Investigation of pharmaceutical metabolites in environmental waters by LC-MS/MS

Emma Gracia-Lor, María Ibáñez, Tatiana Zamora, Juan V. Sancho and Félix Hernández*

Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat, E-12071 Castellón, Spain. Tel.: 964 387366. Fax: 964 387368.

*E-mail: felix.hernandez@uji.es

ABSTRACT

Pharmaceuticals, once ingested, are commonly metabolized in the body into more polar and soluble forms. These compounds might not be completely removed in the wastewater treatment plants and consequently being discharged into the aquatic ecosystem. In this work, a multi-class sensitive method for the analysis of 21 compounds, including 7 widely consumed pharmaceuticals and 14 relevant metabolites, has been developed based on the use of UHPLC-MS/MS in selected reaction monitoring (SRM) mode. The method was validated in six surface waters (SW) and six effluent wastewaters (EWW) at realistic concentration levels that can be found in waters. The optimized method was applied to the analysis of different types of water samples (rivers, lakes and effluent wastewater), detecting nearly all the parent compounds and metabolites investigated in this work. This fact illustrates that not only pharmaceuticals but also their metabolites are commonly present in these types of waters. Analytical research and monitoring programs should be directed not only towards parent pharmaceuticals but also towards relevant metabolites to have a realistic overview of the impact of pharmaceuticals in the aquatic environment.

Keywords: Pharmaceuticals, metabolites, ultra-high performance liquid chromatography, tandem mass spectrometry, multi-class method, surface water, wastewater

1. INTRODUCTION

In the last years, many papers dealing with the presence of pharmaceuticals in the aquatic environment have been reported. Most of the work performed until now has been focused on parent pharmaceuticals, while metabolites have been much less investigated. After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form as the parent compound and/or as free or conjugated metabolites through urine and/or faeces. Metabolism occurs in two phases. The first one involves typically oxidation, reduction, or hydrolysis, and the second phase consists of transferring a polar group to the parent compound or the metabolite to render a conjugate (Khetan and Collins 2007; Mompelat et al. 2009). Obviously, not all pharmaceuticals are metabolised to the same extent. They may be classified in four classes according to the proportions of excreted parent compound (Jjemba 2006), i.e. low excretion (≤ 5%), moderately low (6-39%), relatively high (40-69%), and high excretion compounds (≥ 70%). Among the first group, there are some compounds known as pro-drugs, i.e., inactive substances that after their ingestion are converted to an active form in the body.

Both parent pharmaceuticals and metabolites might not be fully eliminated during the treatment processes in wastewater treatment plants (WWTP) being discharged into the aquatic ecosystems through treated wastewaters. Research is commonly focused on parent compounds, and little is known about the presence of metabolites and on transformation products (TPs) that can be formed during water treatment. In fact, only a few works have reported values of pharmaceuticals metabolites and TPs in the aquatic environment (González Alonso et al. 2010; Langford and Thomas 2011; Kovalova et al. 2012; López-Serna et al. 2012a). Although pharmaceuticals and metabolites are typically found at low concentration levels, the

effects derived from the exposure to a mixture of parent pharmaceuticals and their metabolites are still largely unknown. Moreover, some of the metabolites are still bioactive and may have high stability and mobility in the environment (Gros et al. 2012). Mompelat et al. (2009) have recently reported that only around 30 pharmaceutical by-products (including metabolites and transformation products) have been included in environmental investigations.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with triple quadrupole triple quadrupole (QqQ) analyzer is nowadays the technique of choice for trace analysis of pharmaceuticals due to the high selectivity and sensitivity achieved in selected reaction monitoring (SRM) mode. LC-MS/MS has been commonly used for multi-class determination of pharmaceuticals compounds, normally including only a few metabolites in the target list of analytes (Kasprzyk-Hordern et al. 2007; Batt et al. 2008; Tarcomnicu et al. 2011). In the last two years, this trend is being changing, as more metabolites are included in the analytical methods. For example, Tarcomnicu et al. (2011) have developed a method for the analysis of different pharmaceuticals and four metabolites. Recently, a method based on automated off-line solid phase extraction (SPE) using a triple quadruple-linear ion trap mass spectrometer (QqLIT) has allowed the determination of 8 metabolites (Gros et al. 2012). Another LC-MS/MS method has been reported using QqQ for the analysis of 19 pharmaceutical metabolites and TPs (López-Serna et al. 2012b). In addition, a few papers have been published on investigation of pharmaceutical metabolites and TPs by high-resolution mass spectrometry (HR MS), emphasizing the use of hybrid quadrupole time-of-flight (QTOF) (Martínez Bueno et al. 2007; Hernández et al. 2011; Gómez-Ramos et al. 2011; Ferrer and Thurman 2012). LC-HR MS has been proven to be a powerful and promising approach to investigate these compounds in waters from reported-known

