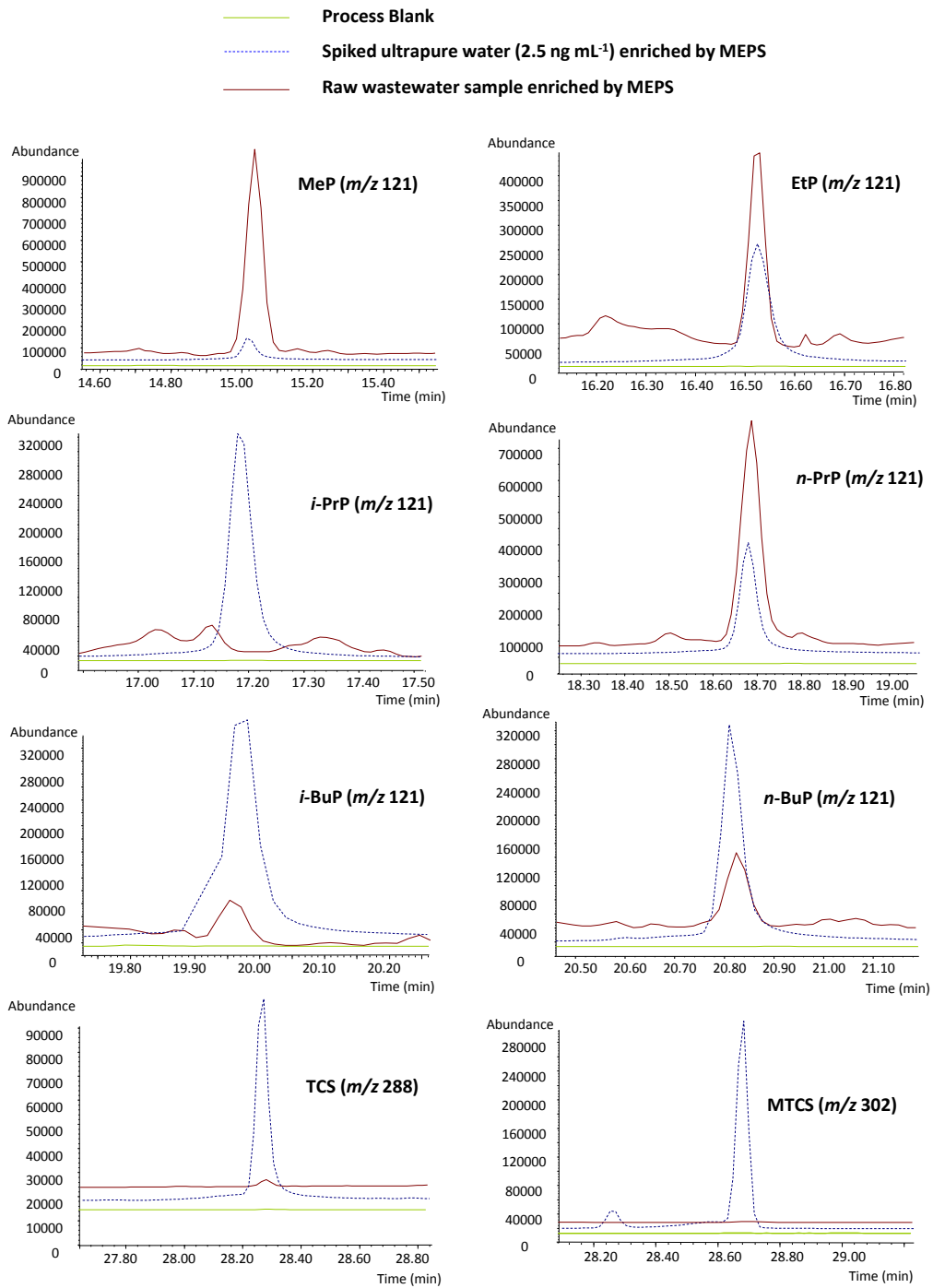
TCS was under its LOQ and MTCS was not detected. Finally, no bactericides were detected in any of the treated water samples, corroborating the high elimination percentages reported during wastewater treatments processes [13,15,45]. As an example, **Figure 4** shows the SIM chromatogram of the influent sample superimposed to a spiked ultrapure water sample (2.5 ng mL⁻¹) and to a blank of the process.

4. CONCLUSIONS

For the determination of six parabens, triclosan and methyl triclosan in water samples the combination of MEPS with GC-MS provides an attractive alternative to laborious standard SPE enrichment. Good precision, trueness and linearity, and acceptable detection limits allow the analysis of sewage water samples. The main advantage of the proposed approach is that sample concentration, analytes enrichment and further introduction of the extract in the GC-MS system are fully automated and integrated in the same device.

The developed methodology requires a very low sample volume and it also shows a low consumption of organic solvents. Moreover, after IS correction, no differences were noticed among the relative yield of the MEPS process for ultrapure and sewage water samples. Further efforts should be focussed on the commercialisation of MEPS syringes packed with other polymeric materials, alternative to silica based sorbents, in order to improve: (1) the absolute extraction efficiency for the most polar of the tested compounds (MeP) and (2) the selectivity of the enrichment step. The experience with SPE sorbents in conventional formats for PCPs determination points to hydrophilic-lipophilic balanced polymers and mixed-mode sorbents (e.g. combination of reversed-phase and ionic exchanger sorbents) as potential candidates to improve the performance of the MEPS technique for medium polarity compounds, e.g. MeP, in complex matrices such as sewage water.

Figure 4. SIM chromatogram of the influent sample superimposed to a spiked ultrapure water sample (2.5 ng mL⁻¹) and to a blank of the process.



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PUBLICACIÓN III.4

MATRIX SOLID-PHASE DISPERSION FOLLOWED BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY FOR THE DETERMINATION OF TRICLOSAN AND METHYL TRICLOSAN IN SLUDGE AND SEDIMENTS

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Abstract

An expeditious method for the determination of triclosan (TCS) and methyl triclosan (MTCS) in sludge and sediment samples is presented. Extraction and clean-up steps were integrated in the same process using matrix solid-phase dispersion as sample preparation technique. Effects of different variables on the efficiency and the selectivity of the sample preparation process are discussed. Under final working conditions, samples (0.5 g) were dispersed with diatomaceous earth (1 g) and transferred to a polypropylene syringe containing 2 g of silica impregnated with sulphuric acid (15%, w/w). Analytes were recovered with 10 mL of dichloromethane. After solvent exchange to ethyl acetate, TCS was converted into the *tert*-butyldimethylsilyl derivative and the extract was analysed by gas chromatography-mass spectrometry, without any additional clean-up. Obtained recoveries, for sludge and sediment samples spiked at different concentration levels, ranged from 86% to 113% with associated standard deviations between 2 and 13%. Limits of quantification (LOQs) of the global method were 6 and 7 ng g⁻¹ for MTCS and TCS, respectively. Both compounds were detected in all the processed sludge samples with maximum concentrations of 191 ng g⁻¹ (MTCS) and 2640 ng g⁻¹ (TCS). The parent bactericide was also found in some sediment samples at concentrations up to 200 ng g⁻¹.

Keywords: triclosan; methyl triclosan; sludge; matrix solid-phase dispersion; gas chromatography; mass spectrometry.

1. INTRODUCTION

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol; TCS) is a broad spectrum bactericide included in body care products (e.g. soaps, deodorants and tooth pastes) and employed also for the treatment of surfaces and sportive clothes. The former application contributes to the direct discharge of this species in the aquatic environment through domestic wastewater. Nowadays, TCS is considered an emerging pollutant, widely distributed in surface and wastewater [1-3], as well as in sewage sludge and sediments [4-6]. In USA, it has been rated as the second most abundant personal care chemical in biosolids elaborated from sewage sludge and employed as fertilisers [7]. TCS shows a moderate endocrine disruption activity [8], it is toxic to certain aquatic organisms [9,10] and it is bio-accumulated by algae and fish [11-13].

The reactivity of TCS in the aquatic environment is another issue of relevance since some of its by-products are more toxic and/or persistent than the parent bactericide. Laboratory assays have demonstrated the formation of chlorinated phenols and dibenzo-*p*-dioxins from TCS, through chlorination and photochemical reactions [14-16]. However, as these compounds may also arise from other sources, the occurrence and the significance of the above reactions under real-life conditions are hard to evaluate. Another TCS by-product is methyl triclosan (MTCS). Since MTCS has no commercial application, there is no doubt about the occurrence of methylation reactions. MTCS ($\log K_{ow}$ 5.3) is more lipophilic and persistent [17] than TCS ($\log K_{ow}$ 4.8); therefore, it can be bio-accumulated in a higher extension than the parent bactericide. Both species have been detected in biota samples [11,18-20]; however, at this moment, it remains unclear if methylation happens before or after TCS intake. As regards sludge from sewage treatment plants (STPs), less information is available in relation to MTCS concentrations [21]. Evaluating the levels of MTCS in sewage sludge is a relevant question in order to (1) fully understand the behaviour of TCS during wastewater treatments, (2) determine the existence of TCS methylation in the aquatic environment (previously to the concentration of TCS in biota) and (3) assess the risk of introducing MTCS in the terrestrial environment, through the disposal of stabilised sewage sludge as fertiliser in agricultural soils. Up to now, this risk has been assessed only for TCS [22,23].

TCS is amenable to gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) based techniques. On the other hand, MTCS is too lipophilic for an efficient ionisation using LC-MS atmospheric pressure ionisation sources; thus, the simultaneous and sensitive determination of both species relies on GC-MS methods. In this case, it is advisable to convert TCS into a less polar derivative to enhance its detectability. Obviously, methylation reagents (e.g. diazomethane) must be avoided when TCS

and MTCS are the target analytes. As regards sample preparation, the development of simplified methodologies for the extraction of TCS and MTCS from sludge still represents a challenging issue. Pressurised liquid extraction (PLE) [2,21], microwave assisted extraction (MAE) [24], Soxhlet [5], sonication [25] and even solid-liquid extraction [26] show good extraction efficiencies for TCS and, in some cases, also for MTCS. However, further time- and solvent-consuming clean-up strategies (based on water dilution of the primary extract followed by solid-phase concentration with reversed-phase sorbents, or normal-phase sorbents combined with size exclusion chromatography) are required to reduce the amount of co-extracted species at a level compatible with the use of capillary GC columns.

In a previous work, we have demonstrated that TCS and MTCS show an excellent stability to concentrated acids; thus, sulphuric acid-impregnated silica was used to remove most of the interferences co-extracted together with target compounds from biota samples [27]. Herein, we investigate the feasibility of on-line combining this clean-up strategy with matrix solid-phase dispersion (MSPD) for the rapid and selective extraction of TCS and MTCS from freeze-dried samples of sludge and sediments. In addition to normal and reversed-phase sorbents, inert materials were also considered to disperse the sample. Extraction solvents were selected on the basis of their affinity for the target analytes and their compatibility with the clean-up methodology. Finally, the optimised method was applied to investigate the levels of TCS and MTCS in a relevant number of sludge and sediment samples.

2. EXPERIMENTAL

2.1. Solvents, standards and sorbents

Dichloromethane, *n*-hexane, ethyl acetate (trace analysis grade solvents) and concentrated sulphuric acid (98%) were purchased from Merck (Darmstadt, Germany). TCS and the derivatisation reagent, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), were provided by Aldrich (Milwaukee, WI, USA). MTCS was acquired from Toronto Research Chemicals (Toronto, Canada). Individual solutions of TCS and MTCS were prepared in ethyl acetate (ca. 1000 µg mL⁻¹). Further dilutions and mixtures of both analytes were made in the same solvent. Isotopically labelled triclosan (¹³C₁₂-TCS; 100 µg mL⁻¹ in nonane) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Diluted solutions of this species, considered as internal surrogate (IS), were made in ethyl acetate.

Calibration standards, containing increasing concentrations of TCS and MTCS and a fixed level (between 50 and 200 ng mL⁻¹) of ¹³C₁₂-TCS, were prepared in ethyl acetate. Before

injection in the chromatographic system, aliquots (1 mL) of the calibration standards were mixed with MTBSTFA (0.1 mL) in order to convert TCS in the corresponding *tert*-butyldimethylsilyl derivative [27].

Florisil (60-100 mesh) and C₁₈ (70-230 mesh) were acquired from Aldrich, and silica (230-400 mesh) from Merck. Silica and Florisil were activated in an oven at 130 °C for 24 hours. Non-activated silica was also tested as sorbent in the MSPD process. Silica impregnated with sulphuric acid (SiO₂-H₂SO₄), at three different concentrations from 5 to 44% (w/w), was prepared by mixing the activated sorbent with the corresponding mass of concentrated acid. Diatomaceous earth was acquired from Aldrich and employed without any additional treatment.

Empty polypropylene syringes (10 mL capacity) and 20 µm polyethylene frits were purchased from International Sorbent Technology (Mid Glamorgan, UK).

2.2. Samples and sample preparation

The extraction experiments involved in this research were carried out with freeze-dried sludge and sediments. In the latter case, samples were previously sieved and the fraction below 60 µm retained for analysis. Optimisation of sample preparation conditions was carried out with a pooled matrix of primary and biological sludge (50:50), with a total carbon content of 32%, spiked with MTCS at 500 ng g⁻¹. TCS was not added, since it had already been detected in the pooled sample at high enough concentrations. Fractions (0.5 g) of the above matrix were mixed with 1 g of anhydrous sodium sulphate and 1-2 g of a suitable sorbent in a glass mortar, with a pestle. After 5 min of homogenisation, the blend was transferred to a polypropylene syringe containing (bottom to top) anhydrous sodium sulphate (1 g) and sulphuric acid-impregnated silica (2 g). A polyethylene frit was placed on top and compounds were eluted by gravity.

Under final working conditions, diatomaceous earth (1 g) and silica impregnated with a 15% (w/w) of sulphuric acid were used as dispersant and clean-up sorbent in the MSPD syringe, respectively. ¹³C₁₂-TCS was added to the sample in the glass mortar and used as IS. The MSPD syringe was first rinsed with 5 mL of *n*-hexane, which was discarded. Then, analytes were extracted with 10 mL of dichloromethane. This extract was evaporated to dryness and reconstituted with 1 mL of ethyl acetate. Before injection in the GC-MS system, 0.1 mL of MTBSTFA (silylation reagent) was added to the final extract (1 mL) in ethyl acetate.

2.3. GC-MS equipment

TCS and MTCS were determined with a GC-MS system consisting of an Agilent (Wilmington, DE, USA) 7890A gas chromatograph connected to a quadrupole type mass spectrometer (Agilent MS 5975C), furnished with an electron impact (EI) ionisation source. The mass analyser was operated in the selected ion monitoring (SIM) mode, with a dwell time of 100 ms per ion. Separations were carried out in an HP-5ms type capillary column (30 m × 0.25 mm i.d., d_f: 0.25 μm) supplied by Agilent. Helium (99.999 %) was used as carrier gas at a constant flow of 1.0 mL min⁻¹. The GC oven was programmed as follows: 110 °C (held for 3 min), increased at 10 °C min⁻¹ to 280 °C (held for 10 min). Ionisation source, mass analyser and transfer line temperatures were set at 230 °C, 150 °C and 290 °C, respectively. Standards and sample extracts were injected in the pulsed splitless mode (30 psi, 2.1 min), maintaining the injection port at 280 °C. The splitless time and the split flow were set at 2 min and 50 mL min⁻¹, respectively.

2.4. Recoveries of the method and blanks

Quantification was performed comparing the ratios between the peak area of each analyte and the IS in the extracts from sludge and sediment samples with those measured for calibration standards, containing the same amount of IS. Retention times and selected ions for each compound are summarised in **Table 1**. Recoveries were calculated as the difference between the concentrations measured for spiked and non-spiked fractions of each matrix divided by the added level of each analyte. Moreover, the absolute recovery of the IS was evaluated as the ratio between the response (peak area) for this species in sample extracts and calibration standards multiplied by 100. Contamination problems were investigated using procedural blanks, corresponding to the whole sample preparation process performed without sample.

Table 1. Performance of the GC-MS system for standards of MTCS and TCS, as silyl derivative, in ethyl acetate.

| | Retention time (min) | Quantification ion (m/z) | Qualifier ions (m/z) | Linearity ^a R ² (1-2000 ng mL ⁻¹) | LOQ (ng mL ⁻¹) |
|------------------------------------|-------------------------|-----------------------------|-------------------------|--|-------------------------------|
| MTCS | 12.56 | 302 | 304,252,254 | 0.9998 | 3 |
| TCS | 16.46 | 345 | 347,200,310 | 0.9996 | 0.5 |
| ¹³ C ₁₂ -TCS | 16.46 | 357 | 359 | | |

^a Evaluated with standards at eight concentration levels.

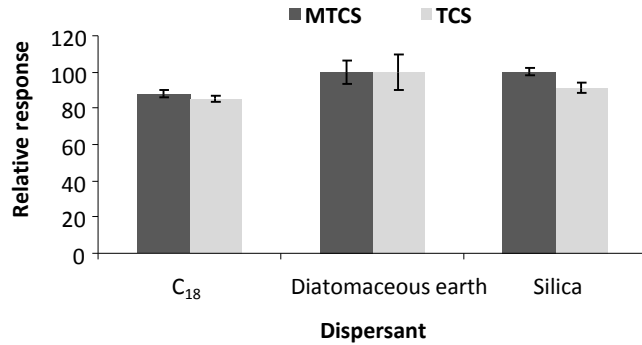
3. RESULTS AND DISCUSSION

3.1. Sample preparation conditions

The performance of MSPD for the extraction of TCS and MTCS from sludge samples is potentially affected by a considerable number of factors. Among them, the extraction solvent and the percentage of sulphuric acid impregnating the layer of clean-up sorbent (silica) are some of the most relevant ones. Also, it must be kept in mind that the choice of extraction solvents is limited by their stability under the strong oxidative conditions existing in the clean-up layer, within the MSPD syringe. Linear hydrocarbons and chlorinated solvents (e.g. dichloromethane) fulfil the above requirement; moreover, they are compatible with the use of GC-MS in the determination step. Finally, the characteristics of the dispersant might also determine the efficiency of the extraction. Reversed-phase materials, particularly C₁₈, are supposed to solubilise the organic fraction of sludge [28,29], improving the yield of the extraction. Normal-phase sorbents interact with matrix components just through adsorption processes. Finally, inert materials simply increase the surface of the sample available to the extraction solvent. In a first series of experiments, C₁₈, activated silica and Florisil, non-activated silica and diatomaceous earth were considered as dispersants. Extractions were carried out using 0.5 g of sludge mixed with 1 g of anhydrous sodium sulphate and dispersed with 2 g of the above materials (1 g in the case of diatomaceous earth). The resulting blend was poured in a polypropylene syringe containing 1 g of anhydrous sodium sulphate (bottom) and 2 g of acidified silica (30% of sulphuric acid). Analytes were extracted using 10 mL of dichloromethane, which were further evaporated to 1 mL. Activated sorbents (silica and Florisil) strongly interacted with TCS, which was not detected in the extract. Data obtained for the rest of dispersants are shown in **Figure 1**. Slightly higher responses were achieved for diatomaceous earth than for C₁₈, whereas no differences were appreciated between the former material and non-activated silica. On the basis of these results, and also considering its lower cost, diatomaceous earth was selected to continue with the study.

Working under conditions reported in the above paragraph, *n*-hexane was tested as alternative to dichloromethane for the extraction of the analytes. Experimentally, it was verified that *n*-hexane (up to 25 mL) was not able to recover either TCS or MTCS. However, chromatograms corresponding to dichloromethane extracts showed a lower baseline when *n*-hexane had been previously passed through the MSPD syringe (figure not shown). Thus, the packed syringe was first rinsed with 5 mL of *n*-hexane, slightly dried by applying pressure and further eluted with 10 mL of dichloromethane.

Figure 1. Effect of the dispersant on the efficiency of the MSPD extraction. Normalised responses obtained for a spiked sludge matrix using 10 mL of dichloromethane as elution solvent (n = 3 replicates).



The percentage of sulphuric acid which impregnates the clean-up layer of silica was deemed as another relevant factor, potentially affecting the selectivity and the yield of extraction. **Figure 2** shows the relative responses obtained for fractions (0.5 g) of the same sludge sample considering percentages of sulphuric acid between 5 and 44%. For the lowest investigated level (5%), extracts showed a pale-yellowish appearance indicating the incomplete oxidation of the organic matter in the MSPD syringe. Moreover, the average response measured for MTCS was slightly lower than those corresponding to the use of silica impregnated with higher percentages of sulphuric acid. Between 15% and 44%, the responses measured for the parent bactericide and its methylated form remained practically constant, confirming their excellent stability under strong oxidative conditions. On the basis of these comments, silica impregnated with a 15% of sulphuric was adopted as clean-up sorbent.

The volume of dichloromethane necessary for the extraction of the target analytes was investigated by collecting consecutive fractions (8 × 2 mL) from the MSPD syringe (**Table 2**). MTCS was observed only in the first two fractions, whereas TCS showed a slower elution profile, being detected up to the 5th fraction. This behaviour is probably related to the higher polarity of TCS in comparison with its methylated by-product. Thus, 10 mL was selected as the optimum volume of dichloromethane.

Figure 2. Responses obtained as a function of the concentration of sulphuric acid impregnating the silica (n = 3 replicates).

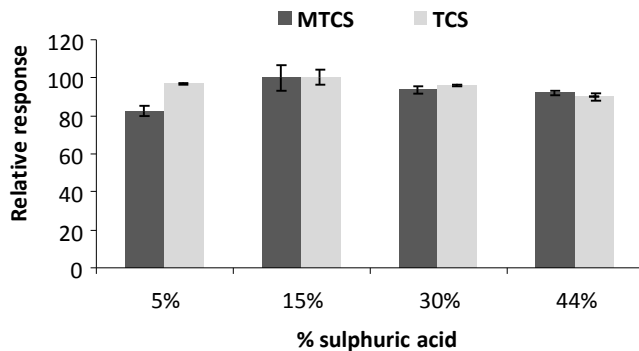


Table 2. Normalised responses as percentages (%), with standard deviations within parenthesis, in the consecutive dichloromethane fractions (2 mL) collected from the MSPD syringe (n = 3 replicates).

| | Fraction number | | | | |
|------|-----------------|------------|-----------|-----------|-----------|
| | 1 | 2 | 3 | 4 | 5 |
| MTCS | 89.3 (2.2) | 10.7 (1.1) | n.d. | n.d. | n.d. |
| TCS | 76.7 (3.3) | 18.0 (1.2) | 3.1 (1.2) | 1.4 (0.8) | 0.8 (0.2) |

n.d. not detected.

3.2. Derivatisation step

TCS can be directly determined by GC-based methods; however, the peak shape for this compound improves considerably after derivatisation of the phenolic moiety. Silylation, particularly formation of *tert*-butyldimethylsilyl derivatives, is one of the most popular reactions to improve the detectability of TCS by GC-MS techniques. On the basis of previous studies, MTBSTFA was selected as derivatisation reagent [27]. Whatever the tested derivatisation conditions (5-60 min, 20-70 °C and 20-200 µL of MTBSTFA), the yield of the reaction was not quantitative in presence of dichloromethane. Consequently, two peaks corresponding to the silylated and non-silylated forms of TCS were observed in the GC-MS chromatograms. The most intense ions in their MS spectra appeared at m/z ratios of 345+347 and 288+290, respectively. The above result is in agreement with the earlier finding of Mol et al. [30], reporting the poor reactivity of MTBSTFA with phenolic species in low polarity solvents. This drawback was overcome introducing a solvent exchange step in the analytical procedure. Dichloromethane extracts (10 mL) were first evaporated to dryness, reconstituted with 1 mL of ethyl acetate and mixed with 0.1 mL of MTBSTFA. Under these conditions, the silylation of TCS was completed in less than 5 min at room temperature.

3.3. Performance of the analytical method

Table 1 summarises some relevant features of the GC-MS system for standard mixtures of MTCS and TCS, the latter as silylated species, prepared in ethyl acetate. GC-MS provided a good linearity (R^2 values higher than 0.999) between 1 and 2000 ng mL⁻¹, and instrumental limits of quantification (LOQs, defined as the concentration of each specie providing a signal 10 times higher than the average baseline noise) of 0.5 and 3 ng mL⁻¹ for TCS and MTCS, respectively.

In order to ensure the absence of analytes inter-conversion processes (TCS methylation and/or MTCS de-methylation) during sample preparation (extraction with simultaneous oxidative treatment followed by dryness evaporation and silylation) two series of extractions, adding around 500 ng of just one of the target species on top of the MSPD cartridge, were performed. In these assays, no sample was used. GC-MS analysis of the corresponding extracts, after dryness evaporation and addition of MTBSTFA, showed a single peak corresponding to the species added to the MSPD cartridge. These data demonstrated the integrity of both analytes during the whole sample preparation process.

MTCS levels in sludge are expected to remain significantly lower than those corresponding to the parent bactericide. Therefore, it is particularly important to guarantee the absence of TCS methylation reactions during the whole sample preparation. In order to further investigate this point, a freeze-dried pooled sludge sample, containing detectable amounts of both analytes, was divided into two fractions: one of them was fortified with TCS at a high level ($50 \mu\text{g g}^{-1}$) and the other processed directly. No differences were noticed in the average responses ($n = 5$ replicates) for MTCS in the extracts from both fractions of the same sample (data not given). All together, the above results clearly demonstrate the absence of significant inter-conversion reactions between TCS and MTCS during sample preparation.

Recoveries of the developed method were first evaluated with samples of primary and biological sludge, from an urban STP plant, fortified at two different levels and aged for one week before extraction. MTCS and TCS were added to these samples at different concentrations, selected on the basis of their reported levels in sludge [21]. Obtained recoveries ranged from 86 to 113%, with associated standard deviations (SDs) below 13 (**Table 3**).

Table 3. Recoveries of the method for spiked samples ($n = 4$ replicates).

| Sample type | TC ^a (%) | Added concentration (ng g^{-1}) | | Recovery (%) \pm SD | |
|------------------|---------------------|--|------|-----------------------|-------------|
| | | MTCS | TCS | MTCS | TCS |
| Secondary sludge | 36 | 50 | 1000 | 100 ± 5 | 107 ± 8 |
| Secondary sludge | 36 | 200 | 3000 | 107 ± 13 | 92 ± 5 |
| Primary sludge | 35 | 50 | 1000 | 113 ± 13 | 90 ± 10 |
| Primary sludge | 35 | 200 | 3000 | 99 ± 9 | 86 ± 3 |
| Sediment | 1.2 | 50 | 100 | 111 ± 9 | 100 ± 2 |
| Sediment | 15.5 | 50 | 200 | 104 ± 9 | 109 ± 2 |

^aTotal carbon content.

Initially, the procedure described in this study was designed to deal with sludge samples. However, on the basis of its simplicity, it was further assessed whether the proposed sample preparation conditions would allow the quantitative extraction of TCS and MTCS from sediment samples. In this case, recoveries were evaluated using two sediments with low and high carbon contents. Found recoveries varied between 100 and 111%, with similar SDs to the ones obtained for sludge (**Table 3**). The absolute average recoveries obtained for the IS ($^{13}\text{C}_{12}$ -TCS) were $86.0\% \pm 8.1$ ($n = 18$ replicates) and $87.2\% \pm 6.6$ ($n = 12$ replicates) for sludge and sediment samples, respectively.

Procedural blanks demonstrated the absence of contamination problems for any of both analytes (**Figure 3**). Thus, LOQs were calculated from signal to noise ratios (S/N) corresponding to MTCS and TCS peaks in non-spiked sludge samples. Values of 6 ng g^{-1} (MTCS) and 7 ng g^{-1} (TCS) were estimated for an S/N value of 10. LOQs previously reported for TCS in sludge by GC-MS methods ranged from 0.49 ng g^{-1} [25] to 100 ng g^{-1} [21]. As regards MTCS, Chen and Bester [21] reached a LOQ of 10 ng g^{-1} for sludge samples combining PLE with size exclusion chromatography as clean-up technique. The main advantages of the approach presented in this research are (1) the significantly lower consumption of organic solvents and (2) the integration of extraction and clean-up processes in the same step. Moreover, most published methods do not consider MTCS. **Table 4** summarises some relevant data related to the sample preparation approach optimised in this work and other strategies applied to the GC-MS determination of TCS and MTCS in sludge samples.

Figure 3. Selected ion chromatograms for a procedural blank (solid line) and a 10 ng mL^{-1} standard (dotted line) of MTCS and TCS.

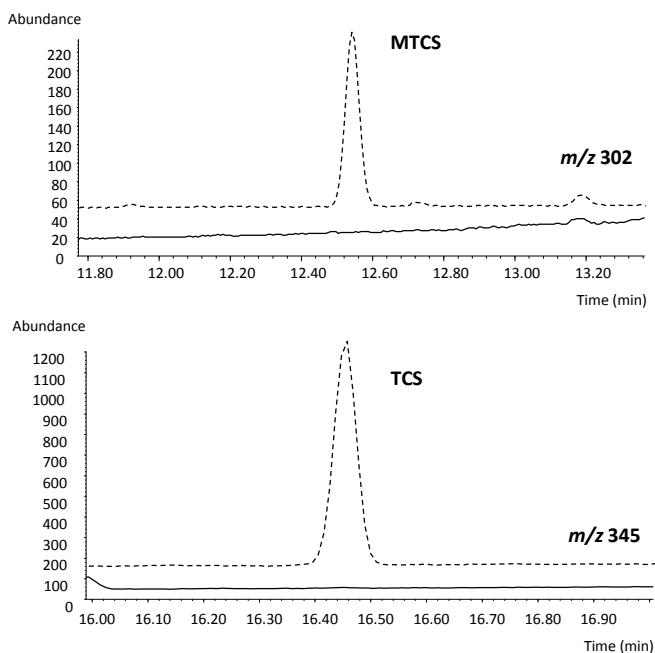


Table 4. Summary of sample preparation conditions for GC-MS determination of TCS and MTCS in freeze-dried sludge samples.

| Analytes | Extraction technique | Clean-up strategy | Estimated solvent consumption (mL) | Derivatisation reaction | Average Recovery (%) | LOQs (ng g ⁻¹) | Ref. |
|-----------|-------------------------|------------------------------|------------------------------------|-------------------------|-------------------------|----------------------------|-----------|
| TCS | PLE | NP-SPE | 10 | Methylation | 100 | 15 ^a | [2] |
| TCS, MTCS | PLE | First, NP-SPE | 240 | None | 55 (MTCS), | 10 (MTCS), | [21] |
| | | Second, SEC | | | 114 (TCS) | 100 (TCS) | |
| | | Third, NP-SPE | | | | | |
| TCS | MAE | First, LLE | 65 | Silylation | 89 | 0.8 | [24] |
| | | Second, RP-SPE | | | | | |
| | | Third, NP-SPE | | | | | |
| TCS | Soxhlet | First, NP-SPE Second, SEC | 180 | None | 94 | 4 | [5] |
| TCS | Solid-liquid extraction | LLE | 50 | Methylation | 116 | 4.5 | [26] |
| TCS | Sonication | RP-SPE | 25 | Silylation | 78 | 0.49 | [25] |
| TCS, MTCS | MSPD | Integrated with MSPD | 15 | Silylation | 105 (MTCS), 94 (TCS) | 6 (MTCS), 7 (TCS) | This work |

^a Estimated as three times the reported LOD.

NP-SPE: normal phase solid-phase extraction.

RP-SPE: reversed phase solid-phase extraction.

SEC: size exclusion chromatography.

3.4. Real samples analysis

The developed method was applied to the determination of TCS and MTCS levels in sludge and sediments. Sludge samples (11 specimens) were supplied by a company which manages STPs from several cities in the Northwest of Spain. All samples correspond to urban sewage plants; however, the information related to their exact locations and the served equivalent population is not available due to a confidentiality agreement. Sediment samples (8 specimens) were collected in small rivers and marine estuaries from the same geographic area; moreover, some samples proceeding from inter-comparison exercises were also processed.

Table 5. Summary of concentrations (ng g^{-1}), with their standard deviations, in real samples ($n = 3$ replicates). TCS and MTCS levels in sediment samples 16 to 19 stayed below the LOQs of the method.

| Code | Sample | Type | MTCS | TCS | Ratio MTCS/TCS |
|------|----------|-------------|--------------|----------------|----------------|
| 1 | | Secondary | 170 ± 9 | 2640 ± 26 | 0.06 |
| 2 | | Secondary | 63 ± 2 | 1565 ± 16 | 0.04 |
| 3 | | Secondary | 44 ± 2 | 2116 ± 20 | 0.02 |
| 4 | | Secondary | 130 ± 9 | 690 ± 21 | 0.19 |
| 5 | | Secondary | 86 ± 3 | 460 ± 7 | 0.19 |
| 6 | Sludge | Secondary | 51 ± 3 | 2230 ± 42 | 0.02 |
| 7 | | Secondary | 191 ± 16 | 983 ± 8 | 0.19 |
| 8 | | Primary | 37 ± 2 | 2620 ± 55 | 0.01 |
| 9 | | Primary | 115 ± 5 | 345 ± 7 | 0.33 |
| 10 | | Primary | 19 ± 2 | 1450 ± 7 | 0.01 |
| 11 | | Disinfected | 15 ± 2 | 272 ± 50 | 0.06 |
| 12 | | River | n.d. | 20 ± 4 | – |
| 13 | Sediment | River | n.d. | 11.4 ± 0.7 | – |
| 14 | | Marine | n.d. | 201 ± 5 | – |
| 15 | | River | n.d. | 8.6 ± 0.8 | – |

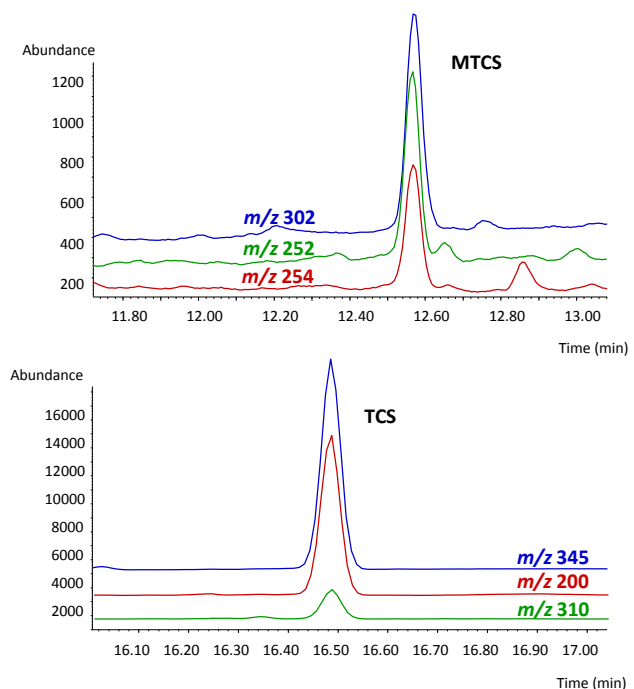
n.d. below the detection limit.

TCS and its methylated by-product were quantified in all the processed samples of sludge. Their concentrations ranged from 15 to 191 ng g^{-1} , in the case of MTCS; and between 272 and 2640 ng g^{-1} , for TCS (Table 5). Figure 4 shows the GC-MS traces corresponding to sample code 7 (Table 5). TCS concentrations stayed at similar, or even slightly lower, levels to those published for sludge samples from Germany [5] and other regions of Spain [31]. They are also lower than the range of TCS levels reported for sludge samples collected in Greece (from 0.19 to 9.85 $\mu\text{g g}^{-1}$) [32] and they remain far below the average value of 10 $\mu\text{g g}^{-1}$

reported for biosolids in USA [7]. The concentrations of MTCS summarised in **Table 5** are also similar to that reported for a single sludge sample collected in Germany [21]; nevertheless, they stayed below the maximum concentration of 450 ng g^{-1} , which has been found in river sediments from a Rhine tributary [33]. The ratio of MTCS/TCS concentrations varied from 0.01 to 0.33 (**Table 5**). Globally, these data prove the methylation of TCS in primary and biological treatment units of STPs. It is also significant that the sample of stabilised sludge (code 11, **Table 5**) contains measurable levels of MTCS and TCS. This sample corresponds to disinfected sludge (mixture of primary and secondary sludge with lime), which is normally disposed as fertiliser in agricultural fields. Thus, this practise contributes not only to TCS, but also to MTCS introduction in the terrestrial environment.

As regards sediment samples, MTCS always remained below the LOQ of the method, whereas TCS was quantified in four of the eight investigated samples at levels from 9 to 200 ng g^{-1} (**Table 5**). The highest level was found in a marine sediment (code 14, **Table 5**) provided by QUASIMEME (Wageningen, The Netherlands) and used in inter-comparison studies focused on the determination of brominated flame retardants. Agüera et al. [4] have also measured TCS levels over 100 ng g^{-1} in marine sediments, collected in the vicinity of treated wastewater discharges.

Figure 4. GC-MS ionic traces obtained for sludge sample code 7 (see **Table 5** for concentrations).



4. CONCLUSIONS

MSPD has been proved as a fast, simple and robust method for the selective extraction of TCS and MTCS from complex environmental samples, such as sludge and sediment. Also, the use of diatomaceous earth has been proposed for the first time as an inert, inexpensive dispersant for the MSPD extraction of TCS and MTCS. The chemical stability of both analytes allows the use of oxidative clean-up processes using sulphuric acid-impregnated silica. On-line combination of extraction and clean-up steps considerably simplifies the sample preparation process. Dichloromethane showed a high extraction efficiency and good compatibility with oxidative conditions so that 10 mL of solvent is enough to quantitatively recover the analytes. Moreover, dichloromethane is a volatile solvent, simplifying the solvent exchange and final extract volume reduction, as the further TCS silylation reaction requires a more polar, non-protic solvent such as ethyl acetate.

The analyses of sludge from several urban STPs demonstrated the ubiquitous distribution of MTCS in this matrix, and thus the occurrence of TCS methylation reactions in STPs. The concentration of the methylated by-product represented between 1 and 33% of that corresponding to TCS. This percentage of methylation must be considered to improve the accuracy of TCS mass balances in STPs and also to assess the potential contamination of agriculture fields with this highly bio-accumulative halogenated diphenyl ether. On the other hand, MTCS was not detected in any analysed sediment, while TCS was found in 50% of these samples at a concentration one order of magnitude lower than in sludge.

Acknowledgements

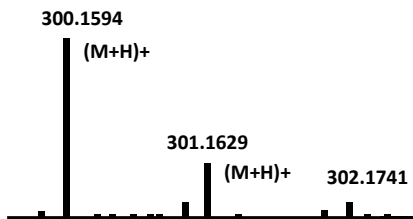
This study has been supported by the Spanish Government and E.U. FEDER funds (project CTQ2009-08377). I.G.M. and J.B.Q. thank their FPU and Ramón y Cajal research contracts to the Spanish Ministries of Education and Science-Innovation, respectively. We acknowledge Labaqua for supplying the sludge samples.

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B. DROGAS DE ABUSO

INTRODUCCIÓN Y ESQUEMAS DE LOS MÉTODOS DESARROLLADOS

Como ya se ha mencionado en el capítulo II, las drogas de abuso y sus metabolitos se introducen de forma continua en las aguas residuales como consecuencia de su excreción por parte de los consumidores. En última instancia, tratamientos de depuración incompletos o inexistentes conllevan su entrada en las aguas superficiales, donde su determinación (en general a concentraciones bajas) requiere el empleo de técnicas analíticas con una adecuada sensibilidad. En aguas residuales sus niveles son más elevados, pero la complejidad de esta matriz obliga a utilizar metodologías especialmente selectivas.

En el momento de abordar la presente tesis, la inmensa mayoría de los trabajos publicados para la determinación de drogas de abuso en aguas utilizaban la SPE como técnica de extracción y la cromatografía de líquidos acoplada a la espectrometría de masas (generalmente en tándem) como sistema de detección. La SPE es simple, robusta y permite alcanzar elevados factores de preconcentración y altas eficacias de extracción. Sin embargo, su selectividad suele ser bastante limitada, y la extracción de otros componentes presentes en las muestras puede afectar significativamente a la ionización de los analitos mediante ESI cuando la determinación se realiza por LC-MS. Como alternativa, el primero de los procedimientos optimizados en la presente tesis doctoral utilizó la cromatografía de gases acoplada a la espectrometría de masas en tándem para la separación y detección de 14 drogas de abuso y metabolitos pertenecientes a 4 familias químicas diferentes. Aun teniendo una amplia tradición en la determinación de este tipo de sustancias en el ámbito clínico y forense, su aplicación medioambiental no había sido abordada, por lo que supuso una opción más económica y menos susceptible a los efectos de matriz que los métodos disponibles hasta entonces en la bibliografía.

Conscientes de la limitación que suponen estos efectos, los siguientes esfuerzos se centraron en el desarrollo de procedimientos de SPE selectivos para llevar a cabo la posterior determinación de los analitos mediante LC-MS/MS con interfase de electrospray. De esta forma, el segundo de los trabajos surgió a raíz de la comercialización de cartuchos tipo jeringa provistos de polímeros impresos molecularmente para la retención de anfetaminas. El protocolo de SPE propuesto por el fabricante se aplicó a la extracción de 5 derivados anfetamínicos en agua residual y las prestaciones analíticas del método resultante se compararon con las obtenidas utilizando otros dos adsorbentes comerciales: Oasis HLB (que ya había sido empleado en el anterior trabajo) y Oasis MCX (previsiblemente más selectivo que HLB).

En el último método desarrollado, el número de analitos a determinar se extendió a 24, incluyendo sustancias y metabolitos pertenecientes a 7 familias químicas diferentes. Para ello se seleccionó el adsorbente Oasis MCX y se optimizó un procedimiento cuya principal diferencia con respecto a otros de los disponibles en la bibliografía radicó en llevar a cabo una elución fraccionada de los analitos: los cannabinoides, junto con los demás componentes ácidos y neutros de la muestra, se eluyeron en una primera fracción; el resto de drogas (básicas) se eluyeron en una segunda, presumiblemente conteniendo menos interferencias y, por ello, menos afectada por los efectos de matriz. La separación y detección se realizó mediante LC-MS/MS (MS para los opioides) empleando un cromatógrafo de líquidos acoplado a un espectrómetro de masas con un analizador híbrido cuadrupolo-tiempo de vuelo.

A continuación, se presentan los esquemas de las metodologías analíticas empleadas en cada uno de estos estudios (**Figuras III.5 a III.7**), así como las publicaciones científicas derivadas de los mismos.

Figura III.5. Esquema para la determinación de drogas de abuso en aguas fluviales y residuales mediante SPE-GC-(EI)-MS/MS (IT).

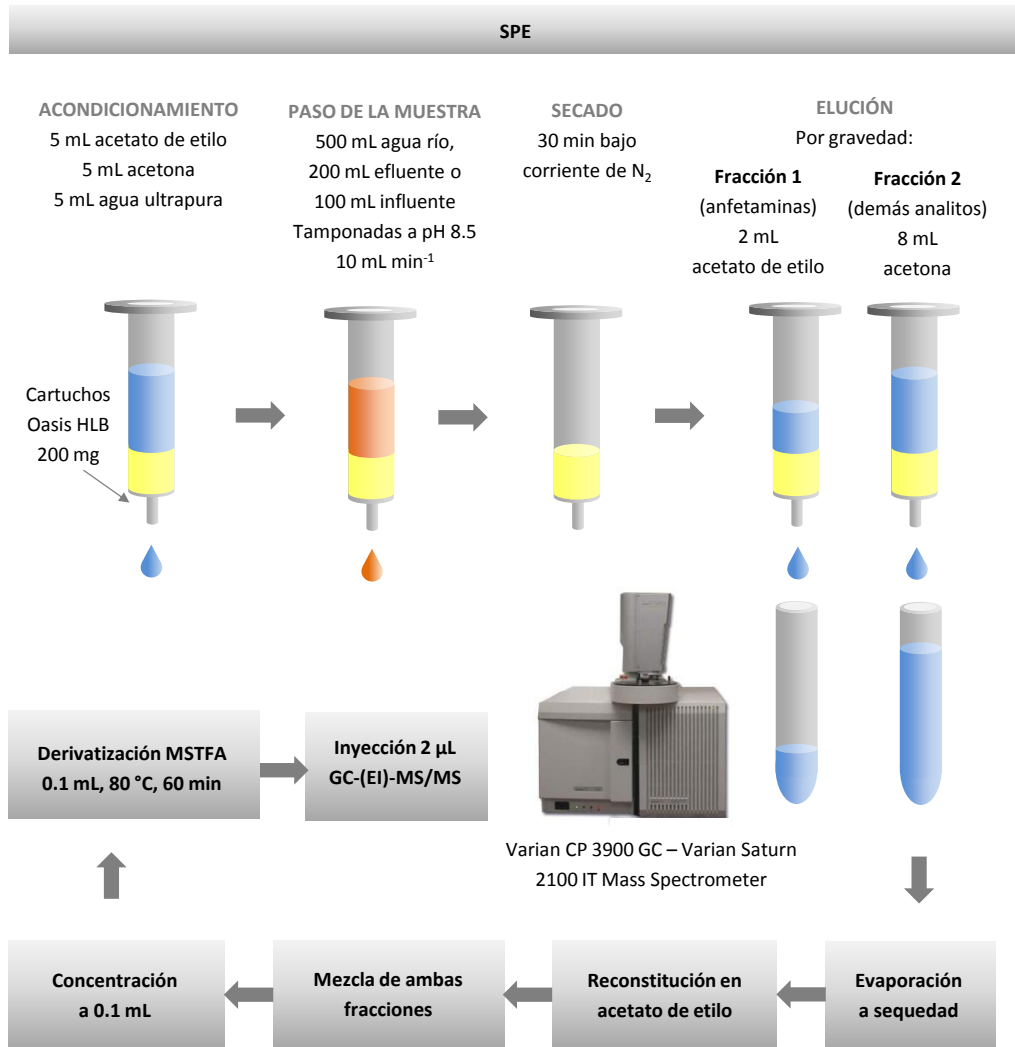


Figura III.6. Esquema para la determinación de derivados anfetamínicos en aguas residuales mediante SPE-LC-(ESI+)-MS/MS (QqQ).

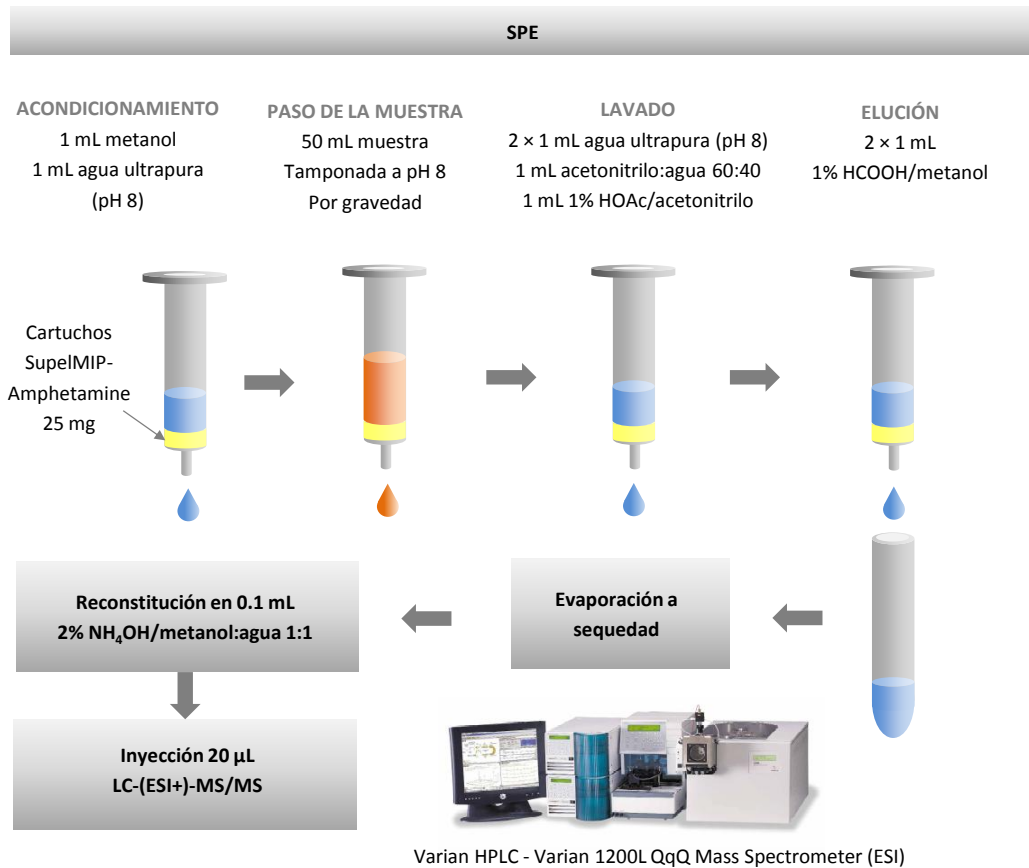
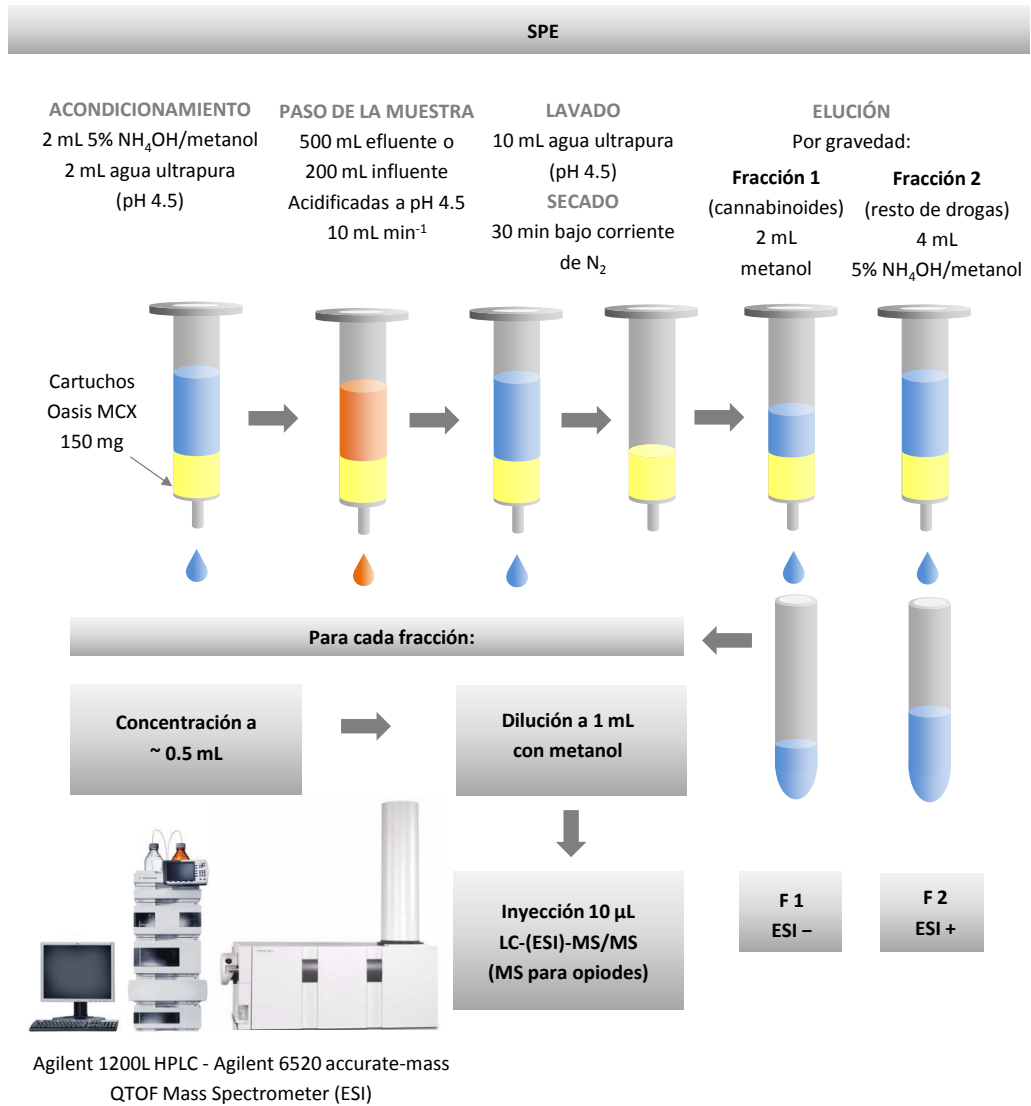


Figura III.7. Esquema para la determinación de drogas de abuso en aguas residuales mediante SPE-LC-(ESI)-MS/MS (MS para opioides) (QTOF).



PUBLICACIÓN III.5

***DETERMINATION OF DRUGS OF ABUSE IN WATER BY SOLID-PHASE EXTRACTION,
DERIVATISATION AND GAS CHROMATOGRAPHY-ION TRAP-TANDEM MASS
SPECTROMETRY***

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DETERMINATION OF DRUGS OF ABUSE IN WATER BY SOLID-PHASE EXTRACTION, DERIVATISATION AND GAS CHROMATOGRAPHY-ION TRAP-TANDEM MASS SPECTROMETRY**Iria González-Mariño, José Benito Quintana, Isaac Rodríguez, Rafael Cela**

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Abstract

An alternative method for the sensitive determination of several drugs of abuse and some of their metabolites in surface and sewage water samples is proposed. Analytes are concentrated using a solid-phase extraction (SPE) sorbent, converted into the corresponding trimethylsilyl derivatives and selectively determined by gas chromatography (GC) with tandem mass spectrometry (MS/MS) detection. Parameters affecting the performance of the extraction, derivatisation and determination steps are systematically investigated. Moreover, the stability of target analytes in sewage water samples is discussed. Under final working conditions, water samples were adjusted at pH 8.5 and concentrated using a 200 mg OASIS HLB SPE cartridge. Analytes were sequentially eluted with ethyl acetate followed by acetone and silylated using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). The reaction was completed in 60 min at 80 °C and the mixture injected directly in the GC-MS/MS system without further purification. In most cases, analytes presented a poor stability in sewage water samples; however, once they are submitted to the SPE process, cartridges can be stored at -20 °C for at least 3 months without significant degradation and/or inter-conversion reactions of illicit drugs. The proposed method provided recoveries over 74% and LODs between 0.8 and 15 ng L⁻¹ for river and treated wastewater samples. In the case of raw wastewater slightly worse recoveries, between 63 and 137%, and similar LODs were attained. Analysis of a limited number of waste and surface water samples confirmed the presence of several illicit drugs in the aquatic environment, with the highest levels and frequency corresponding to benzoylecgonine, the main metabolite of cocaine.

Keywords: illicit drugs; water samples; gas chromatography; ion trap-mass spectrometry; solid-phase extraction; derivatisation.

1. INTRODUCTION

Abuse of illicit drugs has become a serious global problem in our contemporary society. According to the United Nations Office of Drugs and Crime (UNODC), 200 million people consumed any illicit substance during 2005, 110 million used them regularly and 25 million were considered addicted. Facing these data with appropriate solutions requires reliable figures about local consumption and trends. Currently, the above information is inferred from socio-epidemiological studies integrated with population surveys, crime and medical records, drug production and drug seizures. These indicators may lead to inaccurate conclusions since they are too general and most of the information is obtained from the consumers themselves. Moreover, data collection and analysis are time consuming, so updated figures and changing patterns cannot be properly estimated [1,2].

In the past decade, several pharmaceuticals and their metabolites were detected in the water environment. These data was employed by several authors to monitor the consumption of pharmaceuticals in a specific location. Loads of these compounds, and their major metabolites, in waste- and surface waters are calculated and are then related to the local population equivalents (i.e. the number of people served by a given sewage treatment plant (STP) or living in the river's catchment basin). In 2001, Daughton [3] suggested that residues of illicit drugs may be similarly detected in the aquatic environment and, as their metabolism patterns are mostly known, correlated to their consumption. In this way, the first report concerning the presence of illicit substances in water appeared in 2004 [4], but it was not until 2005 when Zuccato et al. [5] related measured levels to local consumption, which was named as *sewage epidemiology*, beginning a trend that has been followed by many other researchers [6-10].

In contrast to classical strategies of screening drugs consumption, analysis of water samples is cheaper, anonymous (avoiding potential conflicts over privacy) and provides real-time data, which would enable detecting changes in drugs usage if a long-term monitoring programme was carried out. In addition, the obtained data are also valuable to evaluate the removal efficiency of illicit substances in STPs and to identify the percentage of them that reach natural waters, where their effects remain mostly unknown. According to recent studies [5-14], some illicit drugs occur in this media at concentrations similar to other emerging contaminants (i.e. the psychiatric drug carbamazepine and the anti-inflammatory diclofenac) so, in future, they might be included in the list of *priority substances* of the EU Water Framework Directive (WDF) [15].

Developed methods for the determination of drugs of abuse in waters are based on a solid-phase extraction (SPE) step followed by the subsequent separation and detection by

liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [16-22]. SPE provides adequate enrichment factors, it is robust and well established in most analytical laboratories. However, with a few exceptions [23], the selectivity of SPE is rather limited; consequently, complex extracts, which normally lead to signal suppression in LC-MS measurements, and even unreliable results in the most critical situations, are obtained [17].

On the other hand, gas chromatography-mass spectrometry (GC-MS) is an inexpensive alternative not suffering from ionisation matrix effects, as compared to LC-MS; it is accessible to most laboratories and has a long tradition for the determination of drugs of abuse in clinical and forensic sciences [17]; however, to the best of our knowledge, it had not been applied yet with environmental purposes. Thus, the goal of this work was the development of an SPE-GC-MS/MS method for the simultaneous determination of several illicit drugs, belonging to four different chemical families, and some of their metabolites in environmental water samples. Experimental parameters were optimised to achieve the maximum efficiency during extraction and derivatisation steps. Moreover, the stability of the analytes during sample storage was discussed. Finally, after validation of the proposed method, it was applied to several river and sewage water samples collected in the NW of Spain.

2. EXPERIMENTAL

2.1. Chemicals and stock solutions

The illicit drugs and metabolites studied were the following: (\pm)-amphetamine (AMP), (\pm)-methamphetamine (MAMP), (\pm)-3,4-methylenedioxyamphetamine (MDA), (\pm)-3,4-methylenedioxymethamphetamine (MDMA), (\pm)-3,4-methylenedioxyethylamphetamine (MDEA), cocaine (COC), cocaethylene (COE), benzoylecgonine (BE), morphine (MOR), codeine (COD), heroine (HER), (\pm)-methadone (MET), (-)- Δ^9 -tetrahydrocannabinol (THC) and (\pm)-11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THCCOOH). All of them were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg mL⁻¹ solutions in acetonitrile (COC, COE and HER) or methanol (the remaining ones), except THCCOOH (0.1 mg mL⁻¹ in methanol). Deuterated compounds were also obtained from Cerilliant (0.1 mg mL⁻¹ in acetonitrile or methanol) and were used as surrogated internal standards (ISs) for quantification of their analogous native analytes. In the case of COE and HER, COC-d₃ and MOR-d₃ were used as ISs as there was no labelled compound available in the laboratory. The structures of analytes and labelled ISs are compiled in **Table 1**.

Mixed working solutions containing all the target analytes or their deuterated analogues were prepared in methanol:water (1:1), when used to fortify water samples, and in

ethyl acetate, when considered to evaluate the performance of the GC-MS system. The above solutions were stored in the dark at -20 °C. Calibration standards with increasing concentrations of analytes and 250 ng mL⁻¹ of ISs were prepared in ethyl acetate containing the appropriate amount of derivatisation reagent.

Methanol and acetonitrile, as well as ammonia solution (25%) and hydrochloric acid (37%), for pH adjustments, were supplied by Merck (Darmstadt, Germany). Acetone and ethyl acetate were from Prolabo (Cerdanyola del Vallès, Spain). Anhydrous sodium sulphate was from Panreac (Castellar del Vallès, Spain).

Standards and sample extracts, both in ethyl acetate, were derivatised before GC-MS determination. Different derivatisation agents were tested in this work: *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and *N*-methyl-bis(trifluoroacetamide) (MBTFA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA); *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco (Bellefonte, Bellefonte, PA, USA); and finally, acetic anhydride was provided by Across Organics (Geel, Belgium).

A key parameter to guarantee reliable results in the analysis of environmental samples is insuring the stability of the analytes during sample storage. Series of experiments were carried out with spiked aliquots of filtered sewage samples, stored under different conditions before being submitted to the analytical procedure. In some cases, sodium azide (Riedel-deHaën, Seelze, Germany) was added to the samples in order to reduce their microbiological activity.

2.2. Samples

Three surface water samples and several STP influents and effluents were employed in this study. The first ones were taken from the rivers Sar, Dos Pasos and Lengüelle in Galicia (NW Spain). Raw and treated wastewaters were collected at five STPs which receive the discharges from the same number of small and medium size cities (from 15000 to 125000 inhabitants) located in the NW Spain. All samples were collected in amber glass bottles, previously rinsed with methanol and ultrapure water, and extracted (SPE) within 24 h after sampling.

2.3. Sample preparation and derivatisation

An SPE procedure was developed to isolate and to concentrate the target compounds from waters. Prior to extraction, samples (500 mL for river water, 200 mL for STP effluent and

100 mL for the influent) were passed through a combination of glass fibre pre-filters and 0.45 μm nitrocellulose filters (Millipore, Bedford, MA, USA) to remove particulate matter. The filtrate was then adjusted to the desired pH, using either HCl or ammonia solutions, spiked with isotopically labelled standards (50 ng each) and, in the case of recovery studies, also with analytes. Oasis HLB 60 mg (3 mL) and 200 mg (6 mL) cartridges (Waters, Milford, MA, USA), were tested.

In the final method, the Oasis HLB 200 mg cartridges were employed. They were sequentially conditioned with 5 mL of ethyl acetate, 5 mL of acetone and 5 mL of ultrapure water. Samples (pH 8.5) were passed through them at a flow rate of approximately 10 mL min^{-1} and, then, sorbents were dried by a continuous nitrogen stream for a minimum of 30 min, wrapped in aluminium foil and stored at $-20\text{ }^{\circ}\text{C}$ until desorption. Just before this step, cartridges were dried again and analytes were eluted in two separated fractions: the first one with 2 mL of ethyl acetate and the second one using 8 mL of acetone. The acetone fraction was evaporated to dryness at $25\text{ }^{\circ}\text{C}$, under a gentle stream of nitrogen, and the residue was mixed with the ethyl acetate fraction. The reconstituted extract was finally concentrated to 0.1 mL and derivatised, in a closed vial equipped with a 0.25 mL insert, by adding 0.1 mL of MSTFA and by heating the mixture at $80\text{ }^{\circ}\text{C}$ for 60 min.

2.4. Gas chromatography-mass spectrometry

Analytes were determined by GC-MS/MS using a Varian CP 3900 gas chromatograph (Walnut Creek, CA, USA) connected to a Varian Saturn 2100 ion-trap mass spectrometer. Injections (2 μL) were made in the splitless mode with a splitless time of 1 min and a split ratio of 50. The injector port was set at $280\text{ }^{\circ}\text{C}$.

Separations were carried out in an HP-5MS type capillary column (30 m \times 0.25 mm i.d., d_f 0.25 μm) supplied by Agilent (Wilmington, DE, USA). Helium (99.999%) was employed as carrier gas using an initial pressure pulse of 25 psi for 1.1 min and then keeping the flow at a constant value of 1.3 mL min^{-1} . The GC oven was programmed as follows: the initial temperature of $90\text{ }^{\circ}\text{C}$ was held for 1 min; next, it was increased to $130\text{ }^{\circ}\text{C}$ at $25\text{ }^{\circ}\text{C min}^{-1}$ and, finally, to $280\text{ }^{\circ}\text{C}$ at $4\text{ }^{\circ}\text{C min}^{-1}$ (held for 5 min). The total run time was 45.10 min and the solvent delay 4.5 min. The GC-MS interface and the ion-trap temperatures were set at 280 and $220\text{ }^{\circ}\text{C}$, respectively.

The mass spectrometer was operated in the electron impact ionisation mode (70 eV) and MS scan (70-500 m/z) in the preliminary experiments; and in resonant MS/MS in the final method, with a filament emission current of 60 μA and a multiplier offset of 100 V. The MS/MS detection conditions, as well as the m/z ratios corresponding to the parent and product ions for each compound and their deuterated analogues are given in **Table 1**.

Table 1. Structures, physicochemical data and experimental parameters employed for the MS/MS determination of the target compounds.

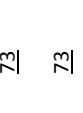
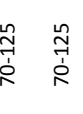
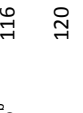
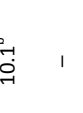
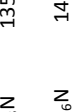

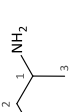
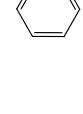


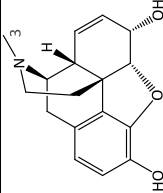
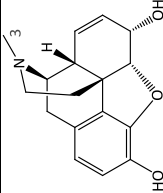
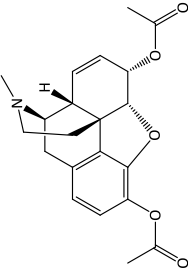
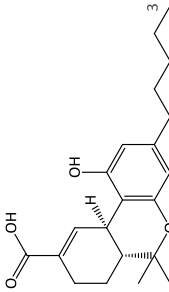
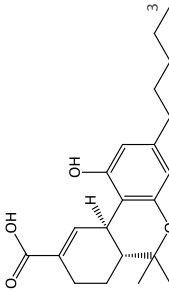
| | Structure ^a | Empirical formula | Monoisotopic MW | pK _a | log K _{ow} | Precursor (m/z) | Scan Range (m/z) | Products ^d (m/z) | Excit. Stor. Level (m/z) ^e | Excit. Ampl. (V) ^f |
|---------------------|---|--|-----------------|--------------------|---------------------|-----------------|------------------|-----------------------------|---------------------------------------|-------------------------------|
| AMP |  | C ₉ H ₁₃ N | 135.10 | 10.1 ^b | 1.76 ^b | 116 | 70-125 | <u>73</u> | 40 | 0.35 |
| AMP-d ₆ |  | C ₉ H ₇ D ₆ N | 141.1 | – | – | 120 | 70-125 | <u>73</u> | 40 | 0.35 |
| MAMP |  | C ₁₀ H ₁₅ N | 149.12 | 9.87 ^b | 2.07 ^b | 130 | 70-135 | <u>73</u> | 40 | 0.35 |
| MAMP-d ₅ |  | C ₁₀ H ₁₀ D ₅ N | 154.12 | – | – | 134 | 70-135 | <u>73</u> | 40 | 0.35 |
| MDA |  | C ₁₀ H ₁₃ NO ₂ | 179.09 | 9.67 ^b | 1.64 ^b | 116 | 70-125 | <u>73</u> | 40 | 0.35 |
| MDA-d ₅ |  | C ₁₀ H ₈ D ₅ NO ₂ | 184.09 | – | – | 120 | 70-125 | <u>73</u> | 40 | 0.35 |
| MDMA |  | C ₁₁ H ₁₅ NO ₂ | 193.11 | 10.32 ^c | 1.81 ^c | 130 | 70-135 | <u>73</u> | 40 | 0.32 |
| MDMA-d ₅ |  | C ₁₁ H ₁₀ D ₅ NO ₂ | 198.11 | – | – | 134 | 70-135 | <u>73</u> | 40 | 0.32 |
| MDEA |  | C ₁₂ H ₁₇ NO ₂ | 207.13 | 10.34 ^c | 2.34 ^c | 144 | 70-150 | <u>73</u> | 40 | 0.32 |
| MDEA-d ₅ |  | C ₁₂ H ₁₂ D ₅ NO ₂ | 212.13 | – | – | 149 | 70-150 | <u>73</u> | 40 | 0.32 |
| MET | | C ₂₁ H ₂₇ NO | 309.21 | 8.94 ^b | 3.93 ^b | 296 | 180-305 | <u>281, 206, 191</u> | 130 | 0.59 |
| MET-d ₃ | | C ₂₁ H ₂₄ D ₃ NO | 312.21 | – | – | 299 | 180-305 | <u>284, 209, 191</u> | 130 | 0.59 |

Table 1 (continuation). Structures, physicochemical data and experimental parameters employed for the MS/MS determination of the target compounds.

| | Structure ^a | Empirical formula | Monoisotopic MW | pK _a | log K _{ow} | Precursor (m/z) | Scan Range (m/z) | Products ^d (m/z) | Excit. Stor. Level (m/z) ^e | Excit. Ampl. (V) ^f |
|--------------------|------------------------|--|-----------------|-------------------|---------------------|-----------------|------------------|-----------------------------|---------------------------------------|-------------------------------|
| COE | | C ₁₈ H ₂₃ NO ₄ | 317.16 | 9.04 ^c | 3.61 ^c | 196 | 75-250 | 168, <u>150</u> , 82 | 70 | 0.42 |
| COC | | C ₁₇ H ₂₁ NO ₄ | 303.15 | 8.61 ^b | 2.30 ^b | 182 | 75-190 | <u>150</u> , 122, 82 | 70 | 0.42 |
| COC-d ₃ | | C ₁₇ H ₁₈ D ₃ NO ₄ | 306.15 | — | — | 185 | 75-190 | <u>153</u> , 125, 85 | 70 | 0.42 |
| BE | | C ₁₆ H ₁₉ NO ₄ | 289.13 | 10.8 ^c | 2.72 ^c | 240 | 75-250 | <u>150</u> , 108, 82 | 60 | 0.33 |
| BE-d ₃ | | C ₁₆ H ₁₆ D ₃ NO ₄ | 292.13 | — | — | 243 | 75-250 | <u>153</u> , 111, 85 | 60 | 0.35 |
| THC | | C ₂₁ H ₃₀ O ₂ | 314.22 | 10.6 ^b | 7.60 ^b | 386 | 280-395 | <u>371</u> , 330, 315 | 170 | 0.98 |
| THC-d ₃ | | C ₂₁ H ₂₇ D ₃ O ₂ | 317.22 | — | — | 389 | 280-395 | <u>374</u> , 330, 315 | 170 | 1.14 |
| COD | | C ₁₈ H ₂₁ NO ₃ | 299.15 | 8.21 ^b | 1.19 ^b | 371 | 175-380 | 355, 281, <u>234</u> | 140 | 1.05 |
| COD-d ₃ | | C ₁₈ H ₁₈ D ₃ NO ₃ | 302.15 | — | — | 374 | 175-380 | 358, 284, <u>237</u> | 140 | 1.05 |

Table 1 (continuation). Structures, physicochemical data and experimental parameters employed for the MS/MS determination of the target compounds.

| Structure ^a | Empirical formula | Monoisotopic MW | pK _a | log K _{ow} | Precursor (m/z) | Scan Range (m/z) | Products ^d (m/z) | Excit. Stor. Level (m/z) ^e | Excit. Ampl. (V) ^f |
|---|--|-----------------|-------------------|---------------------|-----------------|------------------|-----------------------------|---------------------------------------|-------------------------------|
|  | C ₁₇ H ₁₉ NO ₃ | 285.14 | 8.21 ^b | 0.89 ^b | 429 | 230-440 | <u>414</u> , 401, 287 | 180 | 1.1 |
|  | C ₁₇ H ₁₆ D ₃ NO ₃ | 288.14 | – | – | 432 | 230-440 | <u>417</u> , 404, 290 | 180 | 1.1 |
|  | C ₂₁ H ₂₃ NO ₅ | 369.16 | 7.95 ^b | 1.58 ^b | 327 | 200-335 | 284, <u>268</u> | 160 | 1.05 |
|  | C ₂₁ H ₂₈ O ₄ | 344.20 | 4.68 ^c | 6.21 ^c | 473 | 280-480 | <u>355</u> | 240 | 1.75 |
|  | C ₂₁ H ₂₅ D ₃ O ₄ | 347.20 | – | – | 476 | 280-480 | <u>358</u> | 240 | 1.75 |

^a The numbers indicate the position and number of deuterium atoms in the labelled internal standard.