metabolites/TPs to unknown compounds that share common fragments with the parent molecule (Hernández et al. 2011; Ibáñez et al. 2012).

Some works have reported metabolite concentrations higher than the original molecule (López-Serna et al. 2012a; Miao and Metcalfe 2003). This also supports the interest of searching for metabolites to have a wider and more realistic knowledge about the impact of pharmaceuticals in the aquatic environment. To this aim, multi-class methods including pharmaceuticals and metabolites are required, but this type of analysis presents some difficulties, as metabolites are usually more polar than parent compounds. This makes problematic their simultaneous extraction and LC determination, as they are less retained on the SPE cartridges and on the commonly used reversed-phase LC columns. In addition, the low concentrations normally present in waters require the use of highly sensitive methods for their determination. This is especially important in complex environmental matrices where the presence of co-extracted sample matrix components results in ionization suppression or enhancement effects. Although matrix effects can be corrected using isotope-labelled internal standards (ILIS) (Wille et al. 2012), the availability of ILIS reference standards is rather limited in comparison with parent pharmaceuticals. And last but not least, it is necessary to ensure the confident identification of the compound detected. This issue might be problematic for isomeric metabolites that share common fragments with the parent compound, or metabolites that can generate the parent compound as an in-source fragments in the LC-MS instrument (Ibáñez et al. 2012). Under this situation, is necessary to maximize precautions to ensure right identifications, as for example using more than two MS/MS transitions, analysing samples by HR MS techniques, and/or improving the chromatographic separation.

In a previous work (Hernández et al. 2011), five pharmaceutical metabolites were identified in urban wastewater samples by UHPLC-QTOF MS, after detection of the parent pharmaceuticals and subsequent data re-evaluation in a retrospective way. These compounds were N-desmethyl clarithromycin, 14-hydroxy-clarithromycin, fenofibric acid, clopidogrel carboxylic acid and 4-hydroxy omeprazole sulfide. Analysis by QTOF also allowed us to discover the presence of several metabolites of the analgesic dipyrone in urban wastewater (Ibáñez et al. 2012). Based on these previous findings, we decided to widen the study of pharmaceutical metabolites and to develop a multi-residue sensitive method based on LC-MS/MS with triple quadrupole for the simultaneous quantification of relevant metabolites. In addition to those compounds previously detected, the list of target metabolites was completed with nine more compounds were reported in SW and EWW (Miao and Metcalfe 2003; Kasprzyk-Hordern et al. 2007; Tarcomnicu et al. 2011) and taking into account their commercial availability as reference standards. Moreover, parent pharmaceuticals of the metabolites selected were also included in the method as they might not be completely metabolized (Jjemba 2006; Mompelat et al. 2009). The parent compounds clofibrate, fenofibrate and dipyrone were not considered because they are pro-drugs (Gómez et al. 2008; Mompelat et al. 2009) and therefore, they are not expected to be found in the aquatic environment.

The goal of this paper is to investigate the presence of 21 pharmaceuticals, including seven parent compounds and their main metabolites, in environmental waters. To this aim, rapid and sensitive analytical methodology, based on the use of LC-MS/MS with triple quadrupole, has been developed. Validation of the method was made in a notable number of water samples (six different surface water and six different effluent wastewater) trying to cover quite distinct sample compositions and situations that can appear when analyzing real samples. Analyses of environmental water samples has

shown that not only parent pharmaceuticals but also their metabolites are commonly present in the aquatic environment, suggesting that these compounds need to be regularly monitored in waters.