^b Experimental values provided by PhysProp database (Syracuse Research Corporation): <http://www.syrres.com/what-we-do/databaseforms.aspx?id=386>

^c Software estimated values obtained from SciFinder Scholar 2007 database: <http://www.cas.org/products/sfacad/index.html>.

^d Most intense product ions, with the quantification ion being underlined.

^e Excitation storage level.

^f Excitation amplitude.

3. RESULTS AND DISCUSSION

3.1. Derivatisation

3.1.1. Selection of the derivatisation agent

The compounds included in this study contain polar functionalities (hydroxyls, carboxylic acids and amines) that have to be transformed prior to their GC-MS analysis. The aim of an ideal derivatisation reaction is not only decreasing the polarity of the native substances and increasing their volatility, but also improving their stability, chromatographic separation and detectability. To this end, several derivatisation strategies have already been considered in the literature for the GC-MS determination of drugs of abuse and their metabolites in biological samples [24,25]. Among them, silylation and acylation seemed to be the most efficient reactions.

So, in order to obtain the silyl-derivatives, the first derivatising agents contemplated in this study were MSTFA, BSTFA and MTBSTFA. Among them, the last two reagents failed to derivatise some of the aliphatic hydroxyl and the amine groups, even when they were added to the sample at a 40% (v/v) concentration and the mixture was heated at 80 °C for 2 h. On the other hand, MSTFA silylated all the reactive groups (amine, aromatic and aliphatic hydroxyl and carboxyl moieties) including the enolate form of MET. This reaction was performed in softer conditions (20% v/v, 60 °C, 1 h), with the only drawback of leading to relatively low m/z ions in the MS spectra of amphetamines and a single product ion in MS/MS experiments (see section 3.2). Thus, a mixed derivatisation strategy consisting of a first acylation step, in order to derivatise amine and phenolic groups, followed by silylation of the remaining reactive moieties with MSTFA was also considered. In-situ acetylation, by the addition of acetic anhydride to the aqueous samples and extraction of the derivatised analytes into an organic solvent [26], produced still broad, tailing peaks for amphetamines, so it was discarded. The combination between acylation in organic medium (ethyl acetate), using MBTFA, and then silylation with MSTFA (both added at a 20% v/v level and heated at 60 °C for 45 min) produced goods results for all the compounds in terms of peak shape and MS spectra, particularly for amphetamines. Unfortunately, the stability of the acyl-derivatives worked out to be lower than 48 h, even when they were kept at -20 °C.

In view of these results, MSTFA was selected as the sole derivatising agent and the derivatisation conditions were further studied in detail.

3.1.2. Optimisation of the MSTFA derivatisation

Silylation conditions (volume of MSTFA, temperature and time) were simultaneously evaluated using a Box-Behnken response surface design [27]. Derivatisation assays were carried out with aliquots from a pooled SPE extract corresponding to spiked aliquots of wastewater samples, operating in a similar way as described elsewhere for acidic pharmaceuticals [28]. The Box-Behnken design allows the optimisation of three factors with the lowest number of experiments [29]. Thus, all the variables were tested at three different levels: 20, 60 and 100 μL of MSTFA (final volume was always made to 200 μL); 40, 60 and 80 $^{\circ}\text{C}$; and 20, 60 and 100 min, respectively, resulting in a total set of 15 experiments.

Table 2. Experimental domain and relative importance (with their sign) of the main effects associated to each factor in the Box-Behnken design.

| Factor | MSTFA volume (μL) | Temperature ($^{\circ}\text{C}$) | Time (min) |
|---------------|--------------------------------|------------------------------------|------------|
| Low level | 20 | 40 | 20 |
| Central level | 60 | 60 | 60 |
| High level | 100 | 80 | 100 |
| AMP | +++ | - | - |
| MAMP | +++ | - | + |
| MDA | +++ | - | - |
| MDMA | +++ | + | - |
| MDEA | +++ | +++ | +++ |
| MET | +++ | +++ | +++ |
| BE | +++ | -- | - |
| THC | +++ | - | - |
| COD | +++ | --- | --- |
| MOR | +++ | -- | -- |
| THCCOOH | + | - | - |

+++ or --- indicate a statistically significant effect (95% confidence level), positive or negative.

++ or -- indicate that the effect was close to the statistically significance boundary.

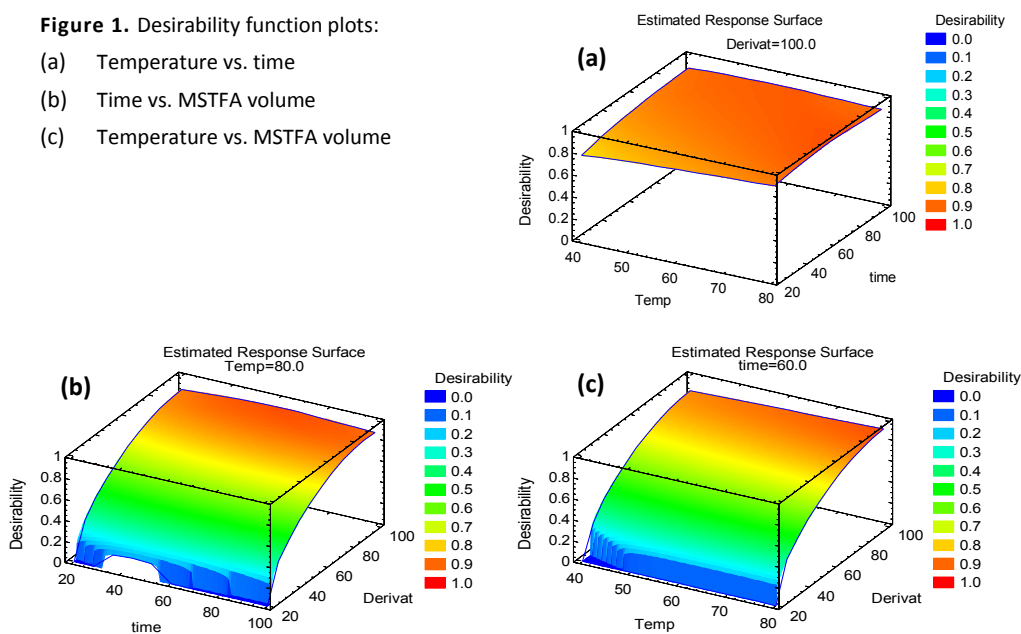
+ or - indicate that the effect was far from being statistically significant.

The analysis of the obtained results (**Table 2**) showed that the volume of the derivatising agent was the most important factor, affecting positively to the response of all the derivatised compounds with the exception of THCCOOH (on which it did not show to have a significant effect at a 95% of confidence level). MET and MDEA silylation was also influenced positively by time and temperature. At last, second order interactions presented small and non-significant effects, meaning that the three variables play independent roles on the

derivatisation reaction. Finally, the optimum conditions were selected using a desirability function [27,28], calculated taking into account only the derivatisation reactive compounds (**Figure 1**). The optimum values predicted by this function were 100 μL of MSTFA, 80 $^{\circ}\text{C}$ and 60 min, which resulted in a complete derivatisation reaction. The derivatives obtained with this optimised procedure turned to be stable for at least one week (see section 3.4).

Figure 1. Desirability function plots:

- (a) Temperature vs. time
- (b) Time vs. MSTFA volume
- (c) Temperature vs. MSTFA volume



3.2. GC-MS/MS

In order to improve the sensitivity and selectivity of the method, fragmentation and MS/MS detection conditions were optimised using a resonant waveform. These parameters are presented in **Table 1**. Additionally, **Figure 2** shows the main MS/MS fragmentation pathways for some representative compounds included in this study.

MS spectra of silylated amphetamines showed a predominant signal at m/z $[\text{M}-91]^+$, corresponding to the loss of the PhCH_2 group. The above parent ion was isolated in the ion trap and submitted to collision induced dissociation (CID), resulting in an only intense product ion at m/z 73, $[\text{Si}(\text{CH}_3)_3]^+$ (**Figure 2a**). In the case of COC and its derivatives (COE and BE), electron impact ionization produced a precursor ion at m/z $[\text{M}-121]^+$ as a result of the loss of

the benzoic group and then, the CID fragmentation of this parent yielded three intense signals at m/z 150, 122 and 82 (**Figure 2b**). Electron impact ionization of the opioids (HER, COD and MOR) caused the loss of one of the acetyl, methyl or trimethylsilyl groups attached to the alcohol moieties and then, the CID of the resulting ion originated a further cleavage in the second alcohol group and, finally, the loss of one of the two OH groups (**Figure 2c**). The most intense signal in the MS spectra of the trimethylsilylated THC and THCCOOH (**Figure 2d**) was the molecular ion, whose MS/MS resulted in three intense signals at m/z $[M-15]^{**}$, corresponding to the loss of one of the methyl groups, m/z $[M-56]^{**}$ (loss of C_4H_8 from the alkyl chain) and m/z $[M-71]^{**}$ (replacement of the whole alkyl chain by hydrogen). Finally, the mass spectra of MET showed a main fragment at m/z 296, whose CID originated three intense ions at m/z 281, 206 and 191, following the fragmentation path proposed in **Figure 2e**.

3.3. Solid-phase extraction

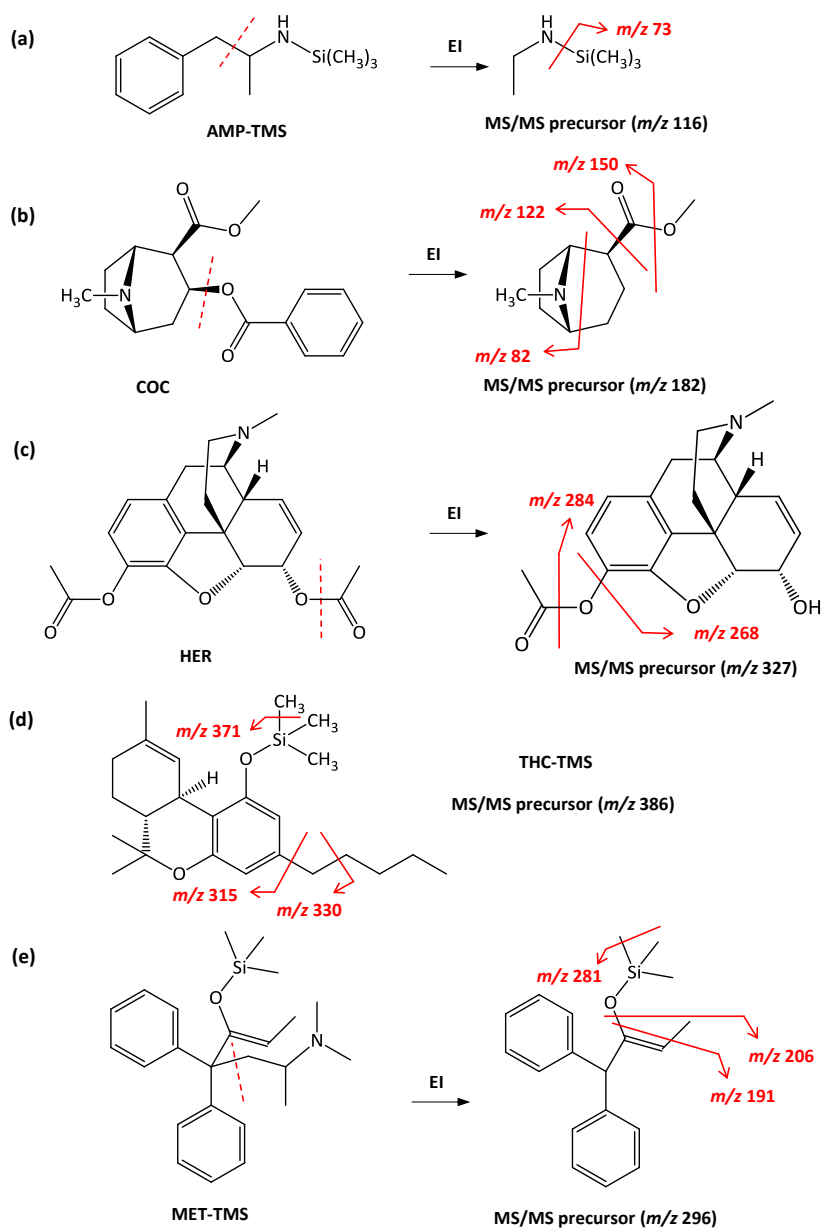
Optimisation of the solid-phase extraction procedure was made with the aim of reaching good extraction recoveries for all the target analytes with different physico-chemical properties. In order to achieve this goal, the Oasis HLB sorbent was selected because its hydrophilic-lipophilic balance provides a good efficiency in the extraction of compounds with a wide range of polarities and acidic characters, as it has been already proved for the multi-residue determination of pharmaceuticals and other emerging contaminants [30], including drugs of abuse [11,12].

Initially, the effect of sample pH (3, 6, 7, 8.5 and 12) on the retention of the analytes was investigated with 500 mL aliquots of ultrapure water, spiked at the 1 ng mL^{-1} level, passed through SPE cartridges containing 60 mg of the Oasis HLB sorbent. Compounds were eluted with 5 mL of ethyl acetate. As it is shown in **Figure 3**, acidic media produced a dramatic reduction on the recoveries of amphetamines (represented by AMP), COD and MOR, which were protonated in a considerable extent (pK_a values between 8.21 and 10.34) and, consequently, became too polar to be retained on the sorbent. On the other hand, a pH of 12 was satisfactory for extracting most of the aforementioned compounds (excepting MOR), but not for isolating COC, HER (likely due to the hydrolysis of the ester bonds) and THCCOOH (bearing two negative charges at this pH value). Intermediate pHs enabled the best overall recoveries, so, for further experiments, samples were adjusted at a pH value of 8.5. Anyway, non-quantitative recoveries were still observed for some of the compounds (e.g. MOR, MET or THCCOOH) so the possibility of analytes breakthrough or incomplete elution was investigated.

Possible breakthrough problems were evaluated by passing spiked aliquots of ultrapure water, adjusted at pH 8.5, through two cartridges connected in series. Considering 60 mg of

sorbent, the breakthrough volumes of AMP, MAMP, BE and MOR remained below 500 mL. On the other hand, at least 1 L volume samples could be concentrated using the 200 mg cartridges (data not given), which were selected to continue with the study.

Figure 2. EI-MS(/MS) fragmentation pathways for five representative analytes; TMS: trimethylsilyl.



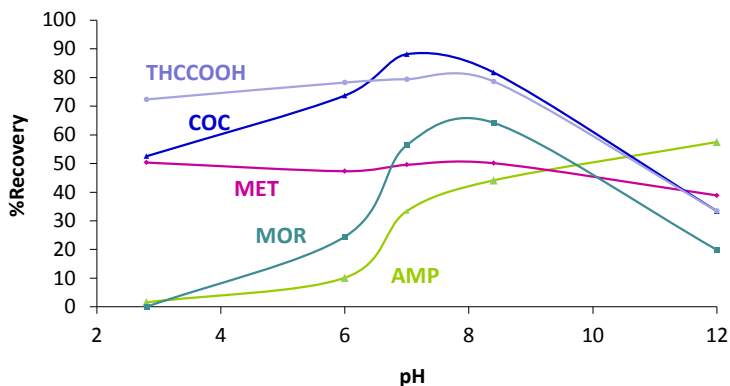


Figure 3. Influence of the pH on the SPE extraction efficiency with Oasis HLB 60 mg cartridges (n = 2).

Finally, different solvents and volumes were considered for the elution step, bearing in mind that aprotic polar solvents are the best suited to perform the further silylation reaction [31]. All compounds could be recovered using just 5 mL of ethyl acetate or acetonitrile. The only exception was THCCOOH. This species showed a low affinity to both solvents, requiring elution volumes over 10 mL. On the other hand, THCCOOH was completely eluted with 6 mL of acetone, but the trimethylsilyl derivatives of the amphetamines could not be found in the extract after derivatisation. The same happened when a standard of amphetamines in acetone was mixed with MSTFA. Likely, there is a reaction between the amino group of these compounds and the carbonyl moiety of the solvent, which prevents their further silylation with MSTFA. In view of these results, a two-steps elution strategy was adopted: first, amphetamines were eluted with 2 mL of ethyl acetate and, subsequently, the remaining compounds were recovered in a separated fraction with 8 mL of acetone. This second eluate was evaporated to dryness prior to its combination with the first one, avoiding by this way the contact between amphetamines and acetone.

3.4. Analytes stability

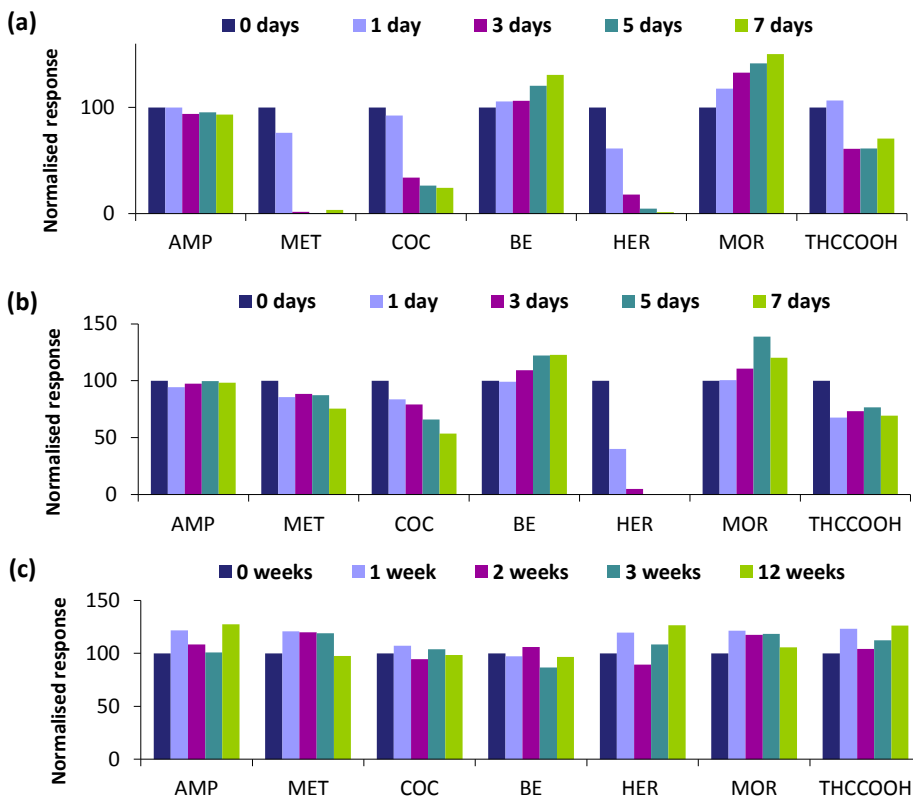
In order to achieve accurate results in the determination of drugs of abuse in the aqueous environment, it is essential to evaluate the stability of these compounds in water samples. In this context, Castiglioni et al. [18] investigated the stability of some illicit drugs and their metabolites in raw wastewater (stored in the dark at 4 °C for 3 days) and found a substantial decrease in the concentrations of COC and COE, accompanied by a parallel upsurge in the amount of their metabolite BE. The same pattern was observed for MOR, whose level was increased as a consequence of the degradation of other opioids. Gheorge et al. [19] proved that acidification of water samples (pH 2) improved the stability of COC and its

metabolite, BE. However, that report did not consider other drug classes and, also, acidified samples would need to be basified again before SPE, leading to a cumbersome procedure, so it was discarded in our study.

Instead, the preservative agent NaN_3 was considered in this research. To this end, raw wastewater was filtered through a combination of glass fibre prefilters and $0.45 \mu\text{m}$ nitrocellulose filters, spiked with target compounds at 100 ng mL^{-1} and divided in two fractions. One of them was poisoned with NaN_3 (0.2%) and both groups of samples were stored in the dark at $4 \text{ }^\circ\text{C}$. Every two days, an aliquot was submitted to the above described sample preparation procedure and analysed. Amphetamines and COD did not undergo any apparent degradation either in presence or in absence of NaN_3 . On the contrary, MET concentration decreased severely in the sample without azide (**Figure 4a**), whereas it presented a better stability in the poisoned one (**Figure 4b**). A high concentration diminution was also detected in the case of COC, COE, THC and THCCOOH (**Figure 4a**), whose degradation was slowed down but not completely stopped by NaN_3 (**Figure 4b**). HER levels fell down remarkably in both samples and, finally, MOR and BE concentrations experimented an increase (mainly in the not poisoned sample) as a consequence of the degradation of COD and HER, and COC and COE, respectively. In view of these results, the possibility of extracting the samples as soon as received, followed by storage of the frozen SPE cartridges was considered. In order to assess the feasibility of this approach, aliquots of the same raw wastewater were spiked with target analytes, processed immediately and the dried SPE cartridges were then kept at $-20 \text{ }^\circ\text{C}$ for different periods: 1-3 weeks (typical analysis time) and, additionally, 12 weeks. **Figure 4c** shows the results (average values for duplicate assays) obtained as a function of the storage time. Within the first 3 months, an acceptable stability was observed for all species (**Figure 4c**). Consequently, this procedure was selected as the best alternative to avoid analytes degradation.

Finally, the stability of the silylated compounds was also evaluated. Four replicates standards of 200 ng mL^{-1} of all analytes were prepared in ethyl acetate, derivatised with 50% MSTFA (heating at $80 \text{ }^\circ\text{C}$ during 60 min) and stored at $-20 \text{ }^\circ\text{C}$ for 1, 2, 4 and 7 days. Then, their GC-MS/MS signals were compared to the ones obtained with a fresh derivatised standard of the same concentration. No significant variations were observed, proving that silylated drugs are stable at $-20 \text{ }^\circ\text{C}$ for, at least, one week (data not shown).

Figure 4. Stability data, mean of two replicates, for some of the illicit drugs involved in this research: (a) wastewater stored at 4 °C; (b) wastewater stored at 4 °C after addition of NaN₃ (0.2%); and (c) aliquots of the same matrix concentrated on Oasis HLB SPE cartridges, which were dried and stored at -20 °C.



3.5. Method performance

Table 3 summarises some data related to the performance of the method. Linearity was investigated by injection of standards solutions at six different concentration levels between 5 and 500 ng mL⁻¹. The R² values for the corresponding graphs varied from 0.9900 to 0.9996. Instrumental precision studies were carried out by five injections of the same standard (50 ng mL⁻¹ level) over a 48 h period, resulting in relative standard deviations (% RSD) between 1.2 and 17.6%. Absolute limits of detection of the GC-MS/MS method (S/N = 3) ranged from 0.1 pg (for THCCOOH) to 34.5 pg (for COE and HER).

Table 3. Instrumental performance, overall internal standard-corrected recoveries ($n = 4$) and LODs for all the compounds.

| IS | GC-MS/MS | | | | SPE-GC-MS/MS | | | | | |
|---------|--------------------|-------------------|-----------------------|--------------------------|--------------|---------------------------------|-----------------------------|-------------|--------------------|----------------|
| | R^2 ^a | %RSD ^b | LOD (pg) ^c | River water ^d | %R (%RSD) | Treated wastewater ^e | Raw wastewater ^f | River water | Treated wastewater | Raw wastewater |
| | | | | (1.6) | | | | | | |
| AMP | 0.9981 | 1.7 | 1.6 | 112.1 (2.1) | 102.7 (3.5) | 106.4 (4.5) | 106.4 (4.5) | 0.8 | 3 | 7 |
| MAMP | 0.9993 | 5.3 | 2.9 | 110.0 (1.2) | 99.2 (3.4) | 119.2 (3.9) | 119.2 (3.9) | 2 | 7 | 7 |
| MDA | 0.9958 | 1.7 | 4.5 | 124.7 (6.2) | 107.4 (7.6) | 103.9 (10.2) | 103.9 (10.2) | 2 | 5 | 7 |
| MDMA | 0.9996 | 4.6 | 5.9 | 119.1 (1.7) | 85.4 (6.6) | 137.2 (2.5) | 137.2 (2.5) | 2 | 6 | 8 |
| MDEA | 0.9980 | 2.0 | 4.8 | 107.1 (1.2) | 93.2 (2.1) | 135.9 (8.7) | 135.9 (8.7) | 2 | 11 | 12 |
| MET | 0.9943 | 2.3 | 7.2 | 121.1 (4.4) | 114.1 (3.2) | 134.2 (3.2) | 134.2 (3.2) | 2 | 6 | 6 |
| COE | 0.9911 | 9.1 | 34.5 | 98.7 (7.1) | 107.0 (19.0) | 98.4 (8.0) | 98.4 (8.0) | 3 | 6 | 6 |
| COC | 0.9976 | 7.7 | 2.8 | 100.9 (7.9) | 115.8 (7.2) | 100.9 (10.2) | 100.9 (10.2) | 1 | 3 | 12 |
| BE | 0.9910 | 10.0 | 2.1 | 120.4 (20.0) | 125.1 (5.7) | 124.1 (9.4) | 124.1 (9.4) | 4 | 4 | 8 |
| THC | 0.9972 | 1.2 | 0.7 | 96.8 (18.6) | 108.4 (19.4) | 106.7 (3.4) | 106.7 (3.4) | 0.9 | 3 | 3 |
| COD | 0.9960 | 3.8 | 2.4 | 96.9 (12.5) | 112.9 (10.0) | 63.4 (10.8) | 63.4 (10.8) | 1 | 4 | 6 |
| MOR | 0.9990 | 3.8 | 4.1 | 91.7 (8.1) | 134.2 (17.7) | 68.8 (6.7) | 68.8 (6.7) | 3 | 11 | 11 |
| HER | 0.9900 | 17.6 | 34.5 | 73.9 (7.7) | 107.9 (25.0) | 97.5 (16.3) | 97.5 (16.3) | 13 | 15 | 15 |
| THCCOOH | 0.9968 | 3.4 | 0.1 | 93.3 (13.6) | 96.7 (5.0) | 66.6 (4.9) | 66.6 (4.9) | 1 | 1 | 1 |

^a 5-500 ng mL⁻¹ calibration.

^b Five injections of a 50 ng mL⁻¹ standard in a 48 h period.

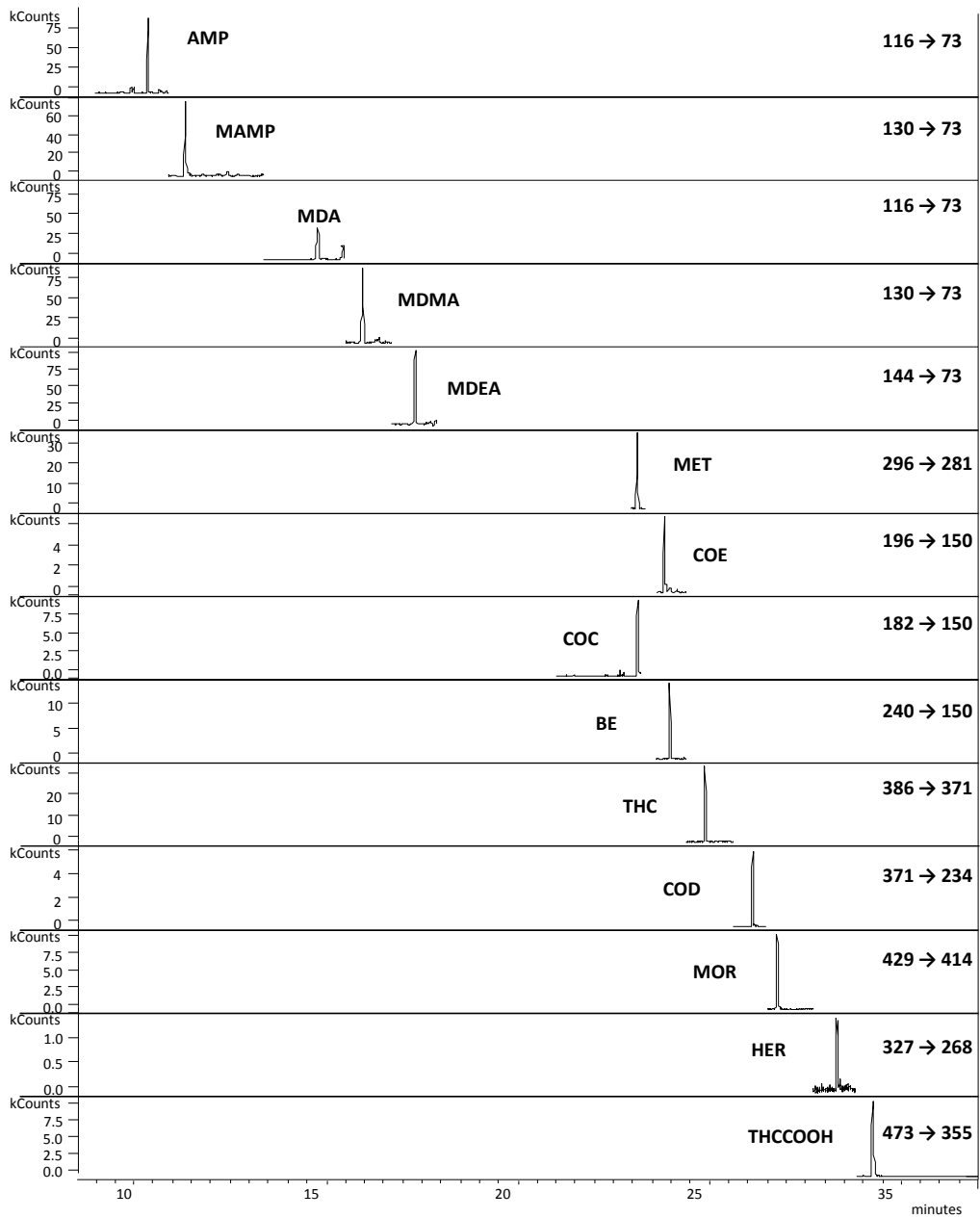
^c $S/N = 3$.

^d SPE of 500 mL samples spiked at 100 ng L⁻¹ level ($n = 4$).

^e SPE of 200 mL samples spiked at 250 ng L⁻¹ level ($n = 4$).

^f SPE of 100 mL samples spiked at 500 ng L⁻¹ level ($n = 4$).

Figure 5. Chromatogram of a river water sample spiked with 100 ng L⁻¹ of each compound.



Recoveries of the whole procedure were evaluated with spiked aliquots (from 100 to 500 mL) of different environmental water samples: river water (100 ng L^{-1}), treated (250 ng L^{-1}) and raw (500 ng L^{-1}) wastewater. A chromatogram of a spiked river is presented in **Figure 5**. The obtained recoveries ranged from 73.9 to 124.7% in the first matrix, between 85.4 and 134.2% in the second one and between 63.4 and 137.2% in raw wastewater. Finally, the estimated LODs of the whole method varied from 0.8 to 15 ng L^{-1} (**Table 3**). These values were calculated considering the signal to noise (S/N) ratios of chromatographic peaks for target compounds in the extracts from the above mentioned samples, and the volume of each matrix submitted to the SPE step. In the same manner, LOQs ($S/N = 10$) ranged between 2 and 60 ng L^{-1} (not given in **Table 3**, but they can be calculated as 3.3 times the LODs).

3.6. Application to real samples

The developed method was applied to determine the levels of the selected illicit drugs in waters from 3 rivers and 5 different STPs in the northwest of Spain. Grab samples were taken in each of these locations, without considering the residence time of the plants. **Figure 6** shows the chromatogram of one of the raw wastewater samples and the MS/MS spectrum of COC compared to that of a pure standard. From this comparison, it is evident that one of the advantages of ion trap-MS/MS systems is their capability to provide unambiguous confirmation of positive samples.

As shown in **Table 4**, COC, BE, COD, MOR and THCCOOH were found in most of the samples, whereas the rest of the compounds normally remained below the LOD. Usually, BE, the metabolite of COC, was the species at higher concentrations (up to 2 ng mL^{-1}) in the samples. This finding matches the data reported by other authors [6,12,14,18,21] and it highlights the widespread consumption of illicit drugs in developed countries.

Although results obtained for grab sewage water are useless to evaluate the efficiency of STPs, the presence of some of the investigate drugs of abuse in one of the processed river samples confirms their capability to reach surface water sources and to migrate into the aquatic environment.

Table 4. Concentrations (n = 4) found in different surface water and municipal wastewater samples. None of the analytes was found above the LODs in Lengüelle River and Dos Pasos Creek.

| | Sar River ^a | Mean concentration in ng L ⁻¹ (%RSD) | | | | | | | | | | | | |
|---------|------------------------|---|---------------------------|-------------------------------|---------------------------|-------------------------------|---------------------------|-------------------------------|---------------------------|-------------------------------|---------------------------|------|------|------|
| | | Treated STP-A ^b | Raw STP-A ^c | Treated STP-B ^b | Raw STP-B ^c | Treated STP-C ^b | Raw STP-C ^c | Treated STP-D ^b | Raw STP-D ^c | Treated STP-E ^b | Raw STP-E ^c | | | |
| AMP | n.d. ^d | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MAMP | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MDA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MDMA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MDEA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MET | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| COE | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| COC | 30 (11) | 43 (25) | 104 (21) | n.d. | 472 (10) | n.d. | 39 (22) | n.d. | 37.2 (7) | n.d. | 61 (26) | n.d. | n.d. | n.d. |
| BE | 316 (11) | 653 (18) | 571 (10) | 122 (11) | 2153 (8) | n.d. | 866 (9) | 164 (7) | 36 (7) | 689 (8) | n.d. | n.d. | n.d. | n.d. |
| THC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 23 (7) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| COD | 149 (49) | n.d. | 115 (26) | 129 (8) | 536 (15) | n.d. | 168 (8) | 426 (32) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MOR | 89 (3) | 140 (10) | 182 (9) | 103 (10) | 194 (4) | n.d. | 101 (6) | 76 (3) | 69 (14) | 90 (11) | n.d. | n.d. | n.d. | n.d. |
| HER | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| THCCOOH | 31 (5) | 77 (9) | 148 (6) | n.d. | 74 (7) | n.d. | 401 (7) | 49 (3) | 36 (6) | 13 (6) | n.d. | n.d. | n.d. | n.d. |

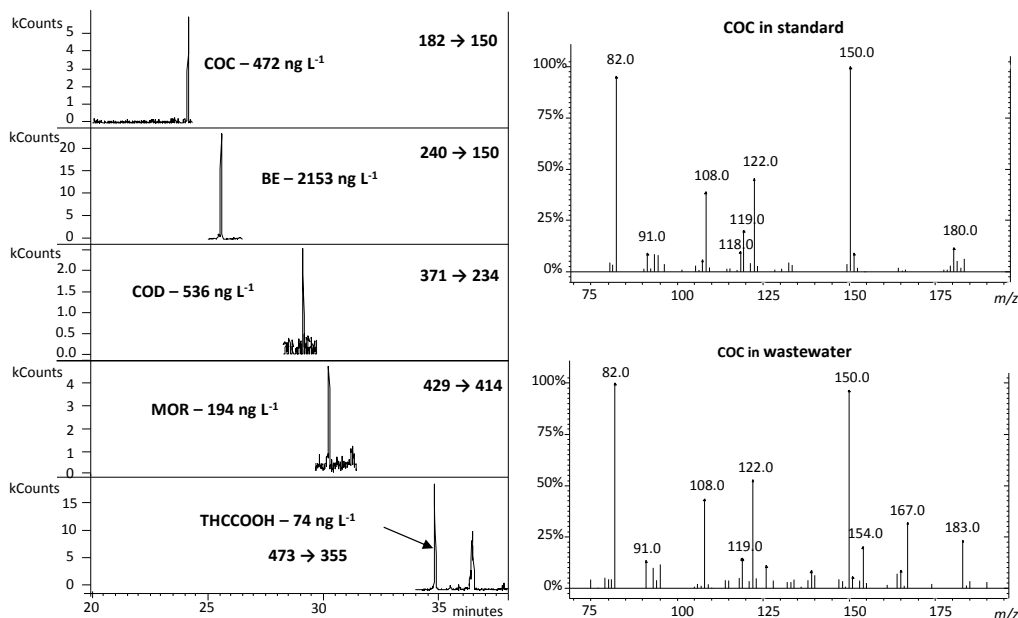
^a SPE of 500 mL samples (n = 4).

^b SPE of 200 mL samples (n = 4).

^c SPE of 100 mL samples (n = 4).

^d n.d.: not detected (<LOD).

Figure 6. Chromatogram of a (non-spiked) raw wastewater sample and MS/MS spectra corresponding to the peak of cocaine in the sample and in a standard.



4. CONCLUSIONS

A method for the determination of drugs of abuse in water samples by GC-MS/MS has been developed for the first time. Samples were extracted by SPE and the SPE cartridges, loaded with the analytes, can then be stored at $-20\text{ }^{\circ}\text{C}$ for at least 12 weeks, avoiding problems of drugs degradation in the sample. After extraction, elution was performed sequentially with two solvents and the concentrated extract was derivatised by silylation with MSTFA. Once optimised, this reaction was capable of derivatising the whole set of analytes considered and the SPE-GC-MS/MS method provided recoveries (63-137%) and LODs ($0.8\text{-}15\text{ ng L}^{-1}$) similar to those reported by SPE-LC-MS/MS, but at a lower cost and without the inconvenient of matrix effects. A further advantage of the ion trap GC-MS/MS instrument is its capability to record full scan MS/MS spectra, which can be used for the unequivocal confirmation of positives. On the other hand, the main drawbacks of the method are that it is not suitable for the analysis of the main metabolite of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which is a quaternary amine, and, to a minor extent, the fact that it is slower than those procedures based on LC-MS, as it requires 60 min for the derivatisation of the analytes.

The final application of the method to grab surface water and sewage samples confirmed COC, BE, THCOOH, MOR and COD as common emerging contaminants entering the aquatic environment.

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PUBLICACIÓN III.6

COMPARISON OF MOLECULARLY IMPRINTED, MIXED-MODE AND HYDROPHILIC BALANCE SORBENTS PERFORMANCE IN THE SOLID-PHASE EXTRACTION OF AMPHETAMINE DRUGS FROM WASTEWATER SAMPLES FOR LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY DETERMINATION

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Abstract

Recent studies have shown that amphetamines and other drugs of abuse residues occur in wastewater. Consequently, several methods have been developed for their determination by solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, a major drawback of these methods is the lack of selectivity during SPE, resulting in reduced sensitivity, due to matrix effects, and, in some cases, in low precision and poor accuracy. In order to tackle this problem, three different SPE alternatives have been evaluated in this work for the determination of five amphetamines: common hydrophilic-lipophilic balance (Oasis HLB), mixed-mode (Oasis MCX) and molecularly imprinted polymers (MIPs) sorbents. Among them, Oasis HLB showed the worst performance, as three amphetamines (MDA, MDMA and MDEA) could not be determined because of interfering signals in the LC-MS/MS chromatogram, and amphetamine recoveries could not be corrected by the use of the deuterated analogous internal standard. Oasis MCX permitted the determination of all the target analytes, but still with strong signal suppression: ca. 70% signal drop with wastewater samples, which, in this case, could be corrected by internal standards providing acceptable trueness (overall recoveries: 101-137%), precision (RSD: 2.0-12%) and limits of detection (LOD: 1.5-5.2 ng L⁻¹). Alternatively, MIPs rendered cleaner extracts with lower matrix effects (ca. 30% signal drop), and thus lower LODs (0.5-2.7 ng L⁻¹) and even better trueness (92-114% overall recovery) and precision (RSD: 1.5-4.9%). The final application of the method with MIP cartridges showed the presence of MDA and MDMA in the 7 analysed wastewaters at the 4-20 ng L⁻¹ level.

Keywords: molecularly imprinted polymers; solid-phase extraction; illicit drugs; liquid chromatography-mass spectrometry; matrix effects; wastewater.

1. INTRODUCTION

Zuccato et al. reported cocaine and its main metabolite for the first time as novel emerging pollutants in sewage and surface water in 2005 [1]. One year later, the same authors extended the analytical methodology to the determination of other illegal drugs of abuse and their metabolites, including amphetamines, cannabinoids, opiates, etc. [2]. The application of the method showed these substances occurring in wastewater at concentrations ranging from the low ng L^{-1} (e.g. amphetamines) up to the $\mu\text{g L}^{-1}$ level (cocaine metabolite). In addition to their environmental concern, the determination of drugs of abuse in wastewater represents a new tool for the estimation of drug consumption patterns, which should be more precise and direct than the actual methodologies based on population surveys, crime statistics, etc. [1,3,4].

Since those first reports, several researchers have dedicated their efforts to the development of analytical methodologies for the measurement of drugs of abuse and to the estimation of their environmental concentrations and fate. Thus, several publications have shown the ubiquity of these new pollutants, as illicit drugs occurrence in wastewater and surface waters in several European countries –Italy [3,5], Switzerland [3], UK [3,6,7], Belgium [8,9], Germany [10], Ireland [11] and Spain [12-14]– and in USA [15] has already been reported. Even some of these drugs and their metabolites have been found to resist drinking water treatment, reaching tap water at the $1\text{-}100 \text{ ng L}^{-1}$ level [13,14]. Moreover, some recent works have also reported illicit drugs associated to airborne particulate matter [16-19].

As recently reviewed by Castiglioni et al. [20], liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the technique of choice for the determination of drugs of abuse in the environment because of its good sensitivity without need for analytes derivatisation. However, LC-MS/MS methods have to face the well-known problem of matrix effects when analysing complex samples. Particularly, strong signal suppression effects (40-90%) have been reported during the analysis of drugs of abuse in wastewater [10,21]. Though deuterated internal standards are available for most of these drugs/metabolites, they cannot always completely compensate this problem [22], and even so, they do not avoid the inherent loss of sensitivity. Thus, more selective sample preparation methodologies that result in a lower amount of co-extracted matrix constituents are highly desirable.

Almost all published methods for the determination of illicit drugs in water environmental samples employ solid-phase extraction (SPE) as the preconcentration

technique. The sorbents employed for SPE are either hydrophilic reversed-phase Oasis HLB [10,23,24] or the mixed-mode modification of them, Oasis MCX [2,22] (or Strata-XC [11]). The second ones have been used to improve the retention of most drugs of abuse, because of their basic properties, but their dual cationic-exchange/reversed-phase character can also be exploited to improve the selectivity of the SPE process, as suggested, for instance, by the manufacturer [25], or as it has already been proved for the determination of basic pharmaceuticals in biological samples [26,27]. A further alternative is the use of molecularly imprinted polymers (MIPs), which have very specific shape- and H-bonding-recognition characteristics. Actually, β -blocker- [28] and NSAID-class selective [29] MIPs have recently been tested for the selective SPE of these two pharmaceutical classes from wastewater in combination with LC-MS/MS, showing an impressive reduction in matrix effects, as compared to other common SPE sorbents.

Thus, taking into account that amphetamine class-selective MIPs are commercially available, this work aims to evaluate the performance of this sorbent as compared to Oasis HLB and Oasis MCX for the extraction and concentration of amphetamine drugs from wastewater samples in combination with LC-MS/MS determination. SPE recoveries, matrix effects, overall method recoveries, repeatability and limits of detection are evaluated.

2. EXPERIMENTAL

2.1. Chemicals and stock solutions

HPLC grade methanol, acetonitrile, acetic acid, and ammonia were supplied by Merck (Darmstadt, Germany), formic acid by Sigma-Aldrich (Steinheim, Germany) and ammonium acetate was from Riedel de Haën (Seelze, Germany). Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, MA, USA).

Amphetamine (AMP), methamphetamine (MAMP), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymetamphetamine (MDMA) and 3,4-methylenedioxyethylamphetamine (MDEA) individual standard solutions (1 mg mL^{-1} in methanol, each) were purchased from Cerilliant (Round Rock, TX, USA). The deuterated internal standards (ISs) individual solutions (0.1 mg mL^{-1} in methanol, each) were also from Cerilliant. The structures and some relevant physico-chemical data of these substances are compiled in **Table 1**.

Stock mixtures of all analytes ($10 \text{ } \mu\text{g mL}^{-1}$) or ISs (also $10 \text{ } \mu\text{g mL}^{-1}$) were prepared in methanol and stored at -20°C until use. Calibration standards with increasing concentrations of analytes and 100 ng mL^{-1} of ISs were prepared in methanol:water (1:1) containing 2% NH_3 .

2.2. Samples

Municipal wastewater grab samples were taken from four different urban wastewater treatment plants (WWTPs) in NW Spain during June 2009. All WWTPs consist of a primary and an aerobic secondary treatment. WWTPs A, B and D receive wastewaters from ≈ 100000 inhabitants and WWTP C from ≈ 20000 inhabitants. A single raw (influent) and treated (effluent) wastewater sample was collected from each WWTP, except from WWTP D, where only the treated wastewater was collected. All samples were collected in amber glass bottles and they were stored in the dark at 4 °C until analysis. According to our experience, amphetamines are stable at this temperature in wastewater for at least one week [30].

Particulate matter was filtered just before extraction through a combination of glass fibre prefilters and 0.45 μm nitrocellulose filters (both from Millipore). Filtration was checked not to lead to losses due to adsorption of analytes on the filter or wastewater suspended matter (data not shown). Filtered samples were adjusted to the desired pH, spiked with labelled standards (10 ng) and, in the case of recovery studies, also with analytes.

2.3. Solid-phase extraction

Oasis HLB protocol: Oasis HLB 60 mg cartridges (Waters, Mildford, MA, USA) were conditioned with 2 mL of MeOH and 2 mL of pH 8 Milli-Q water. Then, 50 mL of sample (adjusted to pH ≈ 8) were percolated through the cartridges at ca. 5 mL min^{-1} . Subsequently, cartridges were washed with 2 mL of pH 8 Milli-Q water and vacuum dried for 10 min. Finally, amphetamines were eluted with 3 mL of MeOH.

Oasis MCX protocol: Oasis MCX 60 mg cartridges (Waters) were conditioned with 3 mL of MeOH and 3 mL of pH 4 Milli-Q water. Then, 50 mL of sample (adjusted to pH ≈ 4) were percolated at ca. 5 mL min^{-1} , and cartridges were sequentially washed with 1 mL of pH 4 Milli-Q water and 1 mL of MeOH. Finally, amphetamines were eluted with 3 mL of MeOH containing 5% NH_3 .

MIP protocol: SupelMIP-Amphetamine 25 mg cartridges (Supelco, Bellefonte, PA, USA) were conditioned with 1 mL of MeOH and 1 mL of pH 8 Milli-Q water. Then, 50 mL of sample (adjusted to pH ≈ 8) were percolated by gravity through the MIP cartridges. Interferences were successively washed off by 2 \times 1 mL pH 8 Milli-Q water, 1 mL acetonitrile:water (60:40) and 1 mL of acetonitrile containing 1% acetic acid, as recommended by the supplier. Elution was finally performed with 2 \times 1 mL MeOH containing 1% formic acid.

The eluates resulting from all the above mentioned SPE protocols were blown down with a gentle stream of nitrogen, reconstituted in 100 μL of MeOH:water (1:1) containing 2% NH_3 and transferred to a vial equipped with a 200 μL insert for LC-MS/MS determination.

2.4. Liquid chromatography-mass spectrometry

The liquid chromatographic system consisted of two ProStar 210 high-pressure mixing pumps (Varian, Walnut Creek, CA, USA), a Metachem Technologies vacuum membrane degasser (Bath, UK), and an autosampler and thermostated column compartment ProStar 410 module (Varian). The LC was interfaced to a triple quadrupole 1200L mass spectrometer equipped with an electrospray interface (Varian). Nitrogen, used as nebulising and drying gas, was provided by a nitrogen generator (Domnick Hunter, Durham, UK). Argon (99.999%) was used as collision gas. Instrument control and data acquisition were performed with the Varian MS Workstation software.

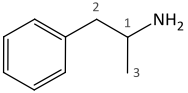
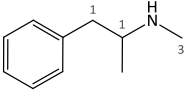
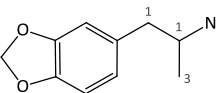
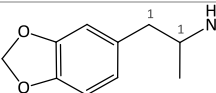
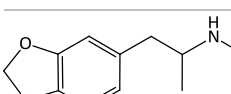
The ESI interface was operated in the positive mode with a needle potential of 5 kV, a source temperature of 50 $^{\circ}\text{C}$, a desolvation temperature of 200 $^{\circ}\text{C}$, a nebulising gas pressure of 50 psi (345 kPa) and a drying gas pressure of 20 psi (138 kPa). Argon pressure in the collision cell was kept at 1.5 mTorr for MS/MS measurements. The mass peak width of the first and last quadrupoles was adjusted to 1.8 and 1.5 amu, respectively.

Selection of the most intense MS/MS transitions was done by infusion of the individual compounds at a concentration level of ca. 1 $\mu\text{g mL}^{-1}$ in MeOH:water (1:1) in ESI positive mode. The intensity of the $[\text{M}+\text{H}]^+$ ion was optimised by varying the capillary voltage and, subsequently, the $[\text{M}+\text{H}]^+$ ion was subjected to MS/MS fragmentation experiments and the most intense product ions were obtained by varying the collision energy with the aid of the automated routines included within the software package. Thus, quantification of all compounds was made by recording the two most intense transitions for each analyte, and just one for each IS, in selective-reaction monitoring (SRM) with a dwell-time of 100 ms per transition, all compounds being included into a single segment. Individual ESI-MS/MS parameters for each compound are summarised in **Table 1**.

LC separation was carried out on a 100 \times 2.1 mm Halo C_{18} (2.7 μm) porous shell column, which consists of a 1.7 μm inert core coated with a 0.5 μm C_{18} layer (Advanced Materials Technology, Nes-Ziona, Israel). Fused core columns provide sharper peaks than conventional 3 μm C_{18} even with our conventional LC instrument [31,32]. The column was protected with a 4 \times 2 mm C_{18} guard cartridge provided by Phenomenex (Torrance, CA, USA) and the temperature was set at 50 $^{\circ}\text{C}$. A dual eluent system of water (A) and methanol (B), both with 5 mM

ammonium acetate, was used. The flow rate was 0.2 mL min⁻¹ and the gradient was as follows: 0 min (2% B), 5 min (2% B), 15 min (100% B), 18 min (100% B), 19 min (2% B) and 26 min (2% B). Injection volume was set at 20 µL. Higher injection volumes cannot be used as they result in broadened peaks.

Table 1. Structures, physicochemical data and experimental parameters employed for the SRM determination of amphetamines.

| Structure ^a | pK _a ^b | log K _{ow} ^b | Monoisotopic MW | Transitions (m/z) | CV / CE ^c | I ₁ / I ₂ ± tol. ^d | |
|---|------------------------------|----------------------------------|-----------------|-------------------|------------------------|---|-----------|
|  | AMP | 9.9 | 1.8 | 135.1 | 136 → 91 136 → 119 | 30 / 14 30 / 6 | 1.1 ± 0.2 |
| | AMP-d ₆ | - | - | 141.1 | 142 → 93 | 30 / 14 | - |
|  | MAMP | 10.4 | 2.1 | 149.1 | 150 → 91 150 → 119 | 30 / 15 30 / 7 | 1.9 ± 0.4 |
| | MAMP-d ₅ | - | - | 154.1 | 155 → 92 | 30 / 16 | - |
|  | MDA | 9.9 | 1.7 | 179.1 | 180 → 163 180 → 105 | 30 / 6 30 / 19 | 4.7 ± 1.2 |
| | MDA-d ₅ | - | - | 184.1 | 185 → 168 | 30 / 7 | - |
|  | MDMA | 10.3 | 1.8 | 193.1 | 194 → 163 194 → 105 | 30 / 8 30 / 20 | 3.8 ± 1.0 |
| | MDMA-d ₅ | - | - | 198.1 | 199 → 165 | 30 / 9 | - |
|  | MDEA | 10.3 | 2.3 | 207.1 | 208 → 163 208 → 105 | 30 / 9 30 / 22 | 3.8 ± 1.0 |
| | MDEA-d ₅ | - | - | 212.1 | 213 → 163 | 30 / 9 | - |

^a The numbers indicate the position and number of deuterium atoms in the labelled internal standard.

^b Software estimated values obtained from Sci Finder Scholar 2007 database:
<http://cas.org/products/scifindr/index.html>.

^c Capillary voltage (V) / Collision energy (eV).

^d Intensity ration between the first and second SRM transition, and tolerances according to 2002/657/EC Decision [33].

2.5. Evaluation of recoveries and matrix effects

Distinction between SPE recoveries, matrix effects at the LC-MS/MS and overall method recoveries was done by spiking samples either before or after SPE with the same amount of analytes. Thus, four aliquots of each sample (raw and treated wastewater from WWTP A) were spiked with the analytes and ISs before SPE and other four were spiked after the SPE (i.e. over

the extract). Also, two samples were measured without analytes spike. Then, the response factor of all the spiked samples, after non-spiked sample signal subtraction (non-spiked samples concentrations were lower than 20 ng L^{-1} in all cases, see section 3.4.), was compared to the response factor of a calibration curve prepared in MeOH:H₂O containing 2% NH₃. In this way, three response factors are obtained: one from the pure standards (R_1), a second one from the samples spiked before SPE (R_2) and a third one from the spike over the extract (R_3). Therefore, the matrix effects percentage (%ME) is calculated as $100 \times R_3/R_1$; the recovery accounting exclusively from the sample preparation (% R_{SPE}) step as $100 \times R_2/R_3$; and finally the overall method recovery percentage (% R_{overall}) as $100 \times R_2/R_1$. Further details on this methodology have already been described in the literature [34-36].

3. RESULTS AND DISCUSSION

3.1. Preliminary considerations

The three SPE protocols compared here were selected according to their reported usage in the literature, as explained in section 1, and to their potential for a more selective extraction of amphetamines from wastewater.

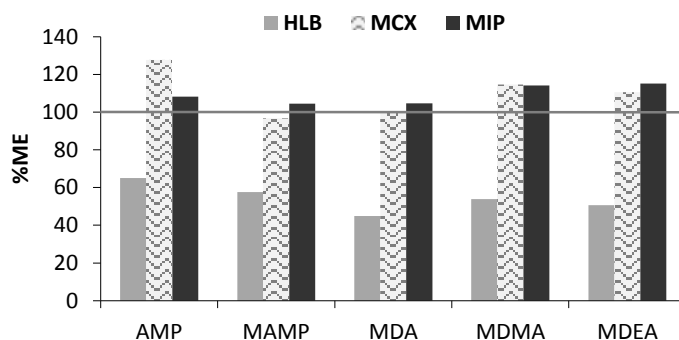
Among them, 200 mg Oasis HLB [23] and 60-150 mg Oasis MCX [2,22] cartridges have been employed in published methods considering amphetamines. Thus, the Oasis HLB protocol was adopted but downscaled to 60 mg cartridges, which are cheaper than the 200 mg ones and have enough capacity for the volume of sample preconcentrated. In the case of Oasis MCX, also 60 mg cartridges were used and the published methods were slightly modified by reducing the elution volume to 3 mL of MeOH (5% NH₃) and by including a washing step using 1 mL of MeOH. This washing step contributes to remove those interfering chemicals which were retained by reversed-phase interactions, while the amphetamines remain retained by an ion-exchange mechanism. Thus, this procedure is expected to reduce matrix effects in LC-MS/MS determination as it has been proved for other basic pharmaceuticals in biological samples [25-27].

Finally, to the best of our knowledge, MIPs had not been tested yet in the determination of amphetamines in wastewater. Thus, the manufacturer recommended protocol was followed in the washing steps, as this is the most critical part to gain selectivity when MIPs are used.

Also, a preliminary test was performed by extracting 20 mL of treated wastewater spiked with 100 ng of each amphetamine, with the final extract reconstituted to 200 μL (100-

fold preconcentration factor) for the three sorbents. The results of this test showed a very good performance of the MIP and MCX materials in comparison with Oasis HLB regarding matrix effects (%ME), **Figure 1**. Moreover, %R_{SPE} was satisfactory in all cases (85-115%, data not shown). However, this preconcentration factor was considered insufficient to reach the required LODs for real water sample analysis and the breakthrough volume of the MIP materials was studied in detail.

Figure 1. Preliminary comparison of matrix effects (%ME) during LC-ESI-MS/MS determination of amphetamines in effluent obtained by the three SPE protocols for a 100-fold preconcentration factor (n = 2).



3.2. Breakthrough volume and sample loading flow on MIPs

Breakthrough volume was studied only in the case of MIPs, as this phenomenon is expected to take place first for these cartridges of only 25 mg than with the 60 mg Oasis HLB and MCX, which are also expected to have a larger surface area [37]. Moreover, the capacity of MIPs is actually limited by the number of specific sites and may thus depend on the sample matrix, when some cross-reactivity with matrix constituents may occur [38]. Hence, the maximum volume that can be loaded was studied with both ultrapure and raw sewage water, spiked with 100 ng of analytes, in the 10-100 mL loading volume range, eluted and made to a final volume of 200 μ L before injection.

As shown in **Figure 2**, no significant drop on recovery (%R_{overall}) was observed even for 100 mL of Milli-Q water (**Figure 2a**), whereas, for raw wastewater, the maximum volume that can be percolated before %R_{overall} decreases is limited to 50 mL of sample (**Figure 2b**). Similar results have been described in the literature [28]. Thus, in order to differentiate between matrix effects and breakthrough, a study of %ME was performed with the 100 mL samples proving that the decrease in %R_{overall} is mostly due to breakthrough of analytes, while matrix effects on the ESI process contribute in less than 25% to the reduction of overall recoveries (**Figure 2c**). Therefore, the volume of samples to be loaded on the MIPs, and for comparison on the other sorbents, was fixed at 50 mL.

Sample loading flow is also considered an important parameter during SPE with MIPs, and, actually, the manufacturer recommends feeding samples by gravity. This implies a quite long sample loading step when 50 mL of water are processed (ca. 50 min). Therefore, the possibility of loading cartridges with the aid of different levels of vacuum (17 and 27 kPa) was investigated, but recoveries dropped to ca. 50% (data not given). In addition, vacuum resulted in the compaction of the sorbent bed, which finally resulted in an even lower flow rate. Thus, the use of vacuum was discarded and samples were loaded on MIPs by gravity.

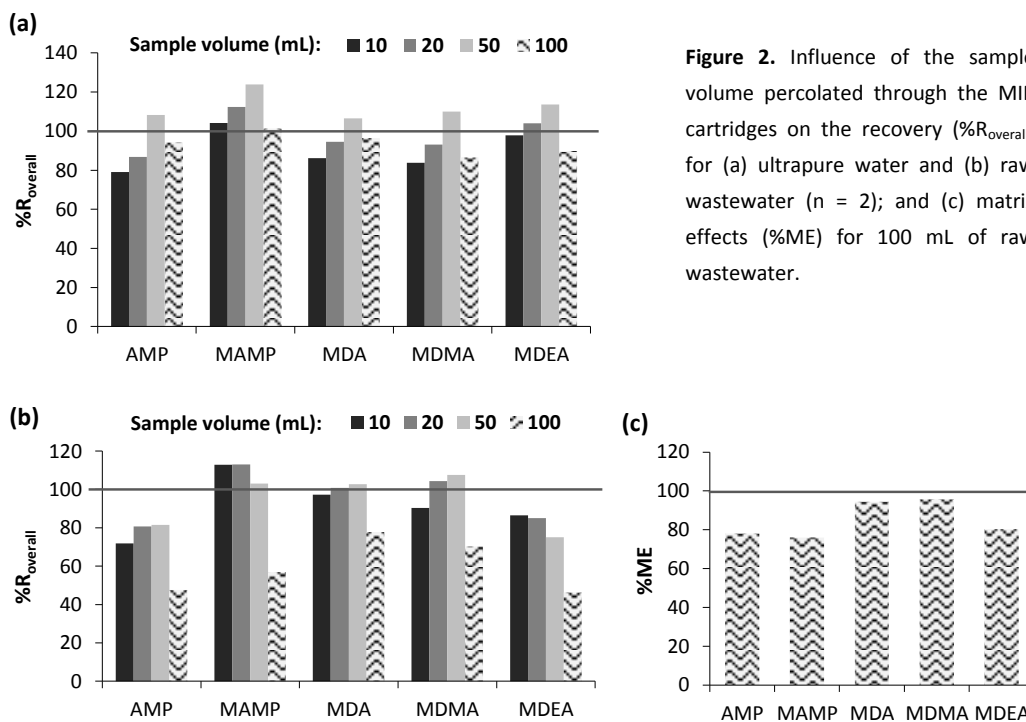


Figure 2. Influence of the sample volume percolated through the MIP cartridges on the recovery (%R_{overall}) for (a) ultrapure water and (b) raw wastewater ($n = 2$); and (c) matrix effects (%ME) for 100 mL of raw wastewater.

3.3. Comparison of final SPE protocols

3.3.1. Evaluation of SPE recoveries and matrix effects

In a first step, absolute (without IS correction) recoveries accounting exclusively from the SPE processes (%R_{SPE}) were calculated (see section 2 for details) from 100 ng L⁻¹ level spiked samples. As shown in **Figure 3a** and **3b**, Oasis MCX sorbents provided the best performance in terms of analytes retention, with the MIP cartridges providing recoveries slightly lower, but still acceptable (> 75%). In the case of Oasis HLB, recoveries for AMP and MAMP were also quite good, but the other three analytes could not be quantified due to very strong signal suppression and interferences occurring on their quantification SRM transitions

for this sorbent (**Figure 4**). Indeed, this latter problem might be partly solved by increasing the resolution of the quadrupoles, but this would result in a substantial decrease of sensitivity.

Regarding matrix effects (**Figure 3c and 3d**), MIPs provide a clearly better performance than any of the Oasis, with %ME values in the 50-90% range. Also, in spite of the 100% methanol washing step, signal suppression was still quite strong for Oasis MCX with the final protocol, implying a 500-fold pre-concentration: %ME at the 20-35% range. Finally, the Oasis HLB protocol is not suitable for MDA, MDMA and MDEA due to very strong signal suppression and interfering signals in the first SRM transition, as mentioned before. These findings are in good agreement with a recent work of Bijlsma et al. [22], who described signal suppression of amphetamines in river water samples pre-concentrated 50-fold on Oasis MCX cartridges producing %ME values of ca. 60%. Additionally, in the case of wastewater, these authors recommended dilution of wastewater since, otherwise, signal suppression was so strong that could not even be compensated by deuterated internal standards [22].

Figure 3. Recoveries ($n = 4$) accounting for the sample preparation step (%R_{SPE}): (a) treated wastewater, (b) raw wastewater; and accounting for matrix effects during the LC-ESI-MS/MS determination (%ME): (c) treated wastewater, (d) raw wastewater. Samples spiked with 100 ng L⁻¹ of analytes.

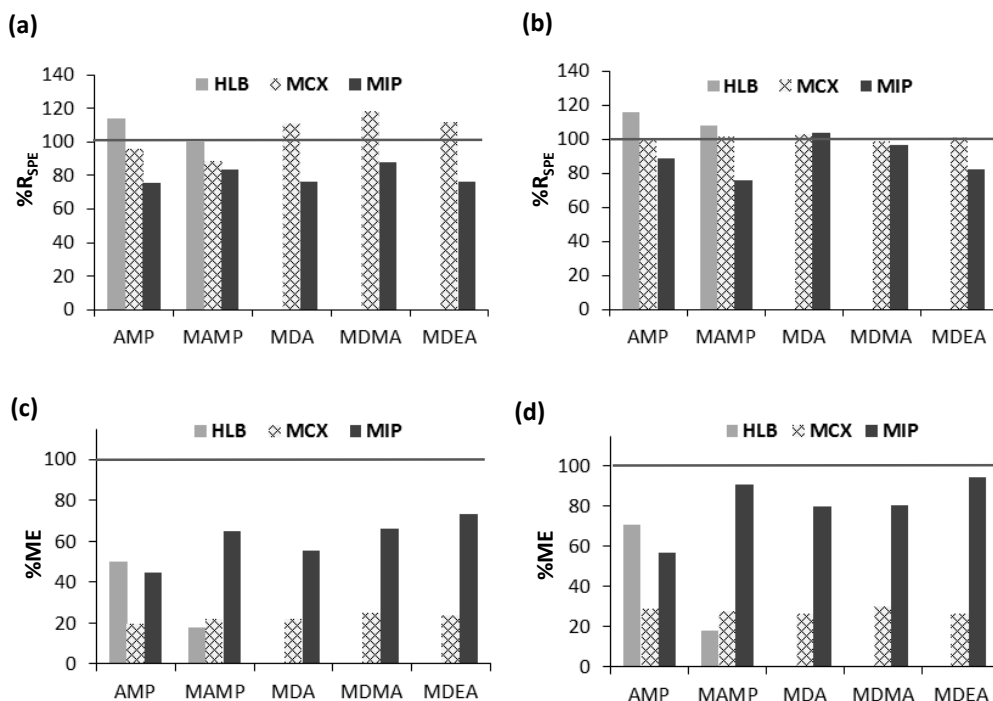
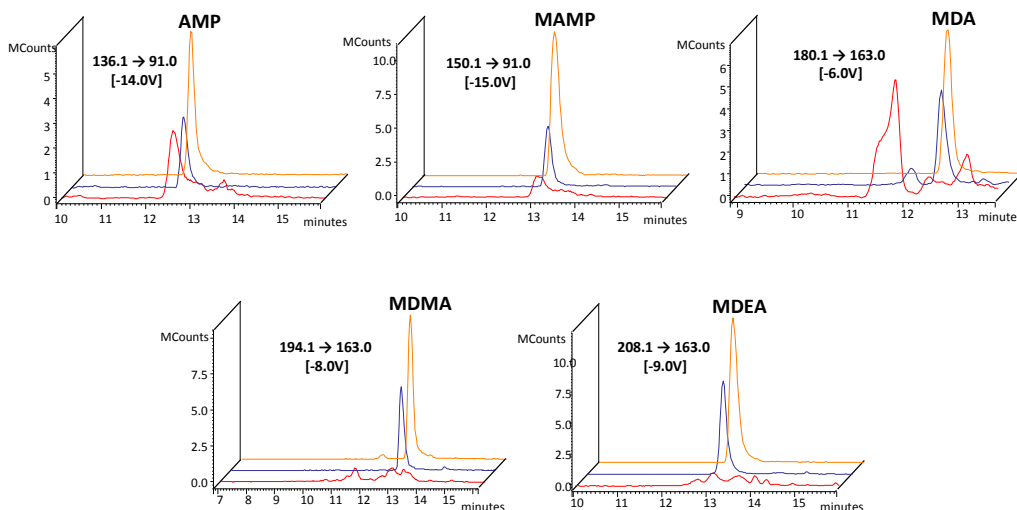


Figure 4. Chromatograms of a treated wastewater sample spiked at the 100 ng L⁻¹ level obtained after SPE with Oasis HLB (red), Oasis MCX (blue) and MIPs (orange).



3.3.2. Overall performance

After comparing the selectivity and capacity of the different sorbents, in terms of absolute %R_{SPE} and %ME, the overall performance was assessed. Thus, internal standard corrected overall method recoveries (%R_{overall}), %RSD and limits of detection (LODs) were calculated with spiked (100 ng L⁻¹ of analytes and ISs) wastewater samples.

As presented in **Table 2**, the performance of the Oasis HLB protocol was unacceptable, as discussed before for MDA, MDMA and MDEA, and because overall corrected recoveries for AMP resulted in a serious overestimation, due to interferences in the transition used for its quantification. Actually, recoveries obtained with the second SRM transition for this analyte were quite good (107-109%) but, since the two transitions ratio was not maintained, confirmation would not be possible due to the lack of further sensitive SRM transitions for this compound. On the other hand, MIP recoveries are slightly better (91-114%) than those obtained with Oasis MCX (101-137%). Regarding precision, MIPs provide again lower RSD values (1.5-4.9%) than Oasis MCX (2.0-11.9%). Anyhow, the accuracy and precision of these two sorbents can be regarded as satisfactory and they show the same range of values as those reported in the literature [2,22,23]. The sensitivity of the method also follows the same trend, with Oasis MCX LODs being approximately twice the obtained with MIPs, (which is clearly a result of matrix effects, as discussed in the previous section) and in the same order of magnitude of other published methods [2,22,23].

Table 2. Internal standard-corrected overall recoveries and LODs obtained with the final protocols.

| | %R _{overall} (%RSD) ^a | | | | | | | | | | | |
|------|---|--------------|-------------|-------------|-------------|-------------|----------------|-----|-----|-----|-----|-----|
| | Treated wastewater | | | | | | Raw wastewater | | | | | |
| | HLB | MCX | MIP | HLB | MCX | MIP | HLB | MCX | MIP | HLB | MCX | MIP |
| AMP | 348.4 (8.7) | 118.7 (11.9) | 104.6 (1.5) | 450.6 (1.9) | 117.5 (6.6) | 98.2 (3.5) | 4.8 | 4.4 | 2.7 | | | |
| MAMP | 111.4 (2.5) | 103.5 (2.0) | 91.6 (4.9) | 111.7 (4.1) | 101.2 (4.2) | 92.0 (1.8) | 7.2 | 1.8 | 0.8 | | | |
| MDA | - | 133.4 (2.9) | 101.9 (2.7) | - | 123.4 (5.1) | 100.5 (4.5) | - | 5.2 | 2.2 | | | |
| MDMA | - | 137.2 (2.6) | 113.9 (3.9) | - | 126.5 (4.5) | 110.9 (4.7) | - | 2.3 | 2.3 | | | |
| MDEA | - | 110.7 (3.1) | 96.5 (2.7) | - | 103.4 (2.8) | 98.7 (1.8) | - | 1.5 | 0.5 | | | |

^a Samples spiked at the 100 ng L⁻¹ level (n = 4).

^b Estimated from spiked raw wastewater samples as S/N = 3.

Table 3. Concentrations (ng L⁻¹) found in different wastewater samples (n = 4) after SPE-LC-MS/MS analysis with MIPs.

| | Mean (%RSD) ^a | | | | | | | | | | | |
|------|--------------------------|------------|----------------|-------------|----------------|------------|----------------|------------|--|--|--|--|
| | Treated WWTP-A | Raw WWTP-A | Treated WWTP-B | Raw WWTP-B | Treated WWTP-C | Raw WWTP-C | Treated WWTP-D | Raw WWTP-D | | | | |
| AMP | n.d. | n.d. | n.d. | 9.1 (4.7) | n.d. | n.d. | n.d. | n.d. | | | | |
| MAMP | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | | | | |
| MDA | 13.4 (35.2) | 13.3 (5.8) | 13.3 (11.7) | 17.4 (16.0) | 19.6 (7.9) | 19.0 (5.0) | 6.0 (7.6) | | | | | |
| MDMA | 9.3 (13.6) | 10.6 (8.0) | n.d. | 4.5 (9.3) | 3.6 (13.9) | 3.7 (15.0) | 6.4 (3.8) | | | | | |
| MDEA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | | | | | |

^a n.d.: not detected (<LOD).