EXPERIMENTAL

2.1. Reagents and chemicals

Reference standards of pharmaceuticals were purchased from Sigma-Aldrich (St Louis, MO, USA). Reference standards of metabolites were obtained from Toronto Research Chemicals (Ontario, Canada), with the exception of carbamazepine 10,11-epoxide, enalaprilat, 4-amino antipyrine and clofibric acid, which were supplied from Sigma-Aldrich. Their chemical structure is shown in **Figure 1**.

Isotopically labelled compounds used as ILIS (omeprazole-d_3, enalaprilat-d_5 and carbamazepine 10,11-epoxide-d_{10}) were from CDN Isotopes (Quebec, Canada).

HPLC-grade methanol (MeOH) was purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). Formic acid (HCOOH, content > 98%), ammonium acetate (NH_4Ac, reagent grade) and ammonia (NH_3, solution 32%, reagent grade) were supplied by Scharlab (Barcelona, Spain).

Individual stock solutions of pharmaceuticals/metabolites (around 500 mg/L) were prepared dissolving an accurately weighted amount in methanol. The individual stock solutions were mixed and diluted with methanol to give a final concentration of around 1 mg/L (40% MeOH, 60% HPLC-grade water, approximately). This mix solution was prepared weekly, based on previous information on omeprazole stability, and it was subsequently diluted with HPLC-grade water to obtain working mixed solutions of pharmaceuticals/metabolites. These solutions were used for spiking