3.4. Application to real samples

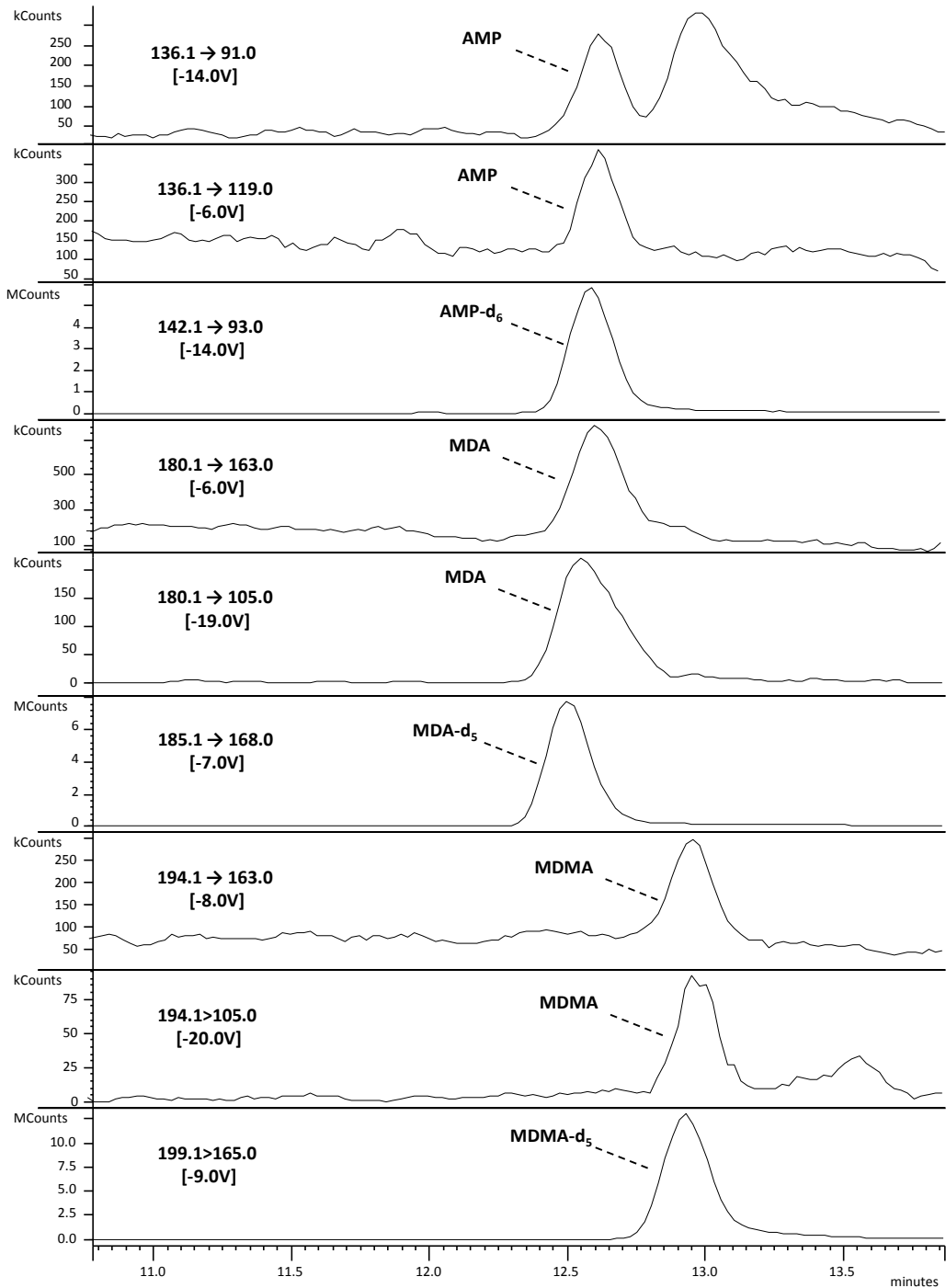
The method based on SPE with MIPs was finally applied to the determination of the concentrations of amphetamines in seven samples from four different WWTPs. As shown in **Table 3**, MDA and MDMA were detected in almost all samples, whereas AMP was detected in just one raw wastewater and MAMP and MDMA remained below the LOD. A chromatogram of a wastewater sample is exemplary presented in **Figure 5**. Among the amphetamines, MDA concentrations ($6\text{-}20\text{ ng L}^{-1}$) are slightly higher than those of MDMA ($4\text{-}11\text{ ng L}^{-1}$), in opposite to the data reported in the literature and to what should be expected, as MDMA (ecstasy) consumption is supposed to be higher [2,12,21-23].

Elimination at WWTPs seems not to be significant, but removals reported in the literature are rather variable [2,12,21-23]. Anyway, these are preliminary data from grab samples that need to be confirmed by the analysis of a larger set of composite samples collected with the appropriate delay accounting for WWTP residence time. However, the obtained data confirm that amphetamines can be detected in wastewater from many different locations.

4. CONCLUSIONS

Three different SPE approaches for the determination of amphetamines in wastewater have been compared. MIPs offer the best performance in terms of selectivity, resulting in lower matrix effects during determination and also in better LODs, accuracy and precision. However, their main drawbacks are (1) vacuum cannot be applied, which increases sample preparation time: ca. 50 min with MIPs by gravity versus ca. 10 min with MCX by vacuum; and (2) the lower capacity as compared to Oasis sorbents. Alternatively, Oasis MCX produces slightly worse figures of merit than MIPs (LODs are twice higher) because their selectivity is lower, even when their mixed-mode character is exploited. Yet, they may turn useful in multiresidue methods where several illicit drugs need to be determined. Finally, Oasis HLB turned out unsuitable for the determination of four of the amphetamines under the experimental conditions tested.

Figure 5. Quantification and confirmation SRM traces of the amphetamines detected in WWTP-B influent and their respective internal standards.



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PUBLICACIÓN III.7

SCREENING AND SELECTIVE QUANTIFICATION OF ILLICIT DRUGS IN WASTEWATER BY MIXED-MODE SOLID-PHASE EXTRACTION AND QUADRUPOLE-TIME-OF-FLIGHT LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

For the first time, a mixed-mode solid-phase extraction, with fractionation of basic analytes from neutral and acidic species during cartridge elution and liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) was combined for the quantitative determination of 24 illicit drugs and metabolites in urban sewage samples. The effects of several sample preparation and instrumental parameters in the sensitivity and selectivity of the quantitative method are thoroughly discussed. Under final working conditions, recoveries above 63% and 82% were attained for all species in raw and treated sewage, respectively; limits of quantification of the method, defined for a signal to noise ratio of 10 ($S/N = 10$), ranged from 2 to 50 ng L⁻¹. Sequential elution of mixed-mode cartridges allowed a significant reduction of matrix effects observed during electrospray ionisation of basic drugs versus those measured for hydrophilic-lipophilic balance reversed-phase sorbents and the same mixed-mode polymer without fractionated elution. Analysis of raw wastewater samples confirmed the ubiquity of cocaine (COC), benzoylecgonine (BE) and 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THCCOOH) in this matrix. The capability of the above methodology to identify new illicit drugs and/or metabolites in sewage samples is also discussed. With this aim, a two steps strategy is proposed. First, high resolution MS chromatograms, acquired throughout each chromatographic run, are automatically searched against an in-house built database, a reduced list of candidate drugs is generated and the corresponding extracted ion chromatograms are obtained. In a further LC run, tandem mass spectrometry (MS/MS) spectra of unknown peaks are acquired using different collision energies and compared with those existing in public libraries, or interpreted, to assign the unknown peak to one of the previously selected candidates.

Keywords: drugs of abuse; cocaine; cannabis; water analysis; time-of-flight (TOF); screening; sewage epidemiology.

1. INTRODUCTION

Abuse of illicit drugs has become a problem of global concern. According to the “World Drug Report 2011” of the United Nations Office of Drugs and Crime (UNODC), between 149 and 272 million people consumed any illicit substance at least once in the past year, and between 15 and 39 million were considered addicted [1]. Because of excretion after consumption and occasional direct disposals into sewage systems, illicit drugs and their metabolites are continuously discharged into wastewaters [2-12]. Since their removal during sewage treatments is usually incomplete, they are released into surface waters [2,9,10,13-15] and they have even reached drinking water sources [13,16-18]. Moreover, analysis of raw wastewater can be used to monitor the consumption of drugs in a specific location. This approach was applied for the first time in 2005 by Zuccato et al. [19] and, since then, other research groups have used it to estimate drug abuse in different countries [10,11,13,17,20-24].

Most procedures developed for the determination of illicit drugs residues in water samples comprise a sample concentration step followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), normally, on triple quadrupole (QqQ) instruments [2,4,9,15,18,24-27]. As regards sample preparation, solid-phase extraction (SPE) is the preferred technique. Analytes are concentrated using either hydrophilic reversed-phase type [3,7,11,28,29] or mixed-mode (reversed-phase plus cation-exchange) materials [2,4,9,24,25,27] and then recovered using an organic solvent or mixture of solvents compatible with the further LC separation. The selectivity of the above approaches is rather limited since the washing step considers only aqueous solutions for the removal of inorganic salts. As a consequence, significant signal suppression effects have been reported during electrospray ionisation (ESI), particularly for wastewater samples with high loads of organic compounds [4,30]. Although deuterated analogues are available to compensate those matrix effects, they certainly result in increased limits of detection (LODs) and quantification (LOQs). Recently, we have shown that an improved SPE protocol can provide cleaner extracts and lower LODs for amphetamine type drugs [31].

Regarding the determination step, LC-MS/MS methods developed with QqQ instruments usually render an unmatched sensitivity. However, for some analytes with low m/z values for their precursor ions, as amphetamine class drugs, it is not possible to obtain

two intense transitions, which are required for their proper identification in the selected reaction monitoring (SRM) mode [30,31]. Similarly, the possibility of interferences from coeluting isobaric compounds can alter SRM transition ratios required for proper identification [32] and, in some cases, a “too rich” MS/MS fragmentation pattern is obtained (e.g. opiate drugs and metabolites [30]) causing a significant loss of sensitivity.

The replacement of QqQ systems by high-resolution/accurate-mass analysers such as hybrid quadrupole-time of flight (QTOF) mass spectrometers can overcome many of those problems and allows the unambiguous identification of a given species from its accurate mass measurements and isotope patterns matching [33-36]. In addition, when working in MS mode as a single TOF, these systems offer the possibility to screen for a theoretically unlimited number of compounds after the LC-MS run (post-target analysis), without the need for reference standards [34,37,38]. This may become very useful for drugs of abuse to detect the consumption of new substances, which appear in the market continuously. Although the quantitative possibilities of LC-QTOF-MS/MS have already been shown for some groups of contaminants in environmental and food samples [38-41], in the field of illicit drugs analysis only its screening capabilities based on unspecific pseudo-MS/MS have been evaluated [42].

Hence, the goal of this study was to develop and to validate a new method for the determination of 24 analytes, corresponding to a wide range of illicit drugs and some of their major urinary metabolites, in wastewater samples, placing special emphasis on its selectivity. Target drugs were selected based on the levels reported in wastewater [43] and recent abuse trends according to the UNODC [1] and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [44]. The method comprises a selective SPE step using a mixed-mode (Oasis MCX) sorbent, which allows the separation of neutral and acidic compounds from basic species during the elution step, reducing matrix effects. The quantitative and screening capabilities of the LC-QTOF-MS/MS system are also discussed. The screening potential was evaluated by performing post-target analysis over the chromatograms of real samples, using an empirical formulae database of 130 drugs (**Table 1**).

Table 1. Personal compound database used in post-target analysis to screen real samples for other potential drugs of abuse different from analytes.

| Compound ID | Compound Name | Formula | Mass (m/z) | ChemSpider ID |
|-------------|---|------------|------------|---------------|
| 1 | (1-(4-hydroxybutyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone | C23H21NO2 | 343.15723 | |
| 2 | (1-(5-hydroxypentyl)-1H-indol-3-yl)(naphthalen-1-yl)methanone | C24H23NO2 | 357.17288 | |
| 3 | 1-(1-Phenylcyclohexyl)piperidin-4-ol | C17H25NO | 259.19361 | 89272 |
| 4 | 1,4-dibenzylpiperazine | C18H22N2 | 266.1783 | 173653 |
| 5 | 11-Hydroxy-THC | C21H30O3 | 330.21949 | |
| 6 | 11-Hydroxy-THC glucuronide | C27H38O9 | 506.25158 | |
| 7 | 1-Benzyl-4-methylpiperazine | C12H18N2 | 190.147 | 667589 |
| 8 | 1-Piperonylpiperazine | C12H16N2O2 | 220.12118 | 85214 |
| 9 | 2C-I (2,5-dimethoxy-4-iodophenethylamine) | C10H14INO2 | 307.00692 | 8442670 |
| 10 | 2C-T-2 (2,5-dimethoxy-4-ethylthiophenethylamine) | C12H19NO2S | 241.11365 | 16787961 |
| 11 | 2C-T-4 (2,5-dimethoxy-4-isopropylthiophenethylamine) | C13H21NO2S | 255.1293 | 21106232 |
| 12 | 2C-T-7 (2,5-dimethoxy-4-n-propylthiophenethylamine) | C13H21NO2S | 255.1293 | 21106233 |
| 13 | 2-Oxo-3-hydroxy-LSD | C20H25N3O3 | 355.18959 | |
| 14 | 3,4-Dihydroxyamphetamine | C9H13NO2 | 167.09463 | 16110 |
| 15 | 3,4-Dihydroxy-ethylamphetamine | C11H17NO2 | 195.12593 | 115341 |
| 16 | 3,4-Dihydroxymethamphetamine | C10H15NO2 | 181.11028 | 141547 |
| 17 | 3-Trifluoromethylphenylpiperazine | C11H13F3N2 | 230.10308 | 4145 |
| 18 | 4-hydroxyindole-3-acetic acid | C10H9NO3 | 191.05824 | 1760 |
| 19 | 4-(3-(1-naphthoyl)-1H-indol-1-yl)butanoic acid | C23H19NO3 | 357.13649 | |
| 20 | 4-hydroxy-3-methamphetamine | C10H15NO2 | 181.11028 | 170725 |
| 21 | 4-hydroxy-3-methoxy-ethylamphetamine | C12H19NO2 | 209.14158 | 114789 |
| 22 | 4-hydroxy-3-methoxymethamphetamine | C11H17NO2 | 195.12593 | 2338803 |
| 23 | 4-hydroxyamphetamine | C9H13NO | 151.09971 | 3525 |
| 24 | 4-Hydroxymetamphetamine | C10H15NO | 165.11536 | 4494 |
| 25 | 4-Methoxyphencyclidine | C18H27NO | 273.20926 | 10526416 |
| 26 | 4-Phenyl-4-(piperidin-1-yl)cyclohexanol | C17H25NO | 259.19361 | 142418 |
| 27 | 5-(3-(1-naphthoyl)-1H-indol-1-yl)pentanoic acid | C24H21NO3 | 371.15214 | |
| 28 | 5-Methoxy-N,N-Diisopropyltryptamine | C17H26N2O | 274.20451 | 133247 |
| 29 | 6-Acetylmorphine | C19H21NO4 | 327.14706 | 16787785 |
| 30 | Ahydroecgonine | C9H13NO2 | 167.09463 | 91897 |
| 31 | Ahydroecgonine methyl ester | C10H15NO2 | 181.11028 | 106705 |
| 32 | AM-2201 | C24H22FNO | 359.16854 | 24751884 |
| 33 | AM-694 | C20H19FINO | 435.04954 | 8064843 |
| 34 | Amphetamine | C9H13N | 135.1048 | 13852819 |
| 35 | Aponorscopolamine | C16H17NO3 | 271.12084 | |
| 36 | Aposcopolamine | C17H19NO3 | 285.13649 | 4925535 |
| 37 | Benzoylcgonine | C16H19NO4 | 289.13141 | 2250 |
| 38 | Benzylpiperazine | C11H16N2 | 176.13135 | 68493 |
| 39 | Bufotenine | C12H16N2O | 204.12626 | 9839 |
| 40 | Carboxy-THC | C21H28O4 | 344.19876 | 97019 |
| 41 | Carboxy-THC glucuronide | C27H36O10 | 520.23085 | |

III. METODOLOGÍA DESARROLLADA. B. DROGAS DE ABUSO

Table 1 (continuation). Personal compound database used in post-target analysis to screen real samples for other potential drugs of abuse different from analytes.

| Compound ID | Compound Name | Formula | Mass (m/z) | ChemSpider ID |
|-------------|---|-------------|------------|---------------|
| 42 | Cathinone | C9H11NO | 149.08406 | 56062 |
| 43 | CB-13 | C26H24O2 | 368.17763 | 7975182 |
| 44 | Cocaethylene | C18H23NO4 | 317.16271 | 2723 |
| 45 | Cocaine | C17H21NO4 | 303.14706 | 2724 |
| 46 | Codeine | C18H21NO3 | 299.15214 | 4447447 |
| 47 | Codeine-glucuronide | C24H29NO9 | 475.18423 | |
| 48 | CP 47,497 | C21H34O2 | 318.25588 | 111910 |
| 49 | D2PM | C17H21NO | 255.16231 | 21106295 |
| 50 | Dehydronorketamine | C12H12CINO | 221.06074 | 142954 |
| 51 | Desoxy D2PM | C17H19N | 237.15175 | 1256 |
| 52 | Desoxypipradol | C18H21N | 251.1674 | 141045 |
| 53 | Dihydroxy-THC | C21H30O4 | 346.21441 | |
| 54 | Ecgonidine | C8H11NO2 | 153.07898 | 16735787 |
| 55 | Ecgonine | C9H15NO3 | 185.10519 | 82586 |
| 56 | Ecgonine methyl ester | C10H17NO3 | 199.12084 | 391939 |
| 57 | EDDP | C20H23N | 277.18305 | 4509491 |
| 58 | EMDP | C19H21N | 263.1674 | |
| 59 | Ephedrine | C10H15NO | 165.11536 | 8935 |
| 60 | Fentanyl | C22H28N2O | 336.22016 | 3228 |
| 61 | Heroin | C21H23NO5 | 369.15762 | 4575379 |
| 62 | HU-210 | C25H38O3 | 386.2821 | 7997318 |
| 63 | Hydromorphone | C17H19NO3 | 285.13649 | 4447624 |
| 64 | Hydroxy-1,4-dibenzylpiperazine | C18H22N2O | 282.17321 | |
| 65 | Hydroxy-1-Benzyl-4-methylpiperazine | C12H18N2O | 206.14191 | |
| 66 | Hydroxy-3-Trifluoromethylphenylpiperazine | C11H13F3N2O | 246.098 | |
| 67 | Hydroxybenzoylecgonine | C16H19NO5 | 305.12632 | 111632 |
| 68 | Hydroxy-benzylpiperazine | C11H16N2O | 192.12626 | |
| 69 | Hydroxycocaine | C17H21NO5 | 319.14197 | 115492 |
| 70 | Hydroxy-fentanyl | C22H28N2O2 | 352.21508 | |
| 71 | Hydroxy-LSD | C20H25N3O2 | 339.19468 | |
| 72 | Hydroxy-mCPP | C10H13CIN2O | 212.07164 | |
| 73 | hydroxy-MDMA | C11H15NO3 | 209.10519 | 112206 |
| 74 | Hydroxy-methoxy-scopolamine | C18H23NO6 | 349.15254 | |
| 75 | Hydroxy-norfentanyl | C14H20N2O2 | 248.15248 | |
| 76 | Hydroxynorketamine | C12H14CINO2 | 239.07131 | 117907 |
| 77 | Hydroxynorketamine-glucuronide | C18H32CINO8 | 425.18164 | |
| 78 | Hydroxy-PCP | C17H25NO | 259.19361 | 9911755 |
| 79 | Hydroxyscopolamine | C17H21NO5 | 319.14197 | |
| 80 | JWH-018 | C25H25NO | 355.19361 | 8536309 |
| 81 | JWH-073 | C23H21NO | 327.16231 | 8647081 |
| 82 | JWH-200 | C25H24N2O2 | 384.18378 | 8221134 |
| 83 | JWH-250 | C22H25NO2 | 335.18853 | 23256117 |
| 84 | JWH-398 | C24H22CINO | 375.13899 | |
| 85 | Ketamine | C13H16CINO | 237.09204 | 3689 |
| 86 | Ketamine-glucuronide | C19H24CINO7 | 413.12413 | |

Table 1 (continuation). Personal compound database used in post-target analysis to screen real samples for other potential drugs of abuse different from analytes.

| Compound ID | Compound Name | Formula | Mass (m/z) | ChemSpider ID |
|-------------|-----------------------------------|-------------|------------|---------------|
| 87 | LSD | C20H25N3O | 323.19976 | 5558 |
| 88 | MBDB | C12H17NO2 | 207.12593 | 111153 |
| 89 | mCPP | C10H13CIN2 | 196.07673 | 1314 |
| 90 | MDA | C10H13NO2 | 179.09463 | 1555 |
| 91 | MDEA | C12H17NO2 | 207.12593 | 10723892 |
| 92 | MDMA | C11H15NO2 | 193.11028 | 1556 |
| 93 | Mephedrone | C11H15NO | 177.11536 | 21485694 |
| 94 | Methadone | C21H27NO | 309.20926 | 3953 |
| 95 | Methamphetamine | C10H15N | 149.12045 | 10379 |
| 96 | Methedrone | C11H15NO2 | 193.11028 | 187475 |
| 97 | Methoxetamine | C15H21NO2 | 247.15723 | 24721792 |
| 98 | Methyl ecgonidine | C10H15NO2 | 181.11028 | 106705 |
| 99 | Methyl-fentanyl | C23H30N2O | 350.23581 | 56081 |
| 100 | Methylone | C11H13NO3 | 207.08954 | 21106350 |
| 101 | Morphine | C17H19NO3 | 285.13649 | 4450907 |
| 102 | Morphine-glucuronide | C23H27NO10 | 477.1635 | 23121293 |
| 103 | N-(3-chlorophenyl)ethylenediamine | C8H11CIN2 | 170.06108 | 9183562 |
| 104 | Norbenzoyllecgonine | C15H17NO4 | 275.11576 | 102710 |
| 105 | Norcocaine | C16H19NO4 | 289.13141 | 519603 |
| 106 | Norcodeine | C17H19NO3 | 285.13649 | 1217 |
| 107 | Norcodeine-glucuronide | C23H27NO9 | 461.16858 | |
| 108 | Norfentanyl | C14H20N2O | 232.15756 | 227671 |
| 109 | Norketamine | C12H14CINO | 223.07639 | 110322 |
| 110 | Norketamine-glucuronide | C18H22CINO7 | 399.10848 | |
| 111 | Nor-LSD | C19H23N3O | 309.18411 | 148419 |
| 112 | Normorphine | C16H17NO3 | 271.12084 | 380506 |
| 113 | Norscopolamine | C16H19NO4 | 289.13141 | 23319128 |
| 114 | Norscopolamine-glucuronide | C22H26NO10 | 464.15567 | |
| 115 | PCAA | C17H25NO2 | 275.18853 | 151498 |
| 116 | PCP | C17H25N | 243.1987 | 6224 |
| 117 | p-Fluorophenylpiperazine | C10H13FN2 | 180.10628 | 2019121 |
| 118 | Phentermine | C10H15N | 149.12045 | 4607 |
| 119 | Pipradol | C18H21NO | 267.16231 | 9681 |
| 120 | p-Methoxyphenylpiperazine | C11H16N2O | 192.12626 | 237180 |
| 121 | p-Methylthioamphetamine | C10H15NS | 181.09252 | 133883 |
| 122 | PMMA | C11H17NO | 179.13101 | 171194 |
| 123 | Psilocin | C12H16N2O | 204.12626 | 4807 |
| 124 | Psilocybin | C12H17N2O4P | 284.09259 | 10178 |
| 125 | Scopolamine | C17H21NO4 | 303.14706 | 10194106 |
| 126 | Scopolamine-glucuronide | C23H28NO10 | 478.17132 | |
| 127 | THC | C21H30O2 | 314.22458 | 2872 |
| 128 | TMA-2 | C12H19NO3 | 225.13649 | 28773 |
| 129 | γ -Butyrolactone | C4H6O2 | 86.03678 | 7029 |
| 130 | γ -Hydroxybutyric acid | C4H8O3 | 104.04734 | 9984 |

2. EXPERIMENTAL

2.1. Standards, solvents and sorbents

(±)-Amphetamine (AMP), (±)-methamphetamine (MAMP), (±)-3,4-methylenedioxyamphetamine (MDA), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-3,4-methylenedioxyethylamphetamine (MDEA), cocaine (COC), cocaethylene (COE), benzoylecgonine (BE), lysergic acid diethylamide (LSD), 2-oxo-3-hydroxy-LSD (O-H-LSD), benzylpiperazine (BZP), 1-(3-chlorophenyl)piperazine (mCPP), 1-(1-phenylcyclohexyl)piperidine (phencyclidine, PCP), fentanyl (FEN), morphine (MOR), 6-acetylmorphine (6-AM), codeine (COD), heroine (HER), (±)-methadone (MET), (±)-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP), ketamine (KET), (-)-scopolamine (SCO), (-)- Δ^9 -tetrahydrocannabinol (THC) and (-)-11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol (THCCOOH) were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg mL⁻¹ or 0.1 mg mL⁻¹ solutions in acetonitrile (ACN) or methanol (MeOH). Scopolamine (SCO) was supplied as pure substance by Sigma-Aldrich (Madrid, Spain). Deuterated compounds were also purchased from Cerilliant (0.1 mg mL⁻¹ in ACN or MeOH) and used as surrogated internal standards (ISs) for the quantification of their analogous native analytes. For those species whose deuterated analogue was not available, a structurally or retention time related IS was used instead (**Table 2**). Mixed standard solutions (containing all the analytes or all the ISs) were prepared in MeOH at 2 mg L⁻¹ and stored in the dark at -20 °C.

LC-grade ACN and MeOH, aqueous ammonia (NH₃) solution (25%), hydrochloric acid (37%) and acetic acid were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained by purifying demineralised water in a Milli-Q system (Millipore, Bedford, MA, USA).

SPE cartridges containing either 200 mg of the Oasis HLB reversed-phase sorbent or 150 mg of the mixed-mode (reversed-phase and cation-exchanger) Oasis MCX material were purchased from Waters (Milford, MA, USA).

2.2. Samples

Several wastewater samples were collected in the course of the study in February 2011 from a sewage treatment plant (STP) serving an urban population of ca. 130000 inhabitants of the northwest of Spain. Grab samples of treated and raw wastewater were taken in different week days and extracted (SPE) within 6 h after sampling in order to avoid analytes hydrolysis [4,5]. Composite samples of raw wastewater were collected in the course of a week by an automatic device working in a time-proportional mode (every 10 min during 24 hours). Again, the combined sample was concentrated within 6 hours after sampling.

2.3. Sample preparation

Prior to extraction, samples (200 and 500 mL for raw and treated wastewater, respectively) were vacuum filtered, first through glass fibre pre-filters and subsequently through 0.45 μm nitrocellulose filters (Millipore, Bedford, MA, USA). The filtrate was adjusted to the desired pH, spiked with isotopically labelled standards (100 ng each) and subjected to the SPE process.

Under final working conditions, samples were adjusted to pH 4.5 and passed through Oasis MCX cartridges (ca. 10 mL min⁻¹) previously conditioned with 2 mL of a MeOH:NH₄OH (95:5) solution and 2 mL of pH 4.5 ultrapure water. Immediately after loading, SPE cartridges were washed with 10 mL of ultrapure water (adjusted to pH 4.5) and dried by a continuous nitrogen stream for 30 min. Finally, analytes were eluted in two separated fractions: cannabinoids (together with neutral/acidic matrix components) were firstly eluted by 2 mL of MeOH, and the remaining (basic) compounds were recovered straight afterwards with 4 mL of MeOH:NH₄OH (95:5). Both fractions were concentrated down separately to ca. 0.5 mL with a gentle stream of nitrogen (99.999%) in a Turbovap II concentrator (Zymark, Hopkinton, MA, USA), adjusted to a final volume of 1 mL with MeOH and injected (10 μL) into the LC-MS system.

2.4. Liquid chromatography-quadrupole-time-of-flight-mass spectrometry

Analyses were performed using an Agilent 1200 Series HPLC comprising a membrane degasser, a binary high-pressure gradient pump, a thermostated LC column compartment and an autosampler. Separations were carried out on a Nucleosil 100-3 C₁₈ HD column (Macherey-Nagel GmbH & Co. KG, Düren, Germany) of 125 \times 2 mm (length \times ID) and 3 μm of particle size, thermostated at 40 °C. The dual eluent system consisted of: (A) 5 mM of ammonium acetate (NH₄OAc) in ultrapure water adjusted at pH 8.5 with NH₃; and (B) 5 mM of NH₄OAc in MeOH made to an apparent pH of 4.5 (by adding the equivalent amount of acetic acid to have such pH in an aqueous solution). The flow rate was set at 0.2 mL min⁻¹ and the gradient programme was as follows: 0 min (2% B), 0.2 min (50% B), 25 min (100% B), 29 min (100% B), 30 min (2% B), 40 min (2% B).

The LC was coupled to an accurate-mass QTOF MS (Agilent 6520) equipped with a dual-ESI ion source. Nitrogen, used as nebulising (9 L min⁻¹) and drying gas (45 psi), was provided by a nitrogen generator (Erre Due srl, Livorno, Italy). Nitrogen of 99.9995% purity, for collision induced dissociation, was purchased from Carburos Metálicos (A Coruña, Spain). The capillary voltage of the ESI was set at 4 kV either in positive or in negative mode. The latter mode was

used for the determination of cannabinoids, whereas remaining analytes were ionised in the positive mode. The temperature of the ESI chamber was set at 275 °C, the drying gas flow at 9 L min⁻¹ and the nebulising gas pressure at 45 psig. The fragmentor voltage was maintained at 140 V for all compounds and the pressure of nitrogen in the collision cell adjusted at 18 mTorr.

Except for opioids, analytes were quantified in the MS/MS mode from the MS/MS base peak extracted ion chromatogram using an accurate mass window of ±20 ppm. Opioids (COD, HER, MOR and 6-AM) were quantified in the MS mode, extracting the [M+H]⁺ ion chromatogram with a ±10 ppm mass window and acquiring also their MS/MS spectra just for confirmation purposes. This decision did not involve any extra analysis, since the QTOF system switches intermittently to single MS during an MS/MS run to allow the continuous calibration of the mass axis. With that aim, one of the ESI nebulisers was continuously infused with a reference solution according to the manufacturer specifications (5 psig), for which in the negative mode the reference masses selected were 112.985587 and 980.016375 *m/z*, and in the positive mode 121.050873 and 922.009798 *m/z*. MS spectra were recorded at 2 spectra per second, and MS/MS spectra at 6 spectra per second in the positive mode and at 2 spectra per second in the negative mode. Spectral data were acquired at 2 GHz (extended dynamic range mode) when used for quantification measurements and at 4 GHz (high resolution mode) for screening purposes. Instrument control, data acquisition and evaluation were performed with the Mass Hunter software (Agilent Technologies). Most relevant MS/MS parameters are summarised in **Table 2**.

2.5. Matrix effects evaluation

Matrix effects during ESI were evaluated spiking an aliquot of the final SPE extracts with 200 ng of all analytes and considering, in addition, non-spiked aliquots from each sample. Hence, the response of the spiked extracts (R_2) after non-spiked sample signal (R_B) subtraction was compared to the response factor of a standard prepared in MeOH (R_1) with the same concentration. Matrix effects percentages (%ME) were calculated as: %ME = $100 \times (R_2 - R_B) / R_1$ [31,45,46].

2.6. Recoveries and real samples analysis

Recoveries (%R) of the whole procedure were evaluated with spiked aliquots of different water samples: ultrapure water, treated wastewater and raw wastewater. Deuterated ISs were added (100 ng) as surrogates in all cases to compensate matrix effects

and losses during sample preparation. Differences between corrected responses (analyte peak area divided by the signal of the IS) for spiked and non-spiked fractions of each sample were compared with calibration curves obtained for standards in MeOH containing the ISs.

Table 2. Experimental parameters used for the quantification of the target analytes and instrumental performance data ^a.

| | IS | Precursor (<i>m/z</i>) | Product (<i>m/z</i>) | CE (V) | Mass error ^b (mDa) (ppm) | | R ^{2c} | RSD ^b (%) | LOQ (pg) |
|-------------|--------------------------|-----------------------------|---------------------------|-----------|--|-----|-----------------|-------------------------|-------------|
| AMP | AMP-d ₆ | 136.1121 | 91.0542 | 8 | 0.2 | 2.2 | 0.9997 | 5.6 | 50 |
| MAMP | MAMP-d ₅ | 150.1277 | 91.0542 | 10 | 0.3 | 3.0 | 0.9995 | 7.7 | 50 |
| MDA | MDA-d ₅ | 180.1019 | 163.0754 | 12 | 1.0 | 6.2 | 0.9947 | 17.8 | 50 |
| MDMA | MDMA-d ₅ | 194.1176 | 163.0754 | 12 | 0.7 | 4.5 | 0.9993 | 8.1 | 30 |
| MDEA | MDEA-d ₅ | 208.1332 | 163.0754 | 12 | 0.7 | 4.1 | 0.9998 | 4.9 | 20 |
| COC | COC-d ₃ | 304.1543 | 182.1176 | 20 | 0.8 | 4.3 | 0.9981 | 5.0 | 20 |
| BE | BE-d ₃ | 290.1387 | 168.1019 | 20 | 0.7 | 4.2 | 0.9995 | 6.3 | 20 |
| COE | COC-d ₃ | 318.1700 | 196.1322 | 20 | 0.4 | 2.1 | 0.9982 | 5.5 | 30 |
| SCO | COC-d ₃ | 304.1543 | 138.0913 | 20 | 0.5 | 3.4 | 0.9986 | 6.9 | 20 |
| LSD | LSD-d ₃ | 324.2070 | 223.1230 | 25 | 0.7 | 3.1 | 0.9997 | 5.5 | 20 |
| O-H-LSD | LSD-d ₃ | 356.1968 | 237.1022 | 25 | 0.6 | 2.7 | 0.9985 | 6.4 | 30 |
| BZP | BZP-d ₇ | 177.1386 | 91.0542 | 25 | 0.2 | 1.9 | 0.9996 | 8.8 | 50 |
| mCPP | BZP-d ₇ | 197.0845 | 154.0418 | 20 | 0.9 | 6.1 | 0.9998 | 8.0 | 50 |
| PCP | PCP-d ₅ | 244.2060 | 86.0964 | 10 | 0.3 | 3.0 | 0.9997 | 7.1 | 20 |
| FEN | FEN-d ₅ | 337.2274 | 188.1434 | 25 | 0.8 | 4.1 | 0.9988 | 5.2 | 20 |
| <u>MOR</u> | <u>MOR-d₃</u> | 286.1438 | 201.0910 | 35 | 0.4 | 1.3 | 0.9981 | 3.6 | 10 |
| <u>6-AM</u> | <u>MOR-d₃</u> | 328.1543 | 165.0699 | 40 | 0.1 | 0.4 | 0.9976 | 5.2 | 10 |
| <u>COD</u> | <u>COD-d₃</u> | 300.1594 | 165.0699 | 40 | 1.2 | 4.0 | 0.9939 | 3.9 | 10 |
| <u>HER</u> | <u>MOR-d₃</u> | 370.1649 | 165.0699 | 40 | 0.3 | 0.7 | 0.9942 | 4.6 | 10 |
| MET | MET-d ₃ | 310.2153 | 265.1587 | 15 | 0.4 | 1.6 | 0.9993 | 2.2 | 10 |
| EDDP | KET-d ₄ | 278.1903 | 234.1277 | 30 | 0.5 | 2.2 | 0.9957 | 6.4 | 20 |
| KET | KET-d ₄ | 238.0993 | 125.0153 | 20 | 0.6 | 4.9 | 0.9993 | 9.5 | 50 |
| THC | THC-d ₃ | 313.2173 | 245.1547 | 35 | 1.8 | 7.5 | 0.9974 | 19.7 | 100 |
| THCCOOH | THCCOOH-d ₃ | 343.1915 | 299.2017 | 22 | 2.0 | 6.8 | 0.9988 | 19.2 | 100 |

^a Underlined compounds were quantified in single MS mode, acquiring their MS/MS for confirmation.

THC and THCCOOH were analysed in ESI-; all remaining compounds in ESI+.

^b Mean of 8 replicates of the same standard (20 ng mL⁻¹) acquired at 2 GHz during a 24 h period.

^c Calibration range LOQ-1000 ng mL⁻¹ (IS 200 ng mL⁻¹).

3. RESULTS AND DISCUSSION

3.1. Liquid chromatography-mass spectrometry

First of all, the two different ionisation modes were tested. Cannabinoids could be determined in both modes but showed higher responses in ESI-, agreeing with previous findings [4,47], whereas remaining analytes, with a basic character, could only be determined in ESI+.