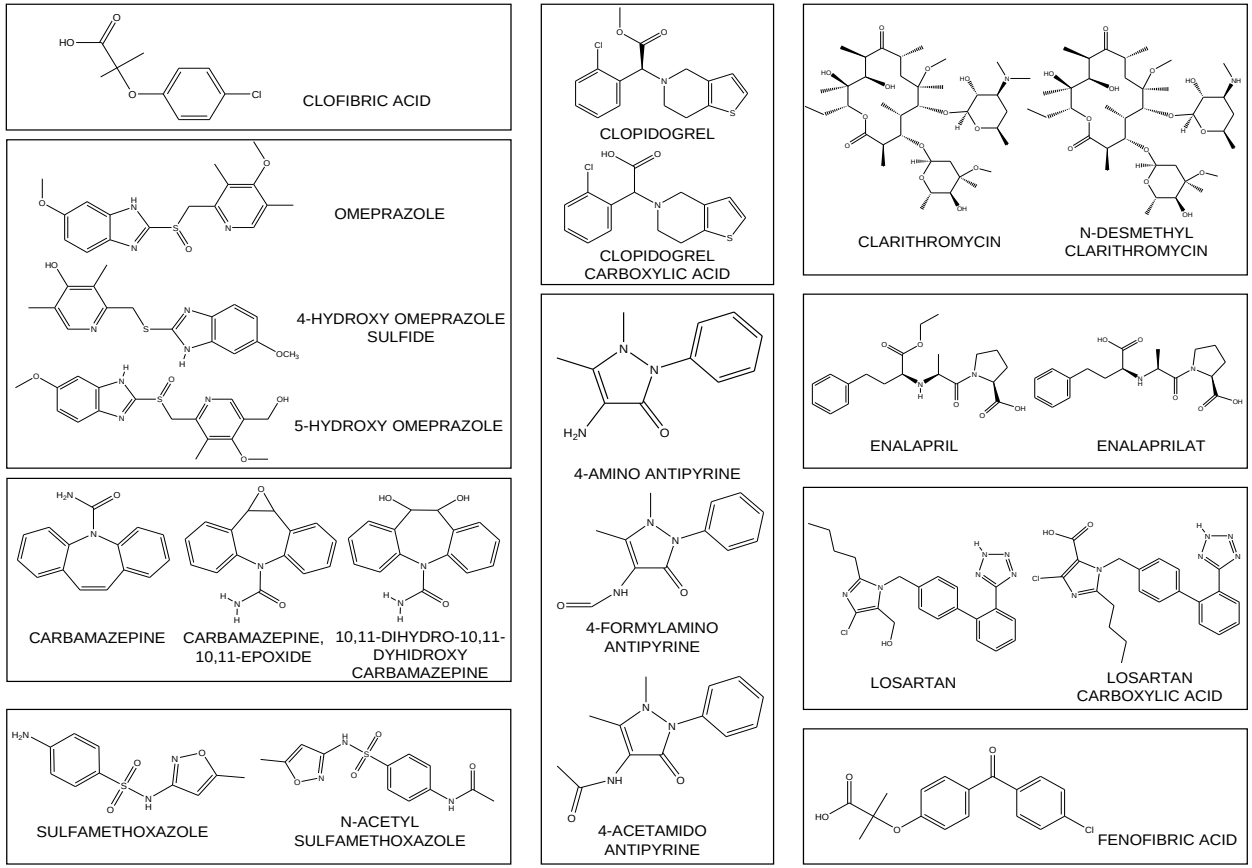

Figure 1 Structures of the selected compounds.

Formatat: VA_Figure_Caption, Centrada

samples in the validation study and also for preparation of calibration standards. In the last case, the standards were prepared in methanol-water (10:90, v/v) to assure the same organic content as in the sample extracts.

Individual stock solutions of ILIS were also prepared in methanol. Mix working solutions at 5 µg/L (for surface water (SW) samples) or at 50 µg/L (for effluent wastewater (EWW) samples) were prepared in HPLC-grade water and used as surrogates.

All standard solutions (stock, intermediate and working solutions) were stored in amber glass bottles at −20 ºC in a freezer.

Cartridges used for SPE were Oasis HLB (200 mg), Oasis HLB (60 mg), Oasis MCX (150 mg) and Oasis MAX (150 mg), from Waters (Milford, MA, USA).

2.2. Liquid chromatography

UHPLC analysis were carried out using an Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was performed using an Acquity UPLC HSS T3 (C18) column, 1.8 µm, 100 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 40 ºC and the sample manager was maintained at 5ºC. Mobile phase consisted of a water/(methanol 0.01% HCOOH) gradient. The methanol percentage was changed linearly as follows: 0 min, 10%; 12 min, 80%; 12 min, 80%; 12.1 min; 10%. Analysis run time was 13 min. The sample injection volume was 100 µL (full loop).

2.3. Mass spectrometry

Formatat: anglès (Regne Unit)

Formatat: Normal, Justificada, Sagnia: Primera línia: 0 cm, Interlineat: Doble

A TQD (triple quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters Corp., Milford, MA, USA) was used. Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N_2 nitrogen LC–MS (Claind, Teknokroma, Barcelona, Spain). The cone gas and the desolvation gas flows were set at 60 L/h and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) at 2×10^{-3} mbar in the T-Wave collision cell. Capillary voltages of −3.0 kV (negative ionization mode) and 3.5 kV (positive ionization mode) were applied. The interface temperature was set to 500 ºC and the source temperature to 120 ºC. Dwell time, inter-channel delay time, and inter-scan delay time were automatically assigned by the system software (MassLynx 4.1, Manchester, UK) using the auto-dwell feature.

2.4. Recommended procedure

A volume of 50 mL of water sample were spiked with the corresponding ILIS mix working solution, giving a final concentration of 0.5 μg/L (surface water) and 5 μg/L (effluent wastewater) for each individual ILIS. Oasis HLB (60 mg) cartridges used for SPE were conditioned with 3 mL MeOH and 3 mL HPLC-grade water before use. Then, the samples were passed through the cartridge by gravity and, after drying under vacuum for 15 minutes, analytes were eluted with 5 mL MeOH. The extract was evaporated to dryness under a gentle nitrogen stream at 40ºC and reconstituted with 1 mL MeOH–water (10:90, v/v). Finally, 100 μL were injected into the UHPLC–MS/MS system under the conditions shown in **Table 1.**

Quantification was made by calibration standards in solvent, using relative responses analyte/ILIS, or absolute responses, depending whether ILIS was used for correction or not.