The LC-QTOF system used in this study does not allow switching the polarity of the ESI source in a single time segment when operating in MS/MS mode. Thus, chromatographic conditions were adjusted to obtain a good separation between the two cannabinoids and the rest of the basic analytes, in order to group them in two different temporal segments. To this end, the organic phase was acidified to an apparent pH of 4.5, whereas the aqueous phase buffer was made to a pH of 8.5. In this way, basic compounds could be effectively retained in the C₁₈ column at low organic content and, at the same time, the organic content gradient was accompanied by a pH gradient, increasing the retention of THCCOOH (pK_a 4.21) and decreasing the retention of MET (the basic drug displaying the highest retention time) so that they could be separated into two well defined segments. The method comprised a first segment (until 17 min) using ESI+, and a second one operating the source in ESI- for the sensitive determination of THC and THCCOOH. As an example, a chromatogram of a 50 ng mL⁻¹ standard is presented in **Figure 1**. In both segments, MS and MS/MS spectra were alternatively recorded using the *m/z* values compiled in **Table 2**. According to the 2002/657/EC Decision [48], one single high-resolution MS/MS transition is enough to fulfil the identification points guideline. Yet, in the case of the four opioid compounds (MOR, 6-AM, COD and HER), their MS/MS collision-induced dissociation leads to a multitude of fragments [30], which compromises the sensitivity of MS/MS quantification. Actually, Boleda et. al [3] decided to use a pseudo-MS/MS transition on a QqQ instrument in order to gain sensitivity in detection, but confirmation still relied on the low yield MS/MS products. Alternatively in this work, opioids were quantified from their single MS [M+H]⁺ narrow-mass extracted ion chromatograms, recording MS/MS spectra for confirmation purposes. Besides MS/MS, and due to the high resolution and mass accuracy of the QTOF system, the [M+H+1]⁺ ion can also be used as a sensitive confirmation ion (for opioids its intensity is ca. 20% of the [M+H]⁺) in order to comply with the 2002/657/EC Decision [48] identification points guideline. An example is shown in **Figure 2**, where the chromatogram of a sample is presented. In the case of COD, the identity of the chromatographic peak can be confirmed by the single MS [M+H+1]⁺ ion and characteristic MS/MS product ions in spite of the presence of other background ions and spectrum complexity.

Figure 1. Chromatogram of a 50 ng mL⁻¹ standard.

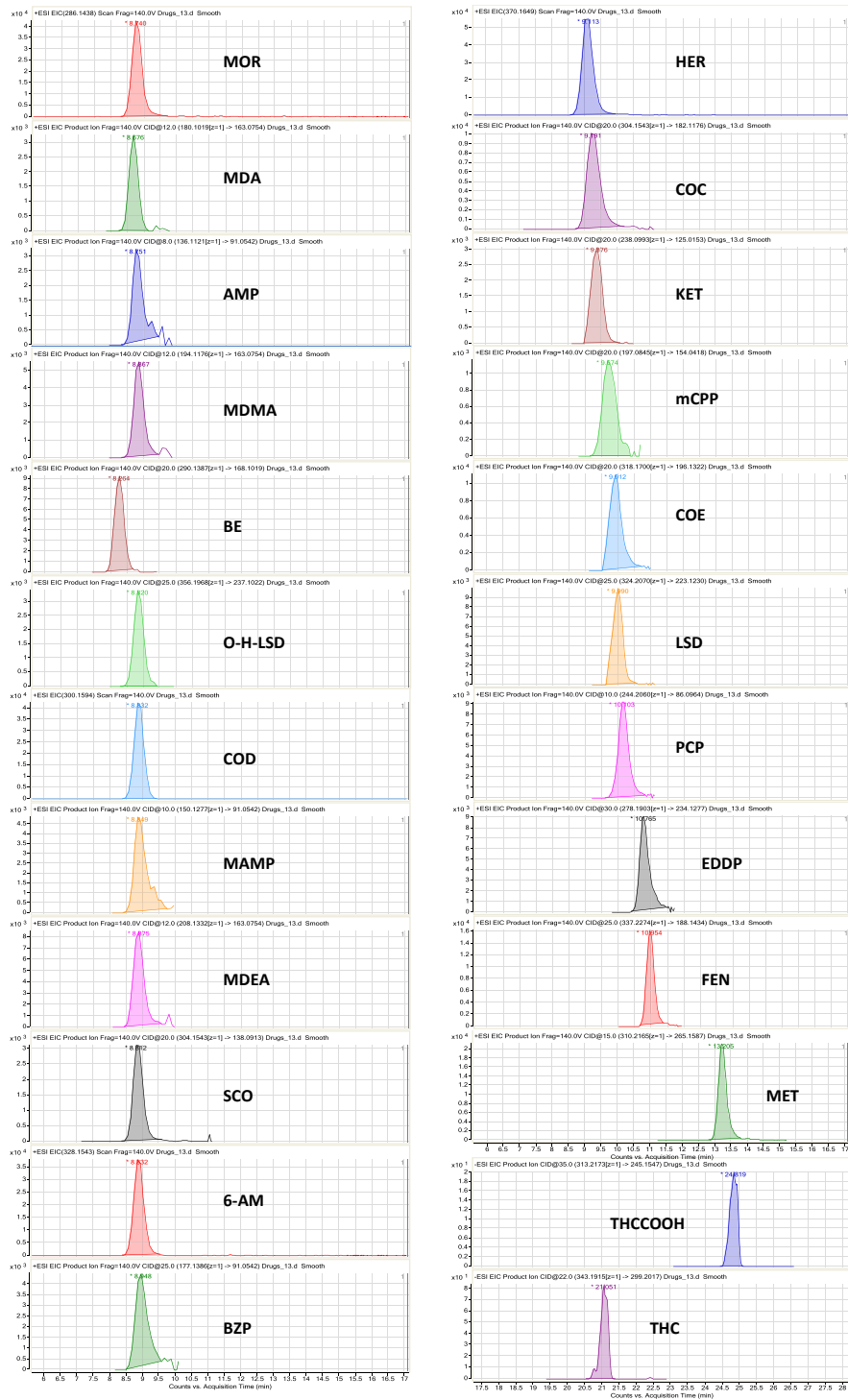
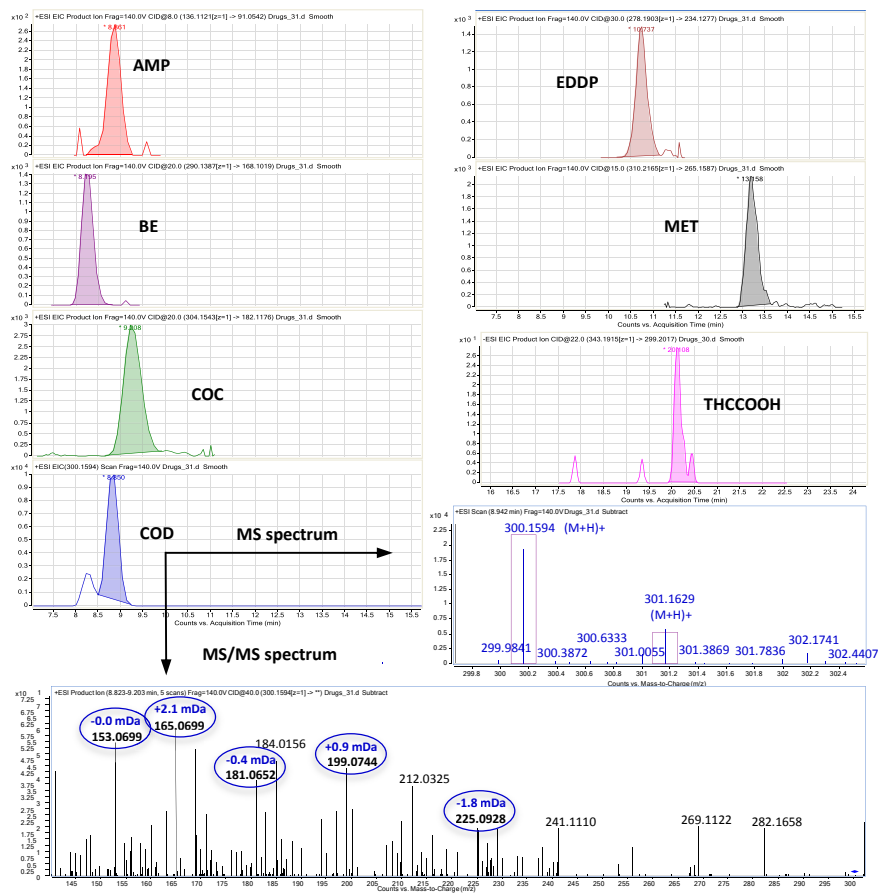


Figure 2. Chromatogram of a wastewater influent sample showing the compounds detected. For codeine, measured in single-MS mode, the accurate-mass MS and MS/MS are presented. Expected and experimental isotopic patterns are presented in the MS spectrum. Confirmation product ions with mass deviation from expected values are highlighted in the MS/MS spectrum.

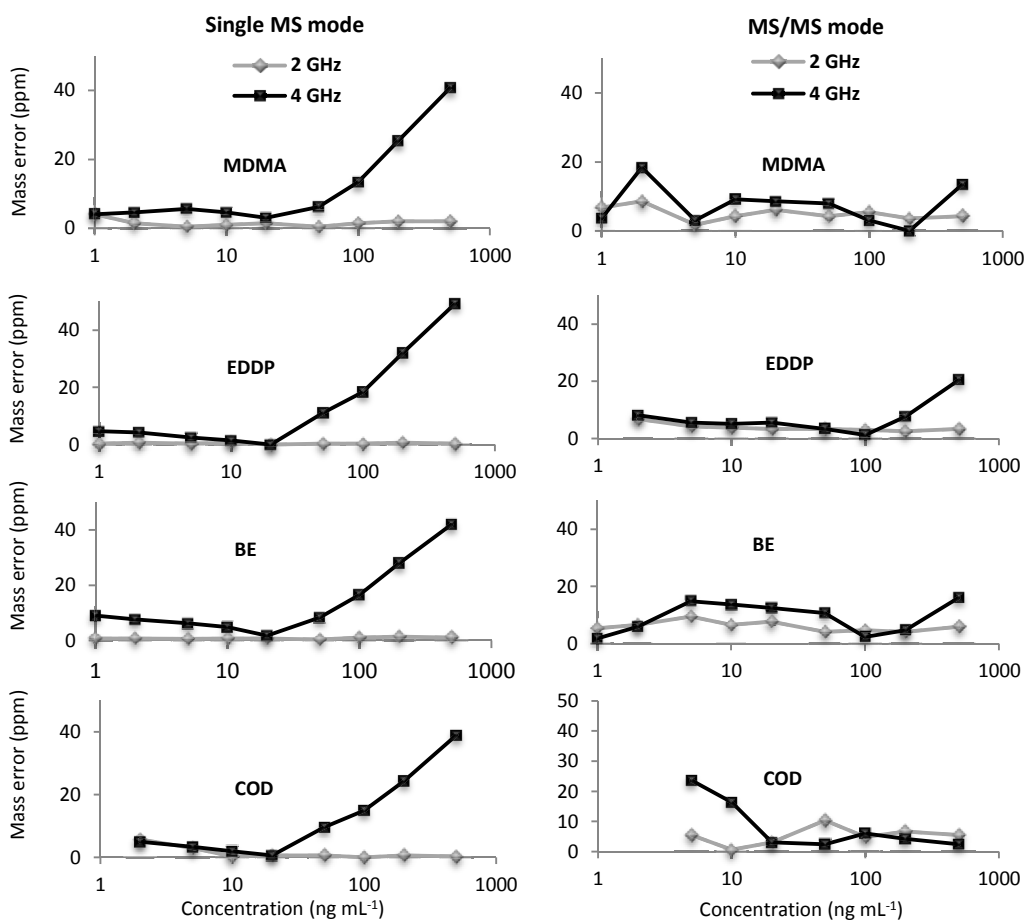


Given that the Agilent 6520 QTOF system uses an Analog to digital converter (ADC) that can be operated either at 4 GHz (highest mass resolving power; FWHM resolution ca. 9500 at m/z 113 and ca. 22000 at m/z 980) or 2 GHz (resolution ca. half of 4 GHz, but expanded linear range), both ADC acquisition modes were compared in terms of mass accuracy in both single MS and MS/MS modes. In single MS mode, at 4 GHz, mass errors increased with the concentration of the target species, reaching the 50 ppm threshold at 500 ng L^{-1} ; on the other hand, at 2 GHz, they stayed below 5 ppm even at concentrations near the LOD (**Figure 3**). In MS/MS operation, though the effect was less significant, still less mass accuracy was provided by the 4 GHz mode. Hence, and particularly taking into account that the four opioids included in this research were quantified in single MS, the ADC was operated at 2 GHz when performing quantitative measurements. As compiled in **Table 2**, in this way the

mass error was not higher than 4 ppm for the analytes determined in single MS mode and lower than 8 ppm in MS/MS mode. Therefore, extracted ion chromatograms used for quantification were taken with a mass tolerance of ± 10 ppm in MS and ± 20 ppm in MS/MS (in the worst case, equivalent to ± 3.7 mDa and ± 6 mDa, respectively) leading to a very low noise baseline.

The LC-MS (/MS) method produced a good linearity in the LOQ-1000 ng mL⁻¹ range and relative standard deviation (RSD) values not higher than 20%, even at levels close to the LOQ (Table 2). Also, the instrumental LOQs of the QTOF instrument were in the 10-100 pg range, which are higher than those reported on UPLC-QqQ-MS/MS instruments (0.05-4 pg) [3,29] but on the same order of magnitude of those achieved with a standard LC-QqQ-MS/MS system (12-530 pg) [4].

Figure 3. Mass accuracy of the QTOF instrument as a function of analyte concentration in MS and MS/MS when the ADC is operated at either 2 or 4 GHz.



3.2. Solid-Phase Extraction

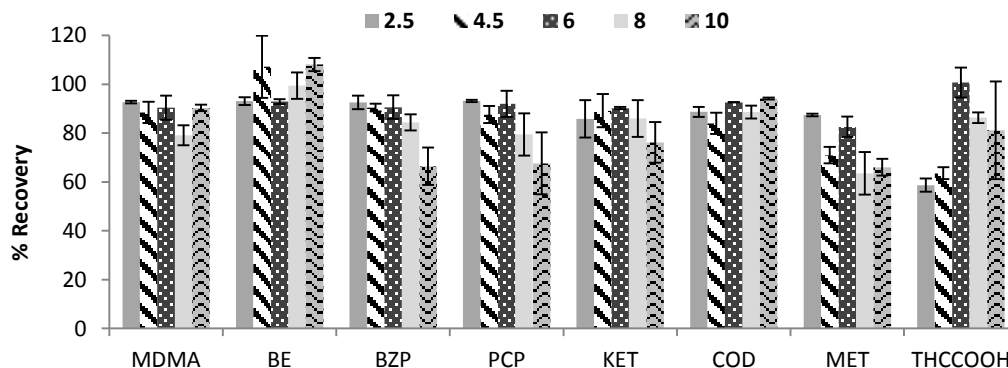
As mentioned in section 1, the Oasis MCX sorbent was selected for the preconcentration of the analytes on the basis of its demonstrated retention efficiency [2,9,25] and its capability to provide more selective extractions than other materials for basic compounds [31].

Initially, the effect of the sample pH on the retention of the analytes was investigated with 200 mL aliquots of spiked ultrapure water (2 ng mL^{-1}) adjusted to different pHs in the range from 2.5 to 10 units. After loading the sample, cartridges were rinsed with 10 mL of ultrapure water adjusted to the corresponding pH and eluted with 10 mL of MeOH:NH₄OH (95:5). Most of the basic analytes, e.g. BE and COD, showed recoveries around 90% within the range of the investigated pH values (**Figure 4**). This trend indicates that even the neutral forms of these species, existing at basic pHs, are efficiently retained in the mixed-mode SPE cartridge through reversed-phase interactions. However, some few compounds (BZP, PCP, KET and MET) showed lower recoveries at pH 10, requiring also the ionic interactions between their positively charged forms and the sulphonic moiety of the sorbent to be quantitatively extracted from the sample. In the case of THCCOOH, recoveries increased, surprisingly, with sample pH. This compound exists only as neutral (pH 2.5) or negatively charged species (rest of tested pHs) interacting with the MCX sorbent just through the reversed-phase mechanism. Consequently, recoveries are not expected to improve with the increase of the pH. However, the trend observed for this compound (**Figure 4**) is likely the consequence of sorption losses for its neutral form ($\log K_{ow} \approx 6.2$) in the walls of sample vessels and connections between the sample and the SPE cartridge at low pHs. On the other hand, at higher pHs, THCCOOH exists as a negatively charged, more polar species ($\log K_{ow} \approx 2.9$ at pH 7) [49], less prone to sorption processes. On the basis of the above results, samples were adjusted at pH 4.5 in order to favour the dual-retention mechanism of basic drugs, which represent 22 of the 24 analytes involved in this research.

Subsequently, breakthrough studies were performed and it was found that 150 mg MCX cartridges can concentrate up to 500 mL of raw wastewater without significant losses for any of the investigated analytes (data not shown). Working sample volumes were finally set at 500 mL in the case of treated wastewater, but reduced to 200 mL for raw samples in order to prevent the bed of sorbent from clogging. In further experiments, the sequential elution of MCX cartridges was optimised. It was found that about 95% of the two cannabinoids were recovered with only two fractions ($2 \times 1 \text{ mL}$) of MeOH, which did not contain any trace of the basic analytes. On the other hand, the successive elution with $4 \times 1 \text{ mL}$ of MeOH:NH₄OH represented around 98% of the basic drugs and metabolites (data not shown). Thus, in the

optimised method, MCX cartridges were eluted first with 2 mL of MeOH and finally with 4 mL of MeOH:NH₄OH (95:5). Both extracts were collected separately, concentrated, made with MeOH to a final volume of 1 mL and analysed in two different LC-MS injections.

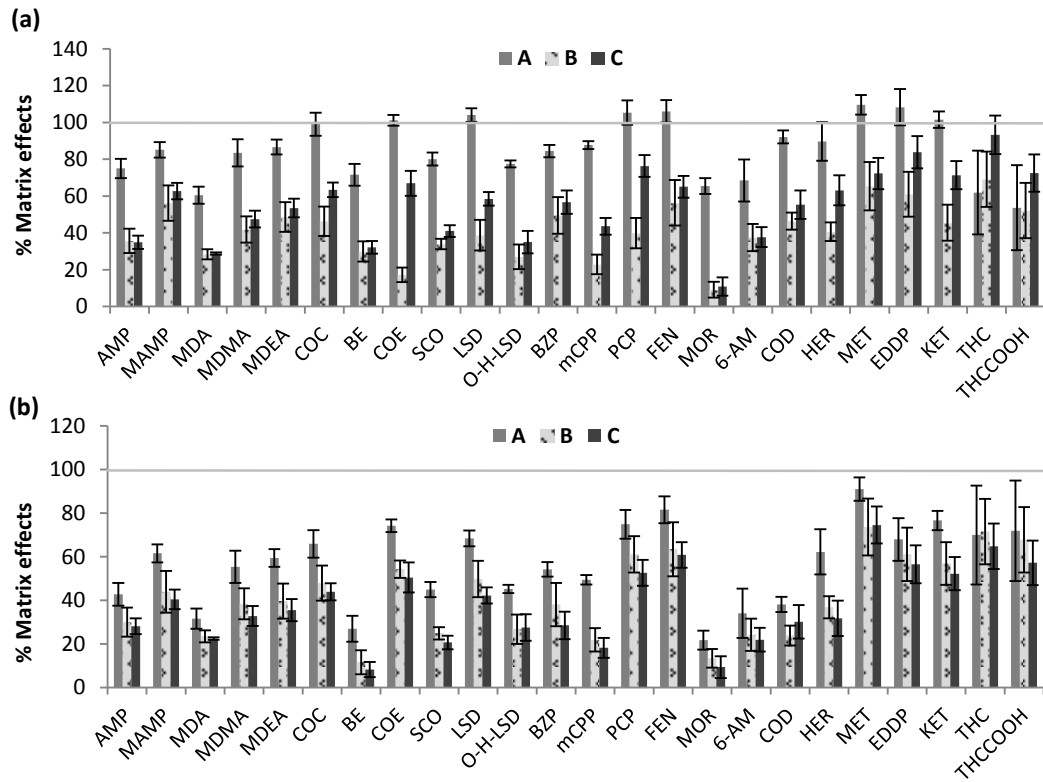
Figure 4. Effect of sample pH on the recoveries of the SPE method, using the MCX sorbent, for some selected compounds.



The above optimised SPE scheme (protocol A) was compared in terms of selectivity (as %ME, see section 2.5) with two other different SPE methods, representing the approaches more frequently used in the literature [43]. In one case (protocol B), acidified samples (pH 4.5) were also concentrated using MCX cartridges, but the whole group of target drugs and metabolites was recovered in the same extract with 5 mL of MeOH:NH₄OH (95:5) [2,9,43]. The third SPE scheme (protocol C) was based on the use of 200 mg Oasis HLB cartridges; in this case, samples were adjusted at pH 8.5, so basic analytes stayed in the neutral form [5], and elution was carried out with 5 mL of pure MeOH.

As it is displayed in **Figure 5a** for an effluent sample after a 500-fold preconcentration, protocol A %ME values were all above 60% for all the basic drugs, whereas in protocols B and C they were as low as 10% in the case of MOR. For 200-fold preconcentrated influents (**Figure 5b**) differences in %ME were lower, but protocol A could still afford a ca. 30% more sensitive detection for basic compounds. These results are a consequence of the fractionated elution protocol A, where many interfering matrix constituents are removed in the first methanolic fraction. Hence, in the case of the two cannabinoid analytes, eluted in that fraction, %ME values are similar with any of the three protocols. Consequently, the SPE method optimised in this work can provide lower LODs/LOQs for all analytes with the exception of cannabinoids.

Figure 5. Matrix effects (%ME) in effluent wastewater (a) and influent wastewater (b) depending on the SPE protocol: A. Oasis MCX with fractionated elution (this work); B. Oasis MCX, single elution; C. Oasis HB.



As shown in **Table 3**, estimated LOQs of the whole method varied from 2 to 20 ng L⁻¹ in effluents and from 5 to 50 ng L⁻¹ in influents, calculated as a S/N of 10. Recoveries (%R) ranged from 76.7 to 118.0% in ultrapure water, from 82.0 to 128.9% in treated wastewater and from 62.9 to 130.8% in raw wastewater. These recovery values and LOQs are in the range of those reported in the literature by SPE and LC-MS/MS [43].

Table 3. Overall internal standard corrected recoveries (n = 3) and LOQs of the whole method for the different matrices considered.

| Compound | %R ^a | | | LOQ (ng L ⁻¹) | |
|----------|------------------------|-----------------------|-----------------------|---------------------------|----------|
| | Ultrapure ^b | Effluent ^c | Influent ^d | Effluent | Influent |
| AMP | 105.6 (10.1) | 116.9 (12.9) | 111.7 (7.0) | 10 | 25 |
| MAMP | 107.1 (2.3) | 106.6 (13.4) | 91.5 (15.3) | 10 | 25 |
| MDA | 109.7 (9.8) | 116.6 (4.1) | 114.2 (12.2) | 10 | 25 |
| MDMA | 106.0 (5.4) | 109.3 (2.6) | 111.4 (9.8) | 6 | 15 |
| MDEA | 105.7 (1.8) | 105.3 (12.7) | 115.4 (7.3) | 4 | 10 |
| COC | 98.9 (7.5) | 91.1 (6.5) | 94.3 (4.2) | 4 | 10 |
| BE | 105.8 (7.0) | 122.7 (9.9) | 121.8 (12.7) | 4 | 10 |
| COE | 102.5 (3.1) | 117.1 (9.9) | 119.3 (3.3) | 6 | 15 |
| SCO | 118.0 (5.8) | 90.7 (8.7) | 100.0 (6.1) | 4 | 10 |
| LSD | 104.3 (3.5) | 112.8 (6.5) | 103.4 (3.9) | 4 | 10 |
| O-H-LSD | 84.4 (11.9) | 84.9 (6.1) | 91.6 (7.4) | 6 | 15 |
| BZP | 103.4 (4.5) | 105.8 (16.8) | 100.9 (8.6) | 10 | 25 |
| mCPP | 108.5 (3.1) | 104.6 (9.4) | 80.4 (15.5) | 10 | 25 |
| PCP | 106.5 (5.0) | 106.3 (6.8) | 111.6 (8.1) | 4 | 10 |
| FEN | 103.8 (4.7) | 109.2 (7.2) | 109.9 (3.5) | 4 | 10 |
| MOR | 99.1 (22.9) | 128.2 (24.4) | 130.8 (22.8) | 2 | 5 |
| 6-AM | 116.4 (11.3) | 82.0 (17.9) | 94.9 (11.7) | 2 | 5 |
| COD | 105.2 (11.9) | 128.9 (12.6) | 94.3 (22.1) | 2 | 5 |
| HER | 83.1 (20.6) | 105.2 (31.2) | 99.0 (29.7) | 2 | 5 |
| MET | 101.9 (4.8) | 117.2 (4.4) | 108.4 (4.3) | 2 | 5 |
| EDDP | 76.7 (21.6) | 102.7 (11.7) | 62.9 (7.7) | 4 | 10 |
| KET | 109.2 (2.8) | 119.1 (8.0) | 115.8 (4.7) | 10 | 25 |
| THC | 90.4 (27.0) | 114.7 (7.9) | 105.4 (20.5) | 20 | 50 |
| THCCOOH | 116.9 (19.1) | 123.8 (8.1) | 107.4 (10.8) | 20 | 50 |

^a Expressed as "Mean (RSD)".

^b SPE of 500 mL ultrapure water samples spiked with 100 ng L⁻¹ of each analyte and 200 ng L⁻¹ of each IS; n = 3 replicates.

^c SPE of 500 mL treated wastewater samples spiked with 200 ng L⁻¹ of each analyte and 200 ng L⁻¹ of each IS; n = 3 replicates.

^d SPE of 200 mL raw wastewater samples spiked with 500 ng L⁻¹ of each analyte and 200 ng L⁻¹ of each IS; n = 3 replicates.

3.3. Application to the quantification of real samples

The developed method was applied to determine the levels of the selected illicit drugs in two treated wastewater grab samples and in five 24 h composite influent samples, all of them collected from the same STP in different days during February 2011.

Mean concentration values for compounds occurring at levels above their LOQ are compiled in **Table 4**. As it is shown, the highest levels corresponded to BE (up to 708 ng L⁻¹), the main metabolite of COC, matching the findings reported by other authors [29,47,50] and highlighting the widespread consumption of this illicit drug. Both, parent drug and metabolite, were quantified in all samples, with significantly higher concentrations in the composite influent samples collected during the weekend. The raw wastewater COC/BE ratio remained quite constant through the different days of the week: it varied from 0.32 to 0.56, slightly higher than the expected excretion ratio of 0.22, although this value has a large uncertainty due to the lack of reliable metabolism studies in humans [43]. THCCOOH could also be determined in all influent samples, confirming the extended abuse of cannabis. On the other hand, MET and its main metabolite EDDP were also quantified in all raw and treated wastewater samples, but their concentrations stayed more constant through the different week days, probably as a result of the use of MET as a medical substitute of heroin in anti-addictive treatment. AMP and COD were also quantified at relatively high values (up to 84.8 and 112.0 ng L⁻¹, respectively) in some of the samples, whereas COE, MDMA and MOR were measured at lower levels.

The concentrations from 24 h-composite influents were translated into mean loads and normalised per 1000 inhabitants-loads (**Table 4**). On the basis of the loads calculated for AMP, BE and THCCOOH, the consumption per 1000 inhabitants of amphetamine, cocaine and cannabis, respectively, was estimated [43]. It accounted for 78.2 mg day⁻¹ 1000 inh⁻¹ for amphetamine, 463 mg day⁻¹ 1000 inh⁻¹ for cocaine and 8500 mg day⁻¹ 1000 inh⁻¹ for cannabis. Assuming an average dose of 30 mg, 100 mg and 125 mg, respectively [43], these data are equivalent to 2.6 doses day⁻¹ 1000 inh⁻¹ of amphetamine, 4.6 doses day⁻¹ 1000 inh⁻¹ of cocaine and 68 doses day⁻¹ 1000 inh⁻¹ of cannabis. The above consumption is within the ranges published for these substances in Europe, with the exception of cannabis, whose maximum published consumption calculated through the sewage epidemiology approach until now had been 61 doses day⁻¹ 1000 inh⁻¹ [43].

Table 4. Mean concentration (n = 3) values for analytes occurring at levels above their LOQ in different real samples ^a.

| C (ng L ⁻¹) | Effluents (grab samples) ^b | | | Influents (24 h-composite samples) ^b | | | | | | | Loads (g day ⁻¹) ^c | Loads per 1000 inh (mg day ⁻¹) ^c |
|-------------------------|---------------------------------------|-------|-------|---|-------|-------|-------|------|-------|--|---|---|
| | W | Th | Tu | W | Tu | W | F | Sa | Su | | | |
| AMP | <LOD | 14.2 | 26.4 | <LOD | 84.8 | 83.5 | 61.7 | 3.2 | 23.5 | | | |
| MDMA | <LOD | <LOD | <LOD | <LOD | <LOQ | <LOD | 25.6 | - | - | | | |
| COC | 22.7 | 30.4 | 111.8 | 97.6 | 205.7 | 294.7 | 187.0 | 10.9 | 79.8 | | | |
| BE | 170.8 | 207.3 | 257.7 | 173.0 | 504.6 | 707.7 | 591.9 | 27.1 | 198.8 | | | |
| COE | <LOD | <LOQ | <LOQ | <LOQ | <LOQ | 24.4 | <LOQ | - | - | | | |
| MOR | <LOD | <LOD | <LOD | <LOD | 17.0 | 19.0 | 26.6 | 0.8 | 5.7 | | | |
| COD | 105.5 | <LOD | 112.0 | <LOD | <LOD | <LOD | <LOD | - | - | | | |
| MET | 20.0 | 15.2 | 23.7 | 15.1 | 31.7 | 33.4 | 29.6 | 1.6 | 11.9 | | | |
| EDDP | 33.9 | 21.0 | 54.6 | 22.3 | 35.5 | 43.1 | 46.7 | 2.5 | 18.0 | | | |
| THCCOOH | <LOD | 32.2 | 98.3 | 55.7 | 228.0 | 147.0 | 101.0 | 7.6 | 56.0 | | | |

^a <LOQ: below limit of quantification; <LOD: below limit of detection; RSD < 30% in all cases.

^b Week day: Tu: Tuesday; W: Wednesday; Th: Thursday; F: Friday; Sa: Saturday; Su: Sunday.

^c Calculated from mean values for the influent samples, considering values lower than LOD and LOQ as equal to LOD/2 and LOQ/2, respectively, for statistical calculation. Loads not calculated for compounds that were below LOQ in more than two samples.

3.4. Screening of other drugs/metabolites using a compound database

As mentioned in section 1, TOF systems provide high resolution spectra that can be used, after the analysis, to search for not preselected analytes (post-target approach). In fact, such possibility was tested by Hernández et al. [42] for screening drugs of potential abuse, but using an unspecific pseudo MS/MS method, named as MS^E by the manufacturer. As no real MS/MS was recorded in that case, the reliability of the results depended on a very efficient chromatographic separation, as UPLC used by the authors of the aforementioned work.

In the present study, the post-target screening approach was also tested in order to find out other possible substances of abuse that may have appeared in the market recently or other metabolites that may be relevant under environmental conditions but would have been missed in the target selection, but using pure MS and MS/MS data. To this end, a database containing more than 130 compounds was constructed, including the most popular illicit drugs of abuse and their metabolites [51,52] and also newly detected substances according to the last reports of UNODC [1] and EMCDDA [44]. The database (**Table 1**) compiles the empirical formulae of the recorded species plus some additional data.

The screening protocol was based on the “Find by Formula” function of the Mass Hunter software provided by the manufacturer. This algorithm automatically searches for the ionised forms and potential adducts of the compounds included in the database (with a defined mass error tolerance of ± 5 ppm) over the real samples, generating the accurate mass extracted chromatograms and comparing their peak spectra with the theoretical ones in terms of mass accuracy, isotopic match and spacing between ions. These three parameters are combined into an overall score, where a value of 100 would represent a perfect match [53]. After a positive match, samples are reanalysed in order to obtain their MS/MS product ion spectra, which can provide relevant structural information necessary for structural confirmation.

Although the 4 GHz option is not recommended for quantitative operation due to detector saturation, leading to m/z shifts at high concentrations (as discussed in section 3.1.), this fact is compensated with the Mass Hunter Qualitative Analysis software for qualitative purposes, as saturated m/z peaks are automatically detected and their spectra automatically taken on the peak tails at a defined percentage below saturation, where mass accuracy is maintained. Therefore, as a first step, an influent wastewater extract was spiked with the 24 target compounds at two concentration levels (10 and 100 ng mL⁻¹, equivalent to 50 and 500 ng L⁻¹ in the sample) and used as benchmark for the screening procedure at both 2 and 4 GHz. The results of this test showed that, at the highest spike level, 83% and 100% of the analytes were detected at 2 GHz and 4 GHz, respectively, whereas at the lowest concentration only

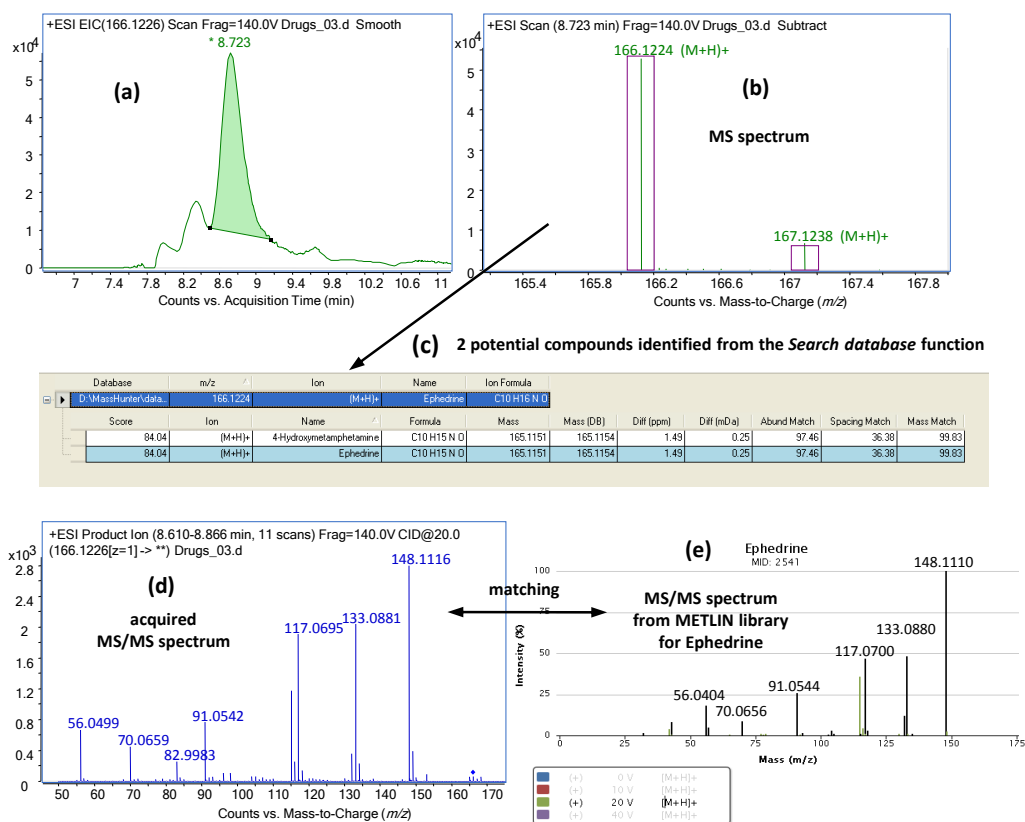
50% and 62% of the compounds were positively identified (**Table 5**). These results highlight one of the main drawbacks of post-target screening: at low concentration levels, the chances to identify new drugs being consumed decreases. Moreover, the highest resolution provides greater possibilities of success in identifying post-target compounds than the 2 GHz mode, but then samples need to be reinjected.