Table 1. MS/MS optimized conditions for selected pharmaceuticals and metabolites

Compound	ESI	Cone (V)	Q Transition (m/z)	C.E. (eV)	q_1 Transition (m/z)	C.E. (eV)	q_2 Transition (m/z)	C.E. (eV)	Q/q_1 [a]	Q/q_2 [a]	IDL (pg)
Carbamazepine	+	25	237.3 > 194.2	25	237.3 > 179.2	35	237.3 > 165.2	40	4.8	7.1	0.3
Carbamazepine 10,11-epoxide	+	15	253.4 > 180.2	20	253.4 > 236.2	10	253.4 > 210.3	20	1.7	10.7	0.9
10,11- Dihydro-10,11-dihydroxy carbamazepine	+	15	271.0 > 180.1	30	271.0 > 253.0	5	271.0 > 236.0	10	1.5	1.7	1.6
Clarithromycin	+	40	748.3 > 158.1	30	748.3 > 83.0	50	590.3 > 158.1 [b]	25	2.2	2.4	0.3
N-Desmethyl clarithromycin	+	25	735.0 > 144.2	20	735.0 > 576.8	20	737.0 > 146.2	15	7.6	168.1	0.4
Clopidogrel	+	25	322.0 > 212.0	15	322.0 > 184.0	25	324.0 > 214.0	15	1.5	3.1	0.3
Clopidogrel carboxylic acid	+	20	308.3 > 198.2	15	308.3 > 77.0	45	310.3 > 200.2	10	1.0	4.1	0.9
Enalapril	+	35	377.4 > 91.1	55	377.4 > 234.2	20	377.4 > 160.2	30	2.1	4.6	1.1
Enalaprilat	+	30	349.5 > 91.1	50	349.5 > 206.3	20	349.5 > 117.2	35	2.0	2.5	10.2
Losartan	+	20	423.2 > 207.0	25	423.2 > 405.2	15	425.2 > 207.0	25	3.0	9.0	0.4
Losartan carboxylic acid	+	25	437.2 > 207.1	30	437.2 > 235.0	20	439.2 > 207.2	20	0.5	1.6	5.7
Omeprazole	+	30	346.3 > 198.1	10	346.3 > 136.1	35	346.3 > 151.1	20	0.9	2.0	2.6
4-Hydroxy omeprazole sulfide	+	20	316.4 > 168.2	25	316.4 > 149.2	25	316.4 > 136.2	25	1.0	1.0	0.5
5-Hydroxy omeprazole	+	15	362.1 > 152.1	35	362.1 > 214.0	20	362.1 > 196.2	30	1.7	3.2	0.5
Sulfamethoxazole	+	40	254.0 > 64.9	50	254.0 > 91.9	30	254.0 > 155.9	20	1.2	49.4	0.7
N-Acetyl sulfamethoxazole	+	30	296.0 > 134.2	20	296.0 > 198.0	20	-	-	2.8	-	0.7
4-Acetamido antipyrine	+	20	246.4 > 83.1	25	246.4 > 228.3	15	246.4 > 104.1	20	1.6	1.6	1.2
4-Amino antipyrine	+	20	204.4 > 56.1	15	204.4 > 83.1	15	204.4 > 94.1	15	5.9	5.6	1.0
4-Formylamino antipyrine	+	25	232.4 > 56.1	25	232.4 > 83.1	20	232.4 > 104.1	20	1.6	1.0	2.9
Clofibric acid	-	20	213.3 > 127.0	15	215.2 > 129.0	10	213.3 > 85.1	10	1.1	5.3	23.2
Fenofibric acid	+	25	319.0 > 233.0	15	319.0 > 138.9	30	319.0 > 121.0	30	1.2	2.6	1.2
Carbamazepine 10,11-epoxide-d_{10}	+	20	263.1 > 190.0	20	-	-	-	-	-	-	-
Enalaprilat-d_5	+	25	354.1 > 96.1	50	-	-	-	-	-	-	-
Omeprazole-d_3	+	30	349.3 > 198.1	10	-	-	-	-	-	-	-

Abbreviations: ES (electrospray ionization), Q (quantification), q (confirmation), C.E. (collision energy), IDL (instrumental detection limit)

[a] Average value for seven standards, from 0.25 to 25 µg/L.

[b] In this case an in-source fragment was used as precursor ion and the cone voltage was 55 V.