Table 5. Post-target screening of drugs of abuse in wastewater results in terms of detection (Y) or non-detection (N) at two concentration levels.

| | Spiked influent | | | |
|-------------------|------------------------|-------------------------|------------------------|-------------------------|
| | 2 GHz MS | | 4 GHz MS | |
| | 10 ng mL ⁻¹ | 100 ng mL ⁻¹ | 10 ng mL ⁻¹ | 100 ng mL ⁻¹ |
| 6-AM | N | Y | N | Y |
| O-H-LSD | Y | Y | N | Y |
| AMP | Y | Y | Y | Y |
| BE | N | Y | N | Y |
| BZP | N | Y | Y | Y |
| COE | Y | Y | Y | Y |
| COC | N | Y | Y | Y |
| COD | Y | Y | N | Y |
| EDDP | Y | Y | N | Y |
| FEN | Y | Y | Y | Y |
| HER | N | N | N | Y |
| KET | Y | Y | Y | Y |
| LSD | Y | Y | Y | Y |
| mCPP | N | Y | N | Y |
| MDA | N | Y | Y | Y |
| MDEA | Y | Y | Y | Y |
| MDMA | Y | Y | Y | Y |
| MET | Y | Y | Y | Y |
| MAMP | N | Y | Y | Y |
| MOR | Y | Y | Y | Y |
| PCP | N | N | N | Y |
| SCO | N | Y | Y | Y |
| THCCOOH | N | Y | Y | Y |
| THC | N | Y | N | Y |
| % Detected | 50% | 83% | 62% | 100% |

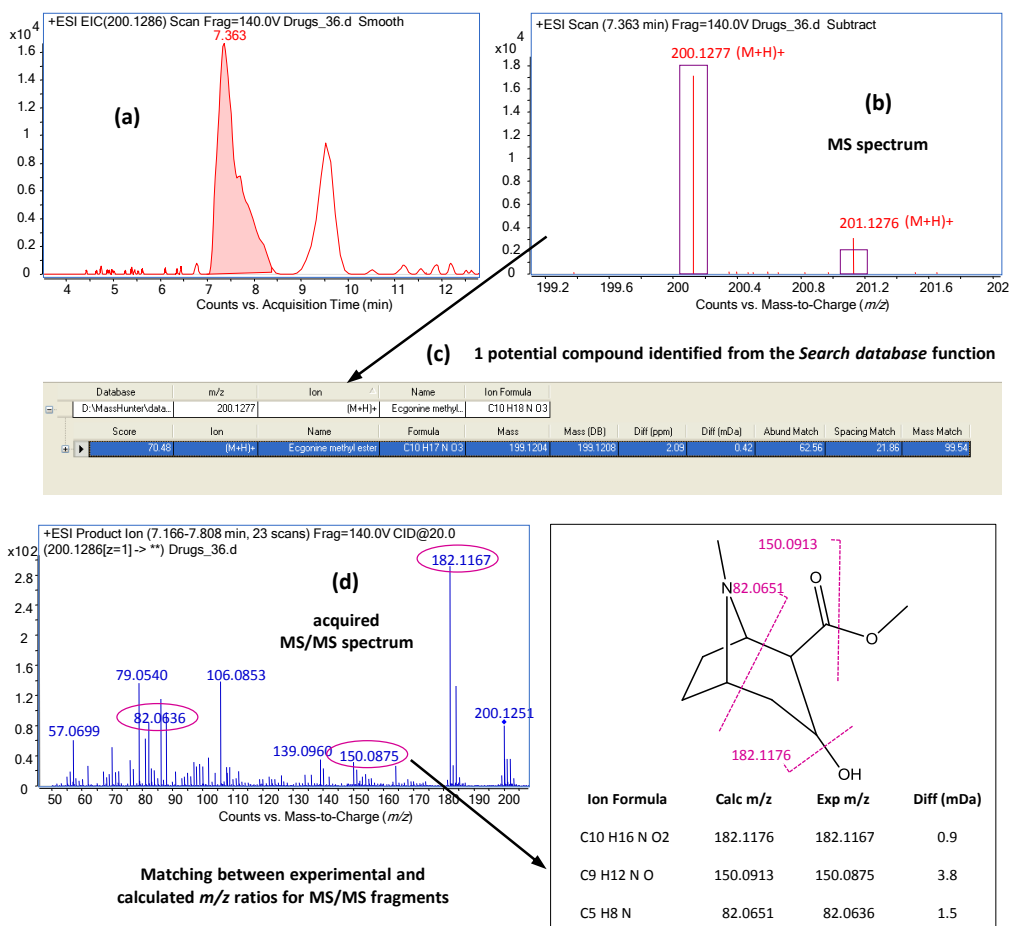
In view of these results, influent samples were reinjected in the 4 GHz mode and the screening protocol was applied. This methodology permitted the identification of ephedrine and ecgonine methyl ester in the influent samples, two substances already reported in wastewater [28,47]. As an example, **Figure 6** shows the identification workflow for ephedrine. First, the extracted ion chromatogram was automatically generated by the software (**Figure 6a**) and its MS spectrum (**Figure 6b**) compared to the theoretical one. In this particular instance, there were two potential positive matches with the database (**Figure 6c**): 4-hydroxymetamphetamine and ephedrine, actually having the same empirical formula. Once candidates were detected, the sample was reinjected and the MS/MS spectra acquired at several collision energies. Then, in this case, the MS/MS spectra (**Figure 6d**) were compared to those available in the METLIN library [54] (**Figure 6e**), so that the compound could be confirmed as ephedrine.

Figure 6. Identification workflow of ephedrine: (a) peak detected; (b) MS spectrum compared to database; (c) database match; (d) MS/MS spectrum acquired and contrasted with (e) METLIN library MS/MS spectrum for ephedrine.

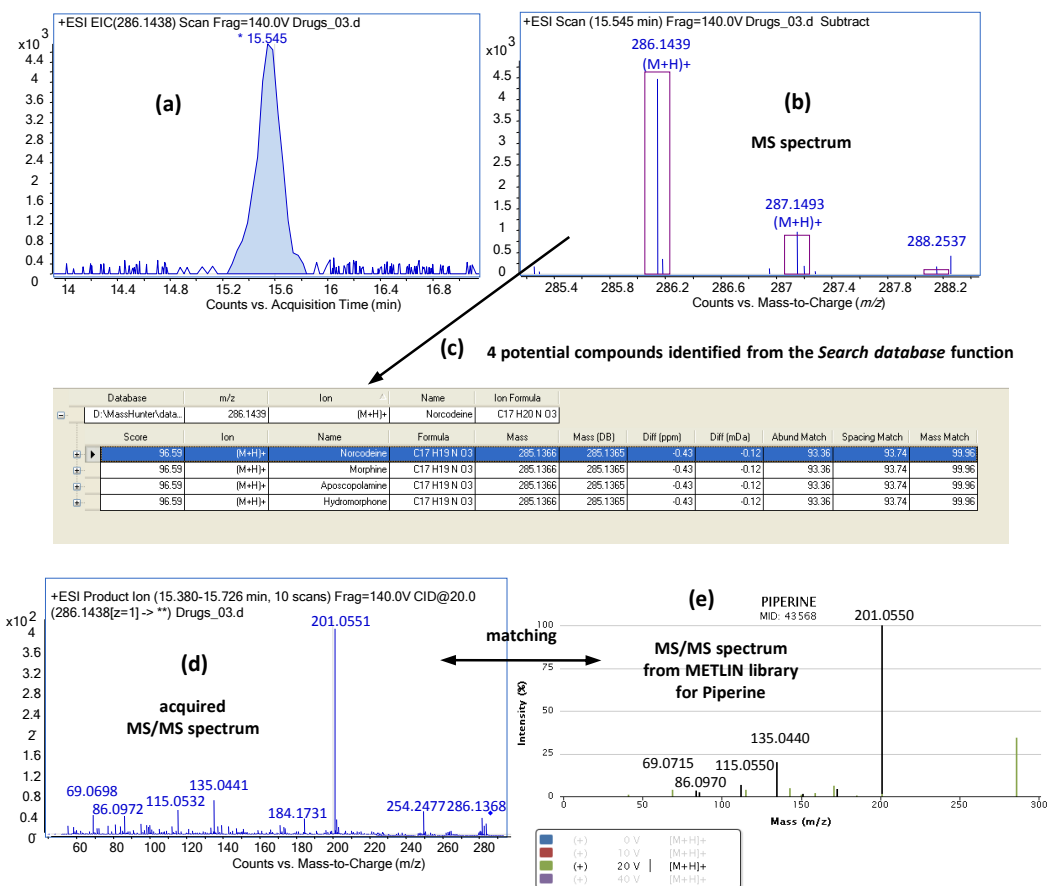


In the case of ecgonine methyl ester (**Figure 7**), no MS/MS spectra are available in the METLIN library, hence its structure was confirmed based on accurate product masses assignments and contrasted with the literature [30].

Figure 7. Sequence of steps followed to confirm the presence of ecgonine methyl ester in raw sewage samples.



On the other hand, an example of a compound initially identified as another potential drug or metabolite in the MS run and finally discarded on the basis of its MS/MS spectrum is presented in **Figure 8**. In this case, the MS/MS spectrum allowed the compound to be identified in the METLIN library as piperine: a natural alkaloid responsible for pungency of pepper and other hot spices.

Figure 8. Identification of piperine in wastewater, initially detected as a possible drug of abuse/metabolite


4. CONCLUSIONS

A new, selective SPE-LC-MS method for the simultaneous determination of 24 drugs of abuse and metabolites in wastewater samples was developed. Analytes were concentrated using mixed-mode Oasis MCX sorbents, improving the selectivity and LODs for basic drugs over other published SPE methodologies by adopting a fractionated elution strategy.

To the best of authors' knowledge, a liquid chromatograph coupled to a hybrid QTOF mass spectrometer was employed for the first time for the quantification of drugs of abuse in waters. Although instrumental LOQs were, in some cases, higher than other values reported with QqQ systems, they were still low enough to allow the determination of several drugs and metabolites in real samples. Moreover, the high mass accuracy and resolution of the QTOF instrument permitted the single MS determination of opioids and better confirmation of low mass amphetamine substances. Finally, the post-target capabilities of the QTOF system were

used for the identification of originally non-target contaminants, such as ephedrine and ecgonine methyl ester.

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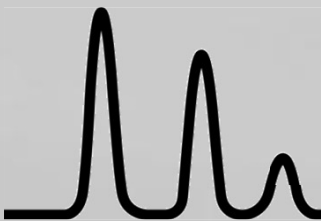
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IV. CONCLUSIONES GENERALES

GENERAL CONCLUSIONS

Las conclusiones derivadas de cada uno de los trabajos han sido detalladas en la publicación correspondiente, por lo que el siguiente capítulo recoge de forma resumida las conclusiones generales más relevantes que se han extraído de la tesis doctoral en su conjunto. Continuando con la estructura seguida a lo largo de la memoria, se dividirán en dos grupos atendiendo a la familia de contaminantes emergentes determinada en cada caso.

A. AGENTES ANTIMICROBIANOS

- A nivel metodológico, se ha confirmado la importancia de reevaluar los efectos de matriz al transferir un método de cuantificación entre dos sistemas LC-MS con interfases de electrospray diferentes. Sólo de esta forma, es posible definir la estrategia de cuantificación más adecuada para cada situación.
- Se ha demostrado la capacidad de la nueva generación de analizadores híbridos cuadrupolo-tiempo de vuelo para evaluar la presencia de compuestos no preseleccionados originalmente como analitos (*post-target analysis*): procesando la información espectral latente en los ficheros de MS, se pudo detectar en muestras reales al derivado monoclorado del *n*-propil parabén, del que no se disponía de patrón y cuya identidad fue confirmada a posteriori mediante ensayos de MS/MS.
- Se ha demostrado la utilidad de la microextracción con adsorbentes empaquetados acoplada *at-line* con GC-MS para la determinación de agentes antimicrobianos en aguas residuales, mediante un método sencillo, con un mínimo consumo de muestra y disolventes orgánicos y totalmente automatizado.
- Similarmente, la dispersión de la matriz en fase sólida se ha revelado como una alternativa rápida, simple y selectiva frente a las metodologías disponibles en la bibliografía (habitualmente basadas en extracción Soxhlet, MAE o PLE) para la extracción de triclosán y metil triclosán en lodos y sedimentos.
- La aplicación de los métodos desarrollados a muestras reales ha permitido confirmar la ubiquidad de los parabenes en las aguas residuales sin tratar, a concentraciones que constituyen un reflejo de su inclusión en los productos de cuidado personal (el metil parabén y el *n*-propil parabén son, con diferencia, los más abundantes).

IV. CONCLUSIONES GENERALES

- La determinación de los niveles de parabenos en los efluentes ha confirmado su rápida degradación, posteriormente corroborada mediante un ensayo de biodegradabilidad aeróbica a escala de laboratorio. Comparativamente, se ha demostrado que sus derivados halogenados son más resistentes a los procesos de degradación, aportando los primeros datos relativos al comportamiento de estos compuestos en las estaciones depuradoras.
- Finalmente, la determinación de triclosán y metil triclosán en muestras de lodo real ha ampliado la escasa información existente en torno a la distribución de este último en matrices sólidas ambientales; adicionalmente, ha servido para confirmar la biometilación del compuesto nativo por la acción de microorganismos contenidos en las aguas residuales.

B. DROGAS DE ABUSO

- Por primera vez, se ha aplicado la cromatografía de gases acoplada a espectrometría de masas en tándem para determinar drogas de abuso en una matriz ambiental (aguas fluviales y residuales), presentándose una alternativa más económica y menos susceptible a los efectos de matriz que los métodos de LC-MS descritos hasta entonces en la bibliografía.
- En relación a estos efectos, se ha demostrado la importancia de incrementar la selectividad en los procesos de preparación de muestra para minimizar su impacto durante la ionización de los analitos en interfases de electrospray. En este sentido, se han propuesto dos procedimientos de SPE especialmente selectivos para la determinación de sustancias ilícitas en aguas residuales: el primero, para la extracción de derivados anfetamínicos mediante polímeros de impresión molecular, y el segundo para la extracción de 24 drogas de abuso y metabolitos mediante adsorbentes poliméricos en modo mixto con elución fraccionada.
- A nivel instrumental, se ha demostrado la validez de la nueva generación de espectrómetros de analizador híbrido cuadrupolo-tiempo de vuelo para la cuantificación de drogas de abuso en aguas mediante MS/MS (hasta la presente tesis doctoral, su aplicación en este campo sólo había sido abordada con fines cualitativos). Aunque su sensibilidad resultó, en algunos casos, inferior a la de los sistemas de triple cuadrupolo, fue suficientemente elevada como para permitir la cuantificación de varias sustancias ilegales en muestras reales.

- Al igual que con los parabenos, se ha confirmado la capacidad de los sistemas QTOF para identificar compuestos no definidos a priori en el método, una vez que el análisis ha finalizado y sin necesidad de disponer de patrones comerciales. De esta forma, ha sido posible detectar en muestras reales potenciales drogas de abuso y metabolitos no preseleccionados originalmente como analitos.
- Finalmente, la cuantificación de varias sustancias en muestras compuestas de influente se ha utilizado para extraer los primeros datos relativos a la prevalencia de su consumo en el noroeste de España de acuerdo con la *Epidemiología de Aguas Residuales*.

The conclusions derived from each work have been detailed in the corresponding published article. Therefore, the present chapter summarises the most relevant conclusions that have been extracted from the whole PhD dissertation. Continuing with the structure followed throughout the text, they are divided in two groups in accordance with the family of emerging pollutants determined in each case.

A. ANTIMICROBIAL AGENTS

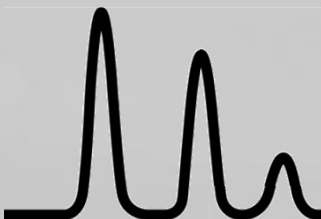
- In the analytical field, it has been confirmed the importance of re-evaluating matrix effects whenever a method is transferred between two LC-MS systems with different ESI interfaces. Only in such a way it is possible to define the most suitable quantification strategy for every single situation.
- It has been demonstrated the ability of the new generation of hybrid quadrupole-time-of-flight mass analysers to evaluate the presence of originally non-target compounds (*post-target analysis*): processing the spectral information from MS data, it was possible to detect the monochlorinated derivative of *n*-propyl paraben (whose standard was not available in the laboratory and whose identity was subsequently confirmed by MS/MS experiments) in real samples.
- It has been demonstrated the usefulness of microextraction by packed sorbents coupled at-line to GC-MS for the determination of antimicrobial agents in wastewater, in a simple, fully automated procedure entailing a minimum sample and solvent consumption.
- Similarly, matrix solid-phase dispersion has been revealed as a fast, simple and selective alternative for the determination of triclosan and methyl triclosan in sludge and sediments, in comparison to other methodologies available in the literature (generally based on Soxhlet extraction, MAE or PLE).
- Application of the developed methods to real samples has enabled confirming the ubiquity of parabens in raw wastewater at levels reflecting their inclusion in personal-care compounds: methyl and *n*-propyl paraben are the most frequently detected and the most abundant species.

- Determination of parabens levels in effluents has confirmed their fast degradation, subsequently corroborated by a lab-scale aerobic biodegradability assay. Comparatively, it has been demonstrated that their halogenated derivatives are more resistant to the degradation processes, providing the first data concerning their behaviour in wastewater treatment plants.
- Finally, determination of triclosan and methyl triclosan in real sludge samples has increased the limited information available relating the occurrence of the latter compound in environmental solid samples; additionally, it has been used to confirm the biomethylation of the native compound by the action of wastewater microorganisms.

B. DRUGS OF ABUSE

- For the first time, gas chromatography coupled to tandem mass spectrometry has been applied to the determination of drugs of abuse in an environmental matrix (surface and wastewater), proving to be an inexpensive and less affected by matrix effects alternative than the LC-MS based methods described in the literature.
- Concerning these effects, it has been proved the importance of increasing selectivity in sample preparation processes in order to minimise their impact during analytes ionisation in electrospray interfaces. In this sense, two highly selective SPE procedures have been developed for the determination of illicit substances in wastewater: the first one, for the extraction of amphetamine-like compounds by means of molecularly imprinted polymers; the second one, for the extraction of 24 drugs of abuse and metabolites by means of mixed-mode polymeric sorbents with fractionated elution.
- Relating to the instrumentation, it has been demonstrated the ability of the new generation of hybrid quadrupole-time-of-flight mass analysers to quantify drugs of abuse in waters by MS/MS (before this PhD dissertation, its applicability in this field had only been performed with qualitative purposes). Although, in some cases, its sensitivity turned out to be lower than the one obtained with triple-quadrupole instruments, it was high enough to allow the quantification of several illicit substances in real samples.

- As in the case of parabens, ability of QTOF systems to identify compounds not originally defined in the method, once the analysis has been performed and without the need for commercial standards, has been proved. In this way, it was possible to detect potential drugs of abuse and metabolites (different from the ones selected as analytes) in real samples.
- Finally, quantification of several substances in composite influent samples was used to estimate the first data concerning the prevalence of their abuse in the northwest of Spain according to the *Sewage Epidemiology* approach.



SIGLAS Y ACRÓNIMOS

ABBREVIATIONS AND ACRONYMS

| | | |
|------------------------|--|--|
| 2-PrOH | <i>2-Propanol</i> | 2-Propanol |
| ACN | <i>Acetonitrile</i> | Acetonitrilo |
| ADC | <i>Analog to digital converter</i> | Convertidor de analógico a digital |
| AED | <i>Atomic emission detector</i> | Detector de emisión atómica |
| APCI | <i>Atmospheric-pressure chemical ionisation</i> | Ionización química a presión atmosférica |
| B | <i>Magnetic sector</i> | Sector magnético |
| BIN | <i>Barrel insert and needle</i> | Inserto de cartucho y aguja |
| BSA | <i>N,O-bis(trimethylsilyl)acetamide</i> | <i>N,O</i> -bis(trimetilsilil)acetamida |
| BSTFA | <i>N,O-bis(trimethylsilyl)trifluoroacetamide</i> | <i>N,O</i> -bis(trimetilsilil)trifluoroacetamida |
| C₂ | <i>Ethylsilane</i> | Etilsilano |
| C₈ | <i>Octylsilane</i> | Octilsilano |
| C₁₈ | <i>Octadecylsilane</i> | Octadecilsilano |
| CE | <i>Collision energy</i> | Energía de colisión |
| CID | <i>Collision induced dissociation</i> | Disociación inducida por colisión |
| DAD | <i>Diode-array detector/detection</i> | Detector/detección de red de diodos |
| DCM | <i>Dichloromethane</i> | Diclorometano |
| DI | <i>Direct injection</i> | Inyección directa |
| DLLME | <i>Dispersive liquid-liquid microextraction</i> | Microextracción líquido-líquido dispersiva |
| DVB | <i>Divinylbenzene</i> | Divinilbenceno |
| EC/CE | <i>European Commission</i> | Comisión Europea |
| EC₅₀ | <i>Effective concentration</i> | Concentración efectiva |
| ECD | <i>Electron capture detector</i> | Detector de captura electrónica |

SIGLAS Y ACRÓNIMOS

| | | |
|----------------|--|---|
| EDCs | <i>Endocrine-disrupting chemicals</i> | Disruptores endocrinos |
| EEC/CEE | <i>European Economic Community</i> | Comunidad Económica Europea |
| EI | <i>Electron impact</i> | Impacto electrónico |
| EMCDDA | <i>European Monitoring Centre for Drugs and Drug Addiction</i> | Observatorio europeo de las drogas y las toxicomanías |
| EPA | <i>Environmental Protection Agency</i> | Agencia para la protección del medio ambiente |
| ESI | <i>Electrospray ionisation</i> | Ionización por electrospray |
| EtOH | <i>Ethanol</i> | Etanol |
| EtOAc | <i>Ethyl acetate</i> | Acetato de etilo |
| FIA | <i>Flow injection analysis</i> | Análisis por inyección en flujo |
| FID | <i>Flame ionisation detector/detection</i> | Detector/detección por ionización en llama |
| FT | <i>Fourier transform</i> | Transformada de Fourier |
| FWHM | <i>Full width at half maximum</i> | Anchura a mitad de altura máxima |
| GC | <i>Gas chromatography</i> | Cromatografía de gases |
| HCl | <i>Hydrochloric acid</i> | Ácido clorhídrico |
| HCOOH | <i>Formic acid</i> | Ácido fórmico |
| HF-LPME | <i>Hollow-fiber liquid phase microextraction</i> | Microextracción en fase líquida con fibra hueca |
| HLB | <i>Hydrophilic lipophilic balance</i> | Balance hidrofílico lipofílico |
| HOAc | <i>Acetic acid</i> | Ácido acético |
| HFBA | <i>Heptafluorobutyric anhydride</i> | Anhídrido heptafluorobutírico |
| HFIP | <i>Hexafluoro-2-propanol</i> | Hexafluoro-2-propanol |
| HPLC | <i>High-performance liquid chromatography</i> | Cromatografía de líquidos de alta eficacia |
| HX | <i>Hexane</i> | Hexano |
| ICR | <i>Ion cyclotron resonance</i> | Resonancia de ion ciclotrón |

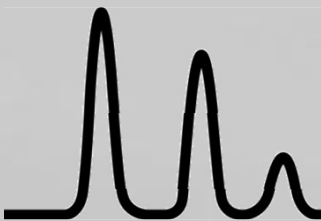
| | | |
|------------------------|---|--|
| IS | <i>Internal standard</i> | Patrón interno |
| ISO | <i>International Organization for Standardization</i> | Organización Internacional para la Estandarización |
| IT | <i>Ion trap</i> | Trampa de iones |
| K_{ow} | <i>Octanol-water partition constant</i> | Constante de partición octanol-agua |
| LC | <i>Liquid chromatography</i> | Cromatografía de líquidos |
| LC₅₀ | <i>Lethal concentration</i> | Concentración letal |
| LIT | <i>Linear ion trap</i> | Trampa de iones lineal |
| LLE | <i>Liquid-liquid extraction</i> | Extracción líquido-líquido |
| LOD | <i>Limit of detection</i> | Límite de detección |
| LOQ | <i>Limit of quantification</i> | Límite de cuantificación |
| LVI | <i>Large volume injector/injection</i> | Inyector/inyección de grandes volúmenes |
| MA | <i>Methacrylate</i> | Metacrilato |
| MAE | <i>Microwave assisted extraction</i> | Extracción asistida por microondas |
| MALLE | <i>Membrane-assisted liquid-liquid extraction</i> | Microextracción líquido-líquido asistida por membranas |
| MBTFA | <i>N-methyl-bis(trifluoroacetamide)</i> | <i>N</i> -metil-bis(trifluoroacetamida) |
| ME | <i>Matrix effects</i> | Efectos de matriz |
| MEC | <i>Measured environmental concentration</i> | Concentración ambiental medida |
| MeOH | <i>Methanol</i> | Metanol |
| MEPS | <i>Microextraction by packed sorbents</i> | Microextracción con adsorbentes empaquetados |
| MIP | <i>Molecularly imprinted polymer</i> | Polímero impreso molecularmente |
| MISPE | <i>Solid-phase extraction with molecularly imprinted polymers</i> | Extracción en fase sólida con polímeros de impresión molecular |
| MS | <i>Mass spectrometry</i> | Espectrometría de masas |

| | | |
|---------------------------|--|--|
| MS/MS | <i>Tandem mass spectrometry</i> | Espectrometría de masas en tándem |
| MSD | <i>Mass selective detector</i> | Detector selectivo de masas |
| MSPD | <i>Matrix solid-phase dispersion</i> | Dispersión de la matriz en fase sólida |
| MSTFA | <i>N-methyl-N-(trimethylsilyl)trifluoroacetamide</i> | <i>N</i> -metil- <i>N</i> -(trimetilsilil)trifluoroacetamida |
| MTBE | <i>Methyl-tert-butyl-ether</i> | Metil- <i>tert</i> -butil-éter |
| MTBSTFA | <i>N-methyl-N-(tert-butyl)dimethylsilyl)trifluoroacetamide</i> | <i>N</i> -metil- <i>N</i> -(<i>tert</i> -butildimetilsilil)trifluoroacetamida |
| MW | <i>Molecular weigh</i> | Peso molecular |
| <i>m/z</i> | <i>Mass/charge ratio</i> | Relación masa/carga |
| NaOAc | <i>Sodium acetate</i> | Acetato de sodio |
| NCI | <i>Negative chemical ionisation</i> | Ionización química negativa |
| NH₄OAc | <i>Ammonium acetate</i> | Acetato amónico |
| NH₄OCOH | <i>Ammonium formate</i> | Formiato amónico |
| NH₄OH | <i>Ammonium hydroxide</i> | Hidróxido amónico |
| NOEC | <i>No observed effect concentration</i> | Concentración sin efecto adverso observado |
| NORMAN | <i>Network of reference laboratories for monitoring of emerging environmental pollutants</i> | Red de laboratorios de referencia para la monitorización de contaminantes ambientales emergentes |
| NP | <i>Normal phase</i> | Fase normal |
| PCPs | <i>Personal care products</i> | Productos de cuidado personal |
| PDMS | <i>Polydimethylsiloxane</i> | Polidimetilsiloxano |
| PFPA | <i>Pentafluoropropionic anhydride</i> | anhídrido pentafluoropropiónico |
| PFPOH | <i>Pentafluoropropionic hydroxide</i> | hidróxido pentafluoropropiónico |
| PLE | <i>Pressurized liquid extraction</i> | Extracción con líquidos presurizados |

| | | |
|----------------------|--|---|
| Pm | <i>Molecular weight</i> | Peso molecular |
| PNEC | <i>Predicted no-effect concentration</i> | Concentración prevista sin efectos |
| POCIS | <i>Polar organic chemical integrative samplers</i> | Sistemas de muestreo pasivo para compuestos orgánicos polares |
| PPCPs | <i>Pharmaceuticals and personal care products</i> | Fármacos y productos de cuidado personal |
| PS | <i>Polystyrene</i> | Poliestireno |
| PTFE | <i>Polytetrafluoroethylene</i> | Politetrafluoroetileno |
| PTV | <i>Programmed temperature vaporizer</i> | Inyector de temperatura programada |
| P_v | <i>Vapour pressure</i> | Presión de vapor |
| PVP | <i>Poly(N-vinylpyrrolidone)</i> | Poli(N-vinilpirrolidona) |
| Q | <i>Quadrupole</i> | Cuadrupolo simple |
| QqQ | <i>Triple quadrupole</i> | Triple cuadrupolo |
| QTOF | <i>Quadrupole-time of flight</i> | Cuadrupolo-tiempo de vuelo |
| R | <i>Recovery</i> | Recuperación |
| R² | <i>Determination coefficient</i> | Coficiente de determinación |
| RP | <i>Reversed phase</i> | Fase reversa |
| RQ | <i>Risk quotient</i> | Cociente de riesgo |
| RSD | <i>Relative standard deviation</i> | Desviación estándar relativa |
| S/N | <i>Signal/noise ratio</i> | Relación señal/ruido |
| SBSE | <i>Stir-bar sorptive extraction</i> | Extracción con barras agitadoras |
| SD | <i>Standard deviation</i> | Desviación estándar |
| SDME | <i>Single drop microextraction</i> | Microextracción con gota suspendida |
| SEC | <i>Size exclusion chromatography</i> | Cromatografía de exclusión por tamaños |
| SFE | <i>Supercritical fluid extraction</i> | Extracción con fluidos supercríticos |

SIGLAS Y ACRÓNIMOS

| | | |
|------------------------|---|--|
| SIM | <i>Selected ion monitoring</i> | Monitorización de un ion seleccionado |
| SPE | <i>Solid-phase extraction</i> | Extracción en fase sólida |
| SPME | <i>Solid-phase microextraction</i> | Microextracción en fase sólida |
| SRM | <i>Selective reaction monitoring</i> | – |
| STP | <i>Sewage treatment plant</i> | Estación depuradora de aguas residuales urbanas |
| t_{1/2} | <i>Half-life time</i> | Tiempo de vida media |
| TC | <i>Total carbon</i> | Carbono total |
| TEA | <i>Triethylamine</i> | Trietilamina |
| TMS | <i>Trimethylsilyl</i> | Trimetilsilil |
| TMSDEA | <i>N-(trimethylsilyl)-N-diethylamine</i> | <i>N</i> -(trimetilsilil)- <i>N</i> -dietilamina |
| TOC | <i>Total organic carbon</i> | Carbono orgánico total |
| TOF | <i>Time of flight</i> | Tiempo de vuelo |
| TrBA | <i>Tributylamine</i> | Tributilamina |
| UNODC | <i>United Nations Office of Drugs and Crime</i> | Oficina de las Naciones Unidas para las Drogas y el Crimen |
| UPLC | <i>Ultra-performance liquid chromatography</i> | Cromatografía de líquidos de ultra eficacia |
| USAE | <i>Ultrasound assisted extraction</i> | Extracción asistida por ultrasonidos |
| USAEME | <i>Ultrasound assisted emulsification-microextraction</i> | Microextracción por emulsión asistida por ultrasonidos |
| UV filters | <i>Ultraviolet filters</i> | Filtros ultravioleta |
| UV-VIS | <i>Ultraviolet-visible</i> | Ultravioleta-visible |
| WFD | <i>Water Framework Directive</i> | Directiva marco del agua |
| WWTP | <i>Wastewater treatment plant</i> | Estación depuradora de aguas residuales (EDAR) |



OTRAS PUBLICACIONES

In-sample acetylation-non-porous membrane-assisted liquid–liquid extraction for the determination of parabens and triclosan in water samples

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Abstract A procedure for the determination of seven parabens (esters of 4-hydroxybenzoic acid), including the distinction between branched and linear isomers of propyl- and butyl-parabens and triclosan in water samples, was developed and evaluated. The procedure includes in-sample acetylation-non-porous membrane-assisted liquid–liquid extraction and large volume injection–gas chromatography–ion trap–tandem mass spectrometry. Different derivatisation strategies were considered, i.e. post-extraction silylation with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide and in situ acylation with acetic anhydride (Ac₂O) and isobutylchloroformate. Moreover, acceptor solvent and the basic catalyser of the acylation reaction were investigated. Thus, in situ derivatisation with Ac₂O and potassium hydrogenphosphate (as basic catalyser) was selected. Potassium hydrogenphosphate overcomes some drawbacks of other basic catalysers, e.g. toxicity and bubble formation, while leads to higher responses. Subsequently, other experimental variables affecting derivatisation–extraction yield such as pre-stirring time, salt addition and volume of Ac₂O were optimised by an experimental design approach. Under optimised conditions, the proposed method achieved detection limits from 0.1 to 1.4 ng L⁻¹ for a sample volume of 18 mL and extraction efficiencies, estimated by comparison with liquid–liquid extraction, between 46% (for methyl- and ethyl-parabens) and 110%

(for benzylparaben). The reported sample preparation approach is free of matrix effects for parabens but affected for triclosan with a reduction of ≈ 40% when wastewater samples are analysed; therefore, both internal and external calibration can be used as quantification techniques for parabens, but internal standard calibration is mandatory for triclosan. The application of the method to real samples revealed the presence of these compounds in raw wastewater at concentrations up to 26 ng mL⁻¹, the prevalence of the linear isomer of propylparaben (*n*-PrP), and the coexistence of the two isomers of butylparaben (*i*-BuP and *n*-BuP) at similar levels.

Keywords Parabens · Triclosan · Polyethylene membranes · Membrane-assisted liquid–liquid extraction (MALLE) · Membrane-assisted solvent extraction (MASE) · Derivatisation · Gas chromatography–mass spectrometry (GC-MS) · Water

Introduction

Triclosan and parabens (alkyl and aryl esters of 4-hydroxybenzoic acid) are employed as bactericides in the formulation of personal care products (PCPs) in amounts ranging between 0.3% and 0.8% [1]. Triclosan is also incorporated as a biocide in sportive clothes, footwear, carpets, plastic toys and kitchenware, and parabens as preservatives in pharmaceuticals and food products [2]. Although for most of them ecotoxicological data are still scarce, nowadays, it is known that all these compounds are weak endocrine disruptors [3, 4]. Moreover, recent studies have suggested a possible relationship between breast cancer and prolonged dermal expositions

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to paraben-containing products [5]. The main concern about triclosan is that it can turn into more toxic and persistent species such as chlorinated phenols, polychlorinated biphenyl ethers and polychlorinated dibenzodioxins [6–8].

As in the case of other chemicals used in PCP formulations, parabens and triclosan are continuously released into the environment through domestic and industrial wastewater and, although most of them (particularly parabens) are completely removed in conventional wastewater treatment plants (WWTP) [9–12], they have been detected in river water [13–16].

Analytical methods for the determination of these compounds in water samples are based on a pre-concentration step, usually solid-phase extraction (SPE), followed by the subsequent determination by gas chromatography–mass spectrometry (GC-MS) [10] or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [13, 17]. Common pre-concentration techniques, such as SPE, are time, solvent and labour consuming. Recently, much effort has been focused on the development of pre-concentration procedures for the determination of parabens and triclosan, which reduce solvent consumption, time and labour effort, such as solid-phase microextraction (SPME) [9, 12, 14], single-drop microextraction (SDME) [18] or ultrasound-assisted emulsification–microextraction (USAEME) [19].

Regarding GC-MS determination, these compounds are often derivatised to improve sensitivity, peak separation and peak symmetry. Silylation has been applied to the derivatisation of parabens and triclosan, mainly using *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) as derivatisation reagent in organic media [9, 12, 20]. Moreover, acylation with acetic anhydride (Ac₂O) has been applied to their derivatisation in aqueous samples [14, 19].

The aim of this work is the development of a method based on membrane-assisted liquid–liquid extraction (MALLE) [21, 22] for the determination of parabens and triclosan in water samples. This technique is carried out by using a non-porous membrane as interface between the sample (donor) and the organic solvent (acceptor), which is not only considered as barrier for particles and macromolecules and for avoiding mixing the two phases but also can provide selectivity in terms of permeation and transport through the membrane. Major advantages are the small amount of solvent required for extraction, the possible exclusion of matrix components and the low cost of membranes. Several derivatisation and extraction conditions were optimised, and in the final method, derivatisation of parabens and triclosan was performed in the aqueous phase with acetic anhydride prior to their GC-MS/MS determination.