In bold the parent pharmaceuticals, in italics the metabolites and with regular format, the ILIS used.

Formatat: Sagnia: Esquerra: 0 cm

Formatat: Superior: 2,25 cm, Inferior: 2 cm

Taula formatada

Formatat: espanyol (Espanya - alfab. tradicional)

2.5. Validation study

Method accuracy (expressed as percentage recovery) and precision (expressed as repeatability in terms of relative standard deviation (RSD)) were estimated by means of recovery experiments in 12 different samples spiked at various concentrations (0.02 μg/L in SW; 0.1 and 0.4 μg/L in EWW). SW samples used for validation were collected in different sites of the Mediterranean Spanish area of Valencia (Mijares and Jucar rivers, Sitjar and Mª Cristina reservoirs, Clot de Burriana lake and Albufera de Valencia). EWW samples were collected from different WWTPs of the same area. For each individual sample, recovery experiments were performed by triplicate, giving a total of 18 data for SW and 18 for EWW at each spiked concentration.

For each sample under study, the limit of quantification (LOQ) was estimated for a signal-to-noise ratio (S/N) of 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. In this way, average LOQ and the interval (both in ng/L) were estimated for both SW and EWW samples**.** True blank samples were not found for several analytes, as they were already present in the samples tested. In these cases, LOQs were estimated from the analyte levels quantified in the non-spiked samples. The instrumental detection limit (IDL) was estimated for S/N = 3 from the chromatogram of the standard at the lowest concentration level tested in the calibration curve.

Linearity of the method was studied by analyzing standard solutions in triplicate at seven concentrations in the range from 0.25 to 25 μg/L (equivalent to 0.005-0.5 μg/L in the water sample). Satisfactory linearity using least squares regression was assumed when the correlation coefficient (r) was higher than 0.99 and residuals lower than 30%

without significant trend, based on absolute responses, except for those compounds that were quantified with ILIS (relative responses).

2.6. Application to environmental samples

The method was applied to twenty-four samples (12 SW and 12 EWW samples). Regarding surface water, they were collected in selected sites of the Spanish Mediterranean area (Castellón and Valencia provinces), corresponding to 4 rivers, 2 reservoirs and 6 lakes. In the case of wastewater, samples consisted on 24-h composite urban wastewater samples and were collected along time from 4 different WWTPs located in the Castellon area. All samples were stored in the dark at <−18 ◦C in polyethylene high-density bottles until analysis. Immediately before analysis, samples were thawed at room temperature.

3. RESULTS AND DISCUSSION

3.1. MS and MS/MS optimization

Full-scan and MS/MS mass spectra were obtained from infusion of 1 mg/L individual standard solutions in methanol/water (50:50, v/v) at a flow rate of 10 μL/min. All compounds were determined in positive ionisation mode, with the exception of clofibric acid. Although this analyte might be analyzed in both positive and negative modes, the latter was preferred because of the better sensitivity reached under this mode. Mass spectrometry parameters, precursor and product ions selected, IDLs and ion ratios (Q/q) used for confirmation are shown in **Table 1**.

All compounds showed an abundant $[M+H]^+$ ion (for clofibric acid. $[M-H]^-$) which was selected as precursor ion. For clopidogrel, clopidogrel carboxylic acid, losartan, losartan carboxylic acid and clofibric acid, the presence of one chlorine atom in their structure allowed the use of two different precursor ions (corresponding to ^{35}Cl and ^{37}Cl isotopes).

For clarithromycin, an additional sensitive transition was obtained selecting an in-source fragment by increasing the cone voltage. The fragmentation of this in-source fragment ion produced a highly abundant product ion, making possible the acquisition of other sensitive transition (see q_2 transition for the mentioned pharmaceutical in **Table 1**).

Three SRM transitions were selected for each compound to assure the reliable confirmation of the compounds detected in water samples. The most sensitive transition was used for quantification (Q) whereas the other two were used for confirmation (q_1 and q_2). For N-acetyl sulfamethoxazole only one confirmation transition could be monitored due to its poor fragmentation.

The method was divided in 24 overlapping retention time windows (one window per compound) and MRM data acquisition rates (dwell time, inter-channel delay time, inter-scan delay times) were automatically optimized.