Experimental

Chemicals

Methyl (MeP), ethyl (EtP), *n*-propyl (*n*-PrP), *n*-butyl (*n*-BuP) and benzyl (BzP) esters of 4-hydroxybenzoic acid, as well as triclosan (TCS), were purchased from Aldrich (Milwaukee, WI, USA). *Iso*-propylparaben (*i*-PrP) and *iso*-butylparaben (*i*-BuP) were obtained from TCI Europe (Zwijndrecht, Belgium). As surrogate internal standards, methyl 4-hydroxybenzoate-2,3,5,6-*d*₄ (MeP *d*₄) and *n*-propyl 4-hydroxybenzoate-2,3,5,6-*d*₄ (*n*-PrP *d*₄) were obtained from CDN Isotopes (Quebec, Canada), and a solution of ¹³C₁₂-triclosan (¹³C₁₂ TCS; 100 μg mL⁻¹ in nonane, 99%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

Individual stock solutions (1,000 μg mL⁻¹) and mixtures of all the analytes (20 μg mL⁻¹) were prepared in methanol and subsequently diluted as necessary. Solutions containing the labelled compounds (5 μg mL⁻¹) were prepared in methanol.

Methanol, hexane, acetonitrile, chloroform, cyclohexane, ethyl acetate, propanol and acetone (all of chromatographic analysis grade), hydrochloric acid and pyridine were purchased from Merck (Darmstadt, Germany). Potassium hydrogencarbonate and potassium hydrogenphosphate were obtained from Aldrich, sodium chloride from VWR Prolabo (Fontenay-sous-Bois, France) and sodium sulphate anhydride from Panreac (Castellar del Vallès, Spain). The derivatisation reagents MTBSTFA and isobutylchlorofomate (iBCF) were supplied by Aldrich and Ac₂O by Acros Organics (Geel, Belgium). Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, MA, USA).

The polymeric material, low density polyethylene (LDPE), was bought in a local supermarket, where it was sold as freezing bags for food (with a membrane thickness of 0.02 mm).

Samples

Surface water and WWTP influent and effluent samples were employed. The WWTP is located near Santiago de Compostela (Galicia, NW Spain) and receives urban and hospital wastewater from ~100,000 inhabitants. Surface water was collected from the river Sar in Galicia (NW Spain), 4 km downstream the WWTP effluent discharge. All samples were taken in amber glass bottles previously rinsed with ultrapure water and methanol and stored in the dark at 4°C for a maximum of 24 h prior to their analysis. Samples were filtered using cellulose acetate membranes (0.45 μm pore size).

Equipment

All analyses were performed using a Varian 450-GC gas chromatograph equipped with an ion trap MS detector Varian-

240-MS and a CP-8400 sampler (Varian Chromatography Systems, Walnut Creek, CA, USA). A Varian 1079 programmed temperature vaporisation (PTV) injector equipped with a Siltek® deactivated liner with frit (Restek, Bellefonte, PA, USA) was used. The PTV LargeVolume injection mode was used with an initial temperature of 45°C and a split flow of 75 mL min⁻¹ (1 min) and raised at 200°C min⁻¹ in splitless mode to a final temperature of 300°C and a split flow of 80 mL min⁻¹. The injection volume was 20 µL. GC analysis was performed on a HP-5ms column (30 m×0.25 mm, 0.25 µm film thickness) (Agilent Technologies, Palo Alto, CA, USA). The oven temperature program was as follows: the initial temperature was 50°C, and this temperature was held for 4 min and then it was increased to 270°C at 10°C min⁻¹, held for 10 min. The helium carrier gas was maintained at a flow rate of 1 mL min⁻¹.

The ion energy used for electron impact ionisation (EI) in the mass spectrometer was 70 eV. Manifold, ion trap, ion source and transfer line temperatures were maintained at 40, 150, 200 and 290°C, respectively. Helium was also used as damping gas at a flow of 2.5 mL min⁻¹.

The ion trap MS operated in the mass range 35–500 *m/z* in the full-scan acquisition mode. For MS/MS analysis, general parameters were fixed as follows: filament delay, 15.80 min; filament emission current, 80 µA; target TIC, 2,000 counts. Specific MS/MS conditions for each analyte are listed in Table 1.

Membrane-assisted liquid–liquid extraction

The LDPE membrane bags were made of by a shrink-wrapping device “CR-200” (Rovebloc, Barcelona, Spain). The tailor-made membrane bags were prepared of 25 mm×10 mm, and its solvent capacity was about 400 µL. After preparing the bags, the overlaying foil borders were cut carefully in order to reduce the polymeric material that

could adsorb analytes. Then, the membrane bags were filled with chloroform to clean them and to check their tightness.

Aliquots of water samples (18 mL) were poured in 22-mL glass vials with crimp cap, containing a magnetic stirrer. After optimisation, 0.2 g potassium hydrogenphosphate and 200 µL of Ac₂O were added to the sample, and the solution was stirred at 500 rpm for 8 min in the capped vial. The membrane bag was attached to a metal funnel and fixed with a Teflon ring (Gerstel, Mühlheim an der Ruhr, Germany), and the funnel was suspended in the opening of the vial (Fig. 1). The bag was filled with 400 µL chloroform, the vial was capped again and the extraction was performed under stirring (500 rpm) at 35°C during 90 min. Finally, the organic phase was withdrawn from the membrane bag and transferred to a 2-mL autosampler vial. This extract did not require any further handling before the PTV-GC-MS/MS analysis.

Extraction efficiencies were estimated by comparison with derivatised compounds prepared by aqueous acetylation and liquid–liquid extraction as follows: a known amount of analytes was spiked over 1.5 mL of Milli-Q water, which contained 0.02 g of potassium hydrogenphosphate and 20 µL of acetic anhydride. The solution was shaken during 5 min. After that, 1.5 mL of chloroform was added, and the mixture was additionally shaken for 3 min. The organic phase was separated and injected in the chromatographic system.

Results and discussion

Derivatisation and extraction conditions

The studied compounds are often determined after derivatisation of the native forms to less polar and more volatile species when GC is the final separation and determination technique. Several post-extraction derivatisation strategies can be considered, such as alkylation with diazomethane or pentafluor-

Table 1 GC-MS/MS working conditions

| Compound | Retention time (min) | Parent ion (<i>m/z</i>) | Excitation storage level (<i>m/z</i>) | Excitation amplitude (V) | Mass range (<i>m/z</i>) | Quantification ions (<i>m/z</i>) |
|-------------------------------------|----------------------|---------------------------|---|--------------------------|---------------------------|------------------------------------|
| MeP <i>d</i> ₄ | 16.14 | 156 | 40 | 31 | 100–166 | 125 |
| MeP | 16.17 | 152 | 40 | 31 | 100–160 | 121 |
| EtP | 17.07 | 166 | 40 | 26 | 100–170 | 121 |
| <i>i</i> -PrP | 17.45 | 138 | 40 | 29 | 100–145 | 121 |
| <i>n</i> -PrP <i>d</i> ₄ | 18.23 | 142 | 40 | 29 | 100–152 | 125 |
| <i>n</i> -PrP | 18.26 | 138 | 40 | 29 | 100–145 | 121 |
| <i>i</i> -BuP | 18.91 | 138 | 40 | 29 | 100–145 | 121 |
| <i>n</i> -BuP | 19.43 | 138 | 40 | 29 | 100–145 | 121 |
| BzP | 23.58 | 228 | 55 | 31 | 75–235 | 183 |
| ¹³ C ₁₂ TCS | 23.62 | 302 | 70 | 46 | 100–315 | 230 |
| TCS | 23.62 | 290 | 70 | 46 | 100–300 | 218 |

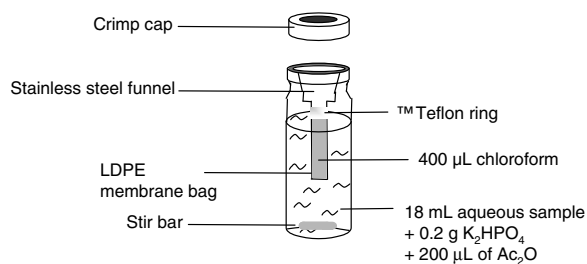


Fig. 1 Experimental setup for MALLE

obenzyl bromide, and silylation with, e.g. bis(trimethylsilyl) trifluoroacetamide or MTBSTFA. A further alternative derivatisation approach that can also increase the extractability of some of the analytes is in situ acylation, which involves the addition of a reagent into the aqueous sample such as acetic anhydride or isobutyl chloroformate. Among these many possibilities, initial derivatisation experiments were performed considering MTBSTFA as post-extraction silylation reagent and Ac₂O and iBCF as in-sample derivatisation agents.

Derivatisation and extraction conditions were studied simultaneously since they are related and their effects cannot be evaluated separately.

Selection of the acceptor solvent

Different organic solvents were evaluated, by duplicated extractions, as acceptor/extraction phase: hexane, acetone, acetonitrile, cyclohexane, ethyl acetate and chloroform. These organic solvents were selected to cover a range of polarity (0–5.8) and water solubility (0–100%). Thus, a Milli-Q water sample of 18 mL spiked with the analytes at the 10 ng mL⁻¹ level was extracted with 400 µL of each solvent for 60 min at 35°C with a stirring speed of 500 rpm. Moreover, the conditions of the different derivatisation reactions were as follows: for acylation, addition to the sample of 3.6 g of potassium hydrogencarbonate and 100 µL of the derivatisation reagent (Ac₂O or iBCF); for silylation, adjustment of the sample to pH2 with hydrochloric acid 1 M and derivatisation at room temperature by addition to the extract of MTBSTFA (20 µL). In both cases, the extracts were evaporated to dryness and reconstituted in ethyl acetate (before being mixing with MTBSTFA in the case of silylation). Experiments were performed in duplicated. As shown in Fig. 2 for the acetylated analytes (similar trends were obtained with the other derivatisation processes), significantly better results were obtained with chloroform for most of the compounds, especially for the most polar parabens (MeP and EtP).

Selection of the derivatisation agent

The post-extraction derivatisation by silylation with MTBSTFA and in situ derivatisation by acylation with Ac₂O

or iBCF were evaluated. Silylation was performed at room temperature on 400 µL of ethyl acetate extract, after the MALLE (extraction time 1 h, acceptor solvent 400 µL chloroform) of a water sample (pH2) containing the analytes (10 ng mL⁻¹), by addition of 20 µL of MTBSTFA. In situ acylation was performed by addition of a base and the acylation reagent Ac₂O or iBCF (100 µL) to a water sample containing the studied compounds (10 ng mL⁻¹) and then subject to MALLE (extraction time 1 h, temperature 35°C, acceptor solvent 400 µL chloroform). In the literature, acylation is normally performed in the presence of hydrogencarbonate or carbonate and pyridine as basic catalysers for the derivatisation with Ac₂O and iBCF, respectively [23–26]. However, the use of carbonate salts in these derivatisation procedures leads to the generation of carbon dioxide bubbles, which can stick on the surface of the MALLE bag and hinder the diffusion of the analytes to the membrane. On the other hand, pyridine, commonly used with iBCF derivatisation, is a very toxic substance. Thus, the use of hydrogenphosphate [14] was considered in this study as an alternative.

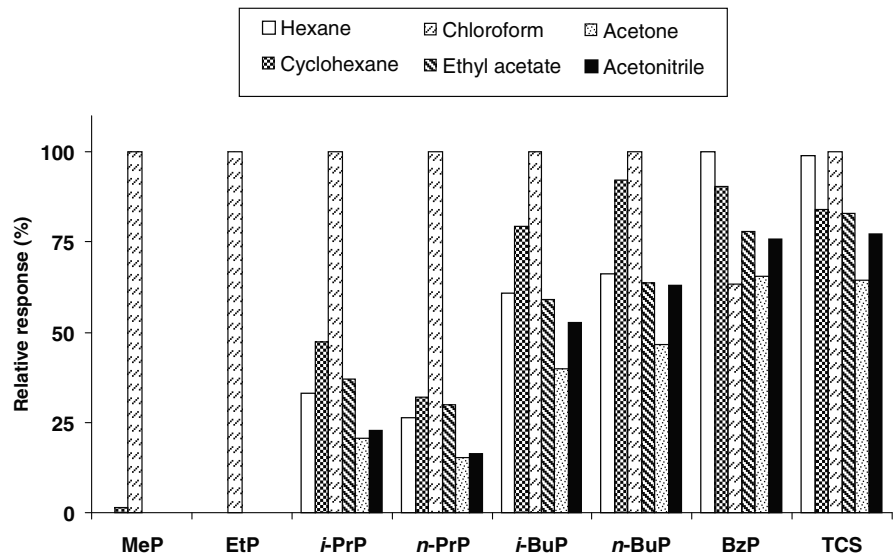
Among the tested derivatisation approaches, the highest yield for the most hydrophobic compounds (TCS and BzP) was obtained by silylation (Fig. 3). On the other hand, for the most polar compound (MeP), post-extraction silylation yielded poor extraction efficiency, whereas better results were obtained by acylation as a result of the increased hydrophobicity of the acylated derivatives. Furthermore, for most of the compounds, the use of hydrogenphosphate during acylation leads to higher extraction efficiencies than the traditional bases: hydrogencarbonate and pyridine (Fig. 3). Hence, as the goal of this study was the simultaneous determination of the eight compounds, Ac₂O with potassium hydrogenphosphate was used in further experiments.

Optimisation of other extraction and derivatisation conditions

Further experiments were performed in order to find the best experimental conditions for the derivatisation and extraction of the studied compounds. The experimental parameters considered were ionic strength (NaCl addition), amount of derivatisation reagent, extraction and derivatisation time. Although the extraction temperature may play a relevant role in the extraction kinetics and yield [27–29], it was kept at 35°C as the boiling point of chloroform is 61°C and higher extraction temperatures could lead to overpressure in the LPDE bag and consequently to solvent losses. On the other hand, lower temperatures are more difficult to control in the laboratory environment and normally would lead to longer extraction times.

In preliminary experiments, the chromatograms showed the presence of the most hydrophobic analytes as a mixture of non-derivatised and derivatised species. This was likely

Fig. 2 Comparison of different acceptor/extraction solvents for the MALLE of parabens and triclosan derivatised with Ac₂O ($n=2$). Results normalised to the highest response. Experimental conditions: in situ derivatisation with 0.2 g K₂HPO₄ and 100 μ L of Ac₂O, concentration level: 10 ng mL⁻¹, extraction time 60 min, stirring speed 500 rpm, temperature 35°C, solvent volume 400 μ L



due to the good extractability of these compounds even in their natural form, which did not allow time enough for their complete acetylation prior extraction. Thus, a pre-stirring step was included after addition of the derivatisation agent to the sample and before the subsequent extraction by MALLE. This parameter was studied together with the effect of salt (NaCl) addition and the amount of Ac₂O by means of a Box–Behnken experimental design (with three central points, i.e. 15 experiments). The experimental domain and the results of the analysis of the experimental design are shown in Table 2. Fortified Milli-Q water samples at 2 ng mL⁻¹ for each compound with 0.2 g of K₂HPO₄ extracted during 60 min at 35°C and 500 rpm were used for the optimisation.

As shown in Table 2, the addition of NaCl had a statistically significant negative effect for the most hydro-

phobic analytes (i.e. *n*-PrP, *i*-BuP, *n*-BuP, BzP and TCS). The volume of Ac₂O was only statistically significant for TCS, and its effect was negative on the extraction yield. Finally, the pre-stirring time showed a non-statistically significant effect of the first-order term but a negative statistically significant effect of the second-order term for the most polar compounds (MeP, EtP, *i*-PrP and *n*-PrP), which means that the optimum for these compounds is found at an intermediate level (ca. 10 min).

Therefore, due to the complexity and different behaviour of the analytes, the system was evaluated by a multiple response optimisation strategy (no further experiments were necessary). This was accomplished by establishing a desirability function, as detailed elsewhere [30–32]. Figure 4 shows the surface plot of the desirability versus the pre-stirring time and the content of NaCl, keeping the volume of Ac₂O at the low

Fig. 3 Comparison of different derivatisation approaches for the derivatisation/extraction of parabens and triclosan ($n=3$). Results normalised to the highest response. Experimental conditions: concentration level 10 ng mL⁻¹, extraction time 60 min, stirring speed 500 rpm, temperature 35°C, solvent 400 μ L of chloroform

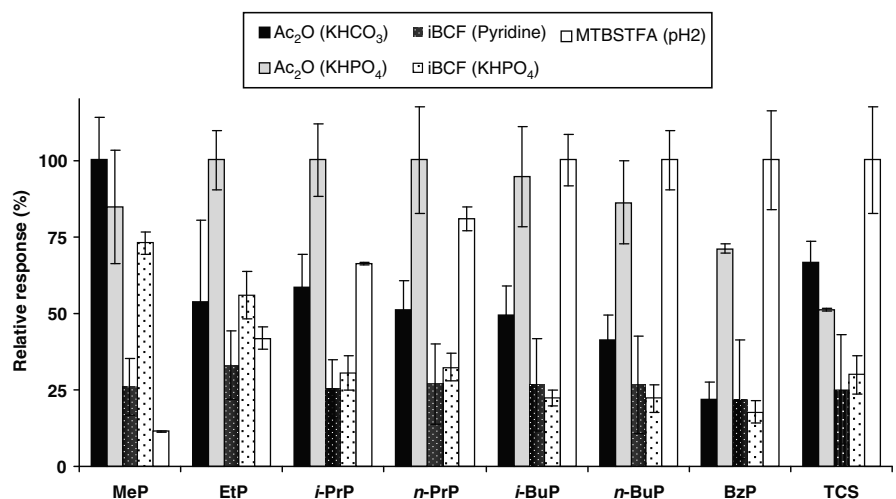


Table 2 Experimental domain and relative importance (with their sign) of the main effects associated to each factor and second-order interactions in the Box–Behnken design

| Factors | NaCl (g) | Ac ₂ O (μL) | Pre-stirring (min) | Interactions | | | | | |
|---------------------|----------|------------------------|--------------------|--------------|----|----|----|----|----|
| Low level | 0 | 20 | 0 | | | | | | |
| Central level | 2.5 | 110 | 10 | | | | | | |
| High level | 5 | 200 | 20 | | | | | | |
| Relative effects | A | B | C | AA | BB | CC | AB | AC | BC |
| MeP | + | – | + | – | + | – | + | + | – |
| EtP | + | – | + | – | + | – | + | + | – |
| <i>i</i> -PrP | – | + | + | – | + | – | + | + | + |
| <i>n</i> -PrP | – | + | + | – | + | – | + | + | + |
| <i>i</i> -BuP | – | + | + | – | – | – | + | + | + |
| <i>n</i> -BuP | – | + | + | – | – | – | + | + | + |
| BzP | – | + | – | – | – | – | – | + | + |
| TCS | – | – | – | ++ | + | – | + | + | + |
| Selected Conditions | 0 | 20 | 8 | | | | | | |

++ or – indicates a statistically significant effect (95% confidence level), positive or negative, respectively; + or – indicates that the effect was not statistically significant

level (20 μL). The highest overall desirability value was obtained at low level of NaCl addition (0.19 g), low level of Ac₂O volume (20 μL) and intermediate pre-stirring time (7.97 min). Thus, for simplicity, the following conditions were selected for further experiments: no salt addition, 20 μL of Ac₂O and 8 min of pre-stirring step.

This procedure was tested in the analysis of raw and treated wastewater spiked with the compounds under the optimised conditions. The analysis of treated wastewater showed a similar signal than the obtained from spiked Milli-Q water. However, the raw wastewater showed a reduction on the signals for PrP, BuP and BzP (data not shown) due to an incomplete derivatisation of the analytes, as underivatised parabens were detected in the chromatogram. Thus, in order to overcome this problem, pre-stirring time and the derivatisation reagent volume factors were re-

evaluated by a univariant study using spiked raw wastewater at 5 ng mL⁻¹. The results of these experiments showed that an increment in the pre-stirring time did not improve the derivatisation (data not shown), while a higher volume of the derivatisation reagent clearly improved the derivatisation and consequently the response, particularly for PrP, BuP and BzP (Fig. 5). Therefore, the volume of Ac₂O was finally fixed on 200 μL.

Finally, the influence of the extraction time was investigated for the extraction of 18 mL Milli-Q water samples spiked at 1 ng mL⁻¹ per compound and submitted to a stirring speed of 500 rpm at 35°C during extraction periods ranging between 15 and 240 min. As depicted in Fig. 6 exemplarily for MeP, *n*-BuP and TCS, the influence of the extraction time was negligible for the less polar compounds (BzP and TCS), the equilibrium was reached for BuP and PrP after 60 min, while the most polar compounds (MeP and EtP) needed a longer extraction time (90 min). Hence, 90 min was selected as optimal extraction time.

Method performance

The method performance was investigated under the optimised conditions: 18 mL of sample with 0.2 g of K₂HPO₄ and 200 μL of Ac₂O, pre-stirred during 8 min, extracted at 35°C, stirring at 500 rpm during 90 min using LDPE bags filled with 400 μL of chloroform. The results concerning detection limits (LODs), precision, extraction efficiencies and calibration data are summarised in Table 3.

LODs were calculated by two different approaches: based on blank MALLE extractions (*n*=6) as blank signal plus three times the standard deviation of the blank, and

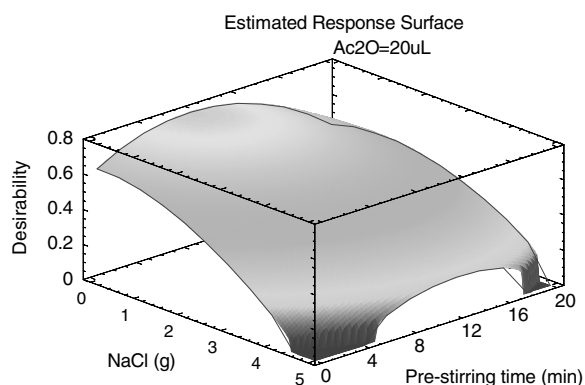
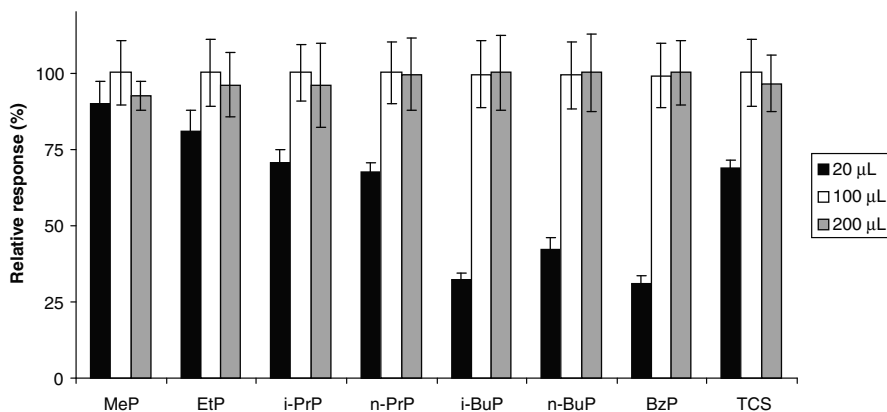


Fig. 4 Global desirability surface plot of NaCl content versus pre-stirring time with an Ac₂O volume of 20 μL

Fig. 5 Effect of the Ac₂O volume on the derivatisation/extraction of parabens and triclosan from raw wastewater ($n=3$). Results normalised to the highest response. Experimental conditions: derivatisation conditions 0.2 g K₂HPO₄, concentration level 5 ng mL⁻¹, extraction time 60 min, stirring speed 500 rpm, temperature 35°C, solvent 400 μ L of chloroform



defined for a ratio signal-to-noise of 3 and calculated on the basis of the calibration water samples. LODs were established based on the highest value of these two approaches for each compound. The obtained LODs ranged from 0.1 to 1.4 ng L⁻¹. These LODs are comparable to those obtained with other microextraction techniques such as SPME (1–25 ng L⁻¹) for a sample volume of 10 mL [9, 12, 14], SDME (1–15 ng L⁻¹) for a sample volume of 3 mL [18] and DLLME or USAEME (2–16 ng L⁻¹) for a sample volume of 10 mL [19, 20].

The precision of the method, expressed as relative standard deviation (RSD), was evaluated by extracting 6 consecutive aqueous samples spiked at 100 ng L⁻¹ with each target analyte. The results varied between 4% and 8%. The calculated calibration curves using internal standards in the range of 10–5,000 ng L⁻¹ gave a high level of linearity for all target analytes with correlation coefficients ranging between 0.994 and 0.998.

Extraction efficiencies were estimated by comparison with derivatised standard obtained by liquid–liquid extraction as described in the “Experimental” section. Extraction efficiencies higher than 89% were obtained for most of the compounds, but for the most polar analytes, MeP and EtP, with recoveries of 46%. These values are considered rather

high, taking into account that MALLE is regarded as a microextraction modality, based on equilibrium processes rather than in exhaustive processes as the classic liquid–liquid extraction and SPE.

Moreover, matrix effects during the extraction were evaluated. Given that membrane-assisted liquid–liquid extraction is an equilibrium technique, competitive adsorption to matrix substances can reduce the effective concentration of the analyte in the aqueous phase and therefore decrease the amount transferred into the organic acceptor phase. Therefore, possible matrix effects were investigated by comparing the responses obtained for Milli-Q water and samples of raw and treated wastewater and expressed as a percentage (Table 4). Treated and raw wastewater samples were spiked with the selected compounds at the 100 and 5,000 ng L⁻¹ level, respectively. Each sample was processed in quadruplicate. Non-spiked aliquots of each sample were also analysed and obtained peak areas subtracted from those corresponding to the spiked ones. In view of the obtained results (Table 4), matrix effects are negligible for parabens, while for TCS, the extraction is affected by the type of sample. Nevertheless, the use of surrogate internal standards compensates these matrix effects and also improves the precision of the method for

Fig. 6 Kinetic study of the derivatisation/extraction procedure for MeP, *n*-BuP and TCS. Experimental conditions: derivatisation conditions 0.2 g K₂HPO₄ and 200 μ L Ac₂O, concentration level 1 ng mL⁻¹, stirring speed 500 rpm, temperature 35°C, solvent 400 μ L of chloroform

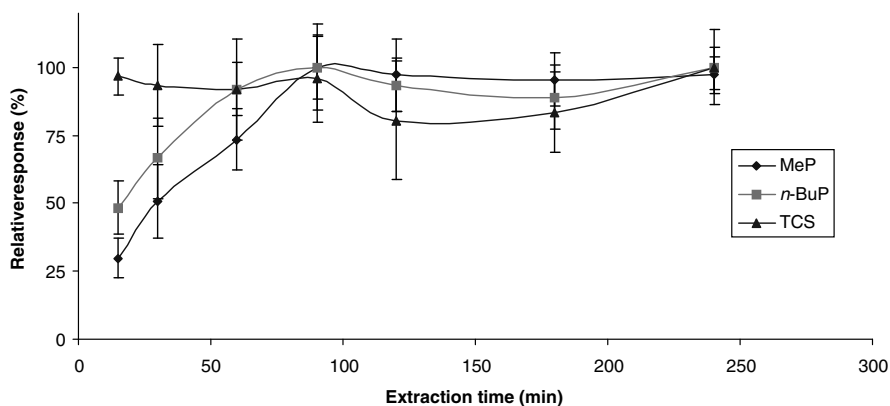


Table 3 Repeatability, linearity and detection and quantification limits of the method

| Surrogate | internal standard | Repeatability (RSD, %) | Extraction efficiency (%) ^a ±SD | Linearity (R^2) ^b | LOD (ng L ⁻¹) ^c | LOQ (ng L ⁻¹) ^c |
|---------------|-----------------------------------|------------------------|--|----------------------------------|--|--|
| MeP | MeP d_4 | 5.0 | 46±12 | 0.9967 | 0.3 | 1.0 |
| EtP | MeP d_4 | 5.4 | 46±8 | 0.9968 | 1.4 ^d | 4.6 ^d |
| <i>i</i> -PrP | <i>n</i> -PrP d_4 | 6.8 | 89±12 | 0.9980 | 0.1 ^d | 0.2 ^d |
| <i>n</i> -PrP | <i>n</i> -PrP d_4 | 5.3 | 104±11 | 0.9973 | 0.7 | 2.2 |
| <i>i</i> -BuP | <i>n</i> -PrP d_4 | 4.3 | 96±9 | 0.9973 | 0.4 | 1.2 |
| <i>n</i> -BuP | <i>n</i> -PrP d_4 | 6.2 | 104±12 | 0.9975 | 0.6 | 1.9 |
| BzP | <i>n</i> -PrP d_4 | 8.3 | 110±10 | 0.9938 | 0.6 | 2.0 |
| TCS | ¹³ C ₁₂ TCS | 5.8 | 98±15 | 0.9963 | 1.1 | 3.6 |

^a Relatives to those obtained by liquid–liquid extraction ($n=6$), see text for details

^b 10–5,000 ng L⁻¹, eight levels in duplicate

^c Calculated as three times (LODs) or ten times (LOQs) the standard deviation of the blanks ($n=6$)

^d Calculated for a signal-to-noise ratio of 3 (LODs) or 10 (LOQs)

all analytes (Table 4). Thus, quantification should be performed by internal standard calibration in the case of TCS, but it is not mandatory for parabens.

Analysis of real samples

In total, six samples, two surface and four wastewater samples were analysed using the present method. Blanks were subtracted for concentration calculations, and internal standard calibration was used for quantification. As shown in Table 5, the highest concentrations were found in raw wastewater samples, with MeP and *n*-PrP being the analytes of higher concentration. A chromatogram of a raw wastewater sample is shown in Fig. 7. In wastewater samples, a clear reduction in the parabens concentration was observed during the water treatment. This behaviour is in agreement with the tendency described in the literature [9, 10]. The study of both isomers of PrP and BuP showed that *i*-BuP and *n*-BuP occurred in the same concentration range, while PrP was found mainly as the linear isomer (*n*-PrP), in agreement with the only previous work that considers these isomers by González-Mariño et al. using SPE and LC-MS/MS [13].

Conclusions

A method for the simultaneous determination of seven parabens and triclosan in water samples by means of in-sample acetylation–non-porous MALLE followed by LVI-GC-MS/MS determination has been developed and validated. For the extraction of parabens and TCS, acetylation with Ac₂O in presence of K₂HPO₄ proved to be the best-suited derivatisation procedure as it increases their extractability, and in combination with chloroform as extraction solvent, showed the highest extraction yields. The method permits the determination of analytes at the low nanogram per litre level (LODs < 1.4 ng L⁻¹). The major advantages of this procedure are the small amount of solvent required for extraction (400 µL), the small volume of sample consumed (18 mL), the possible exclusion of matrix components resulting in clean extracts, the high potential for automation and the low cost of membranes. Actually, the cost of these lab-made LPDE membranes is about 1 Euro for 1,000 membranes; so, they can be considered as inexpensive disposable devices, eliminating the risk of cross-contamination problems. Finally, the method was validated with real samples, showing a good

Table 4 Matrix effects in wastewater samples

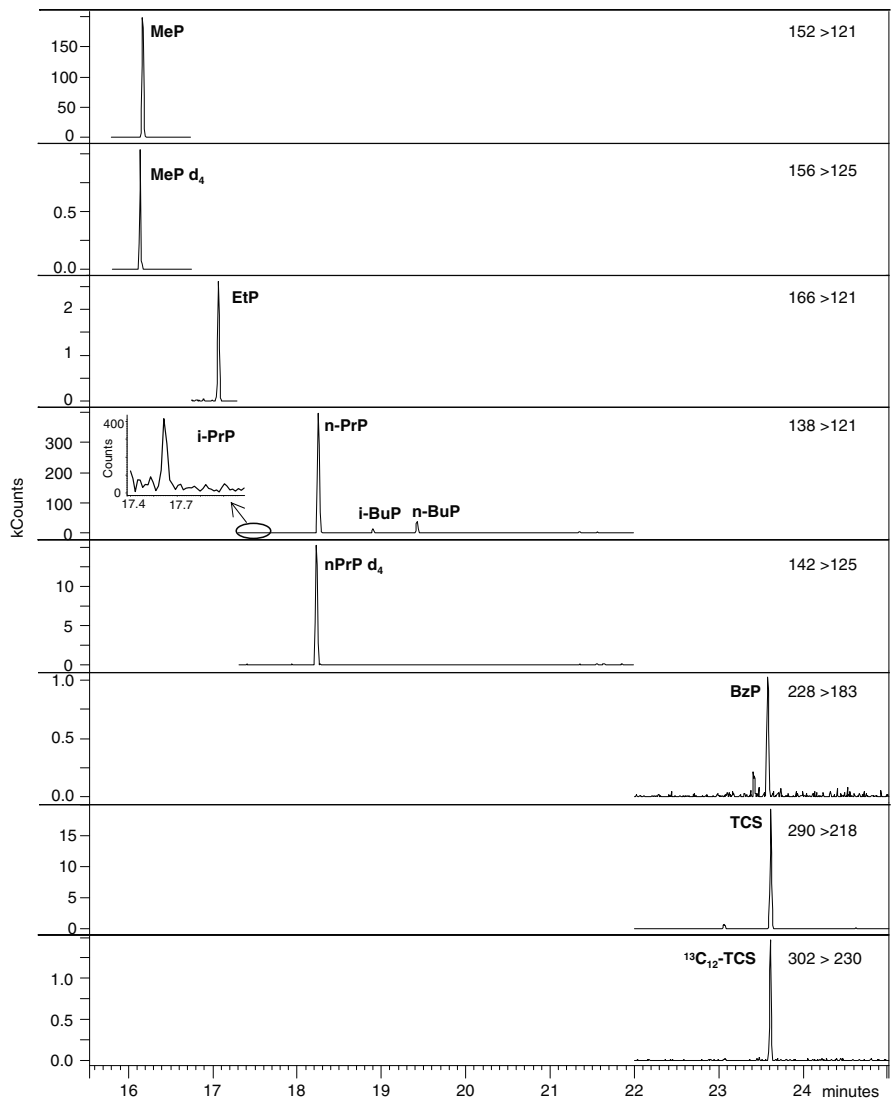
| | Matrix effects (RSD) | | Corrected matrix effects (RSD) | |
|---------------|----------------------|----------------|--------------------------------|----------------|
| | Treated wastewater | Raw wastewater | Treated wastewater | Raw wastewater |
| MeP | 105 (14) | 103 (14) | 101 (13) | 99 (8) |
| EtP | 102 (11) | 105 (10) | 97 (11) | 101 (6) |
| <i>i</i> -PrP | 90 (12) | 92 (9) | 84 (7) | 104 (3) |
| <i>n</i> -PrP | 88 (13) | 88 (11) | 83 (7) | 101 (3) |
| <i>i</i> -BuP | 92 (12) | 89 (9) | 91 (6) | 100 (2) |
| <i>n</i> -BuP | 92 (11) | 85 (6) | 91 (9) | 95 (3) |
| BzP | 92 (10) | 74 (2) | 91 (10) | 83 (6) |
| TCS | 63 (25) | 59 (18) | 90 (17) | 96 (2) |

Table 5 Concentration (ng L⁻¹)±standard deviation of parabens and triclosan found in river and wastewater samples (n=4 replicates of the same sample)

| | October 2009 | | | January 2010 | | |
|---------------|--------------|---------|--------------|--------------|---------|-----------|
| | River | Treated | Raw | River | Treated | Raw |
| MeP | nd | nd | 26,194±1,388 | 54±8 | nd | 6,810±267 |
| EtP | 30±6 | 57±10 | 1,943±111 | 29±2 | nd | 480±14 |
| <i>i</i> -PrP | nd | nd | 5.4±0.5 | 0.8±0.1 | 0.9±0.1 | 2.2±0.2 |
| <i>n</i> -PrP | nd | nd | 1,737±113 | 105±7 | nd | 1,227±63 |
| <i>i</i> -BuP | nd | nd | 106±6 | 4.8±0.7 | 2.7±0.1 | 40±3 |
| <i>n</i> -BuP | nd | nd | 172±7 | 6.4±0.5 | 2.4±0.3 | 88±5 |
| BzP | nd | nd | nd | 2.4±0.1 | 2.1±0.1 | 4.7±0.2 |
| TCS | 58±5 | 178±15 | 423±22 | 138±32 | 141±20 | 1,142±23 |

nd not detected

Fig. 7 GC-MS/MS chromatogram of a real (non-spiked) raw wastewater sample



performance and its applicability in the analysis of surface water and wastewater samples.

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