3.2. Chromatographic optimization

In order to optimize chromatographic separation, both methanol and acetonitrile solvents as well as different HCOOH and NH_4Ac contents were evaluated. Acetonitrile was discarded because sensitivity decreased for most compounds in comparison with MeOH. Regarding the modifiers, the use of NH_4Ac led to worse sensitivity compared

with HCOOH. Besides, the addition of HCOOH favoured the retention of acidic compounds in the LC column, such as losartan carboxylic acid or clofibric acid. Thus, for losartan, the retention time shifted from 6.28 min (NH_4Ac 5 mM) to 9.19 min (HCOOH, 0.01%). This behaviour was similar for clofibric acid (from 6.62 min to 9.28 min). On the contrary, for a few analytes the chromatographic run time decreased when HCOOH was added, especially for clarithromycin, which eluted the latest. However, enalapril and its metabolite presented a worse peak shape when acid was present in the mobile phase. This situation is quite usual when compounds with very different physico-chemical characteristics are simultaneously analysed, and obviously a compromise has to be reached. Finally, the addition of HCOOH was tested in both organic and aqueous solvents, obtaining best results (in terms of sensitivity) when this modifier was added only to the MeOH.

Once the mobile phase was selected (water/(MeOH 0.01% HCOOH)), two UHPLC C18 columns were compared (HSS T3 and BEH, both 10 cm). The results were similar showing that both columns are suitable for the retention of a broad group of compounds with different polarity. Finally, HSS T3 column was selected because the analytes were more retained and peak shape was better (narrower peaks) for a few compounds such as losartan carboxylic acid and enalaprilat.

After testing several injection volumes (20, 50 and 100 µL), the optimum was found to be 100 µL due to the increased sensitivity without affecting the peak shape. Column temperature was maintained at 50 ºC to improve peak shape of enalapril (Gracia-Lor et al. 2010).

3.3 Solid phase extraction optimization

Metabolites are usually more polar than parent compounds, making their simultaneous extraction more problematic. Therefore, the optimization of the SPE step is especially important in this case. In this work, the extraction efficiency of three cartridges was checked (Oasis HLB (200 mg), Oasis MCX (150 mg) and Oasis MAX (150 mg)), using HPLC-grade water spiked with the analytes. Oasis HLB can be used for a wide range of target compounds with quite distinct polarities, while MCX is suitable for compounds with basic groups and MAX for acidic compounds. Oasis HLB was tested at neutral pH, while MCX required acidification of the water sample (pH 2) to ensure protonation of basic compounds, and MAX required working at basic medium (pH 11) in order to fully deprotonate acidic compounds. After loading the samples, the cartridges were dried under vacuum for 15 min. The elution was carried out with 8 mL MeOH (HLB), with 4 mL MeOH followed by 4 mL MeOH 5% NH_4OH (MCX), or with 4 mL MeOH followed by 4 mL MeOH 5% HCOOH (MAX).

Our results showed that recoveries for N-desmethyl clarithromycin and carbamazepine 10,11-epoxide were lower using MCX (around 30%). The first one is expected to be efficiently retained on MCX cartridge due to the protonation of the amino group. A possible explanation for these unexpected results might be that the elution with 4 mL MeOH 5% NH_4OH was not enough to break its strong retention in the cartridge, yielding to poor recoveries. On the contrary, the acidification of the water sample would generate the diol group through the epoxide ring opening of carbamazepine 10,11-epoxide, being partially converted into 10,11-dihydro-10,11-dihydroxy carbamazepine, and leading to low SPE recoveries.

In general, recoveries with HLB and MAX were quite similar, although the first one showed more reproducible figures for all the compounds (data not shown). Therefore, HLB cartridges were selected for subsequent experiments. Then, a

179 comparison between Oasis HLB containing 60 mg and 200 mg was carried out, eluting
180 with 5 and 8 mL MeOH, respectively. As similar results were obtained for all
181 compounds, Oasis HLB 60 mg was selected to economize the amount of stationary
182 phase. Finally, this cartridge was tested adjusting the pH of the sample loaded at three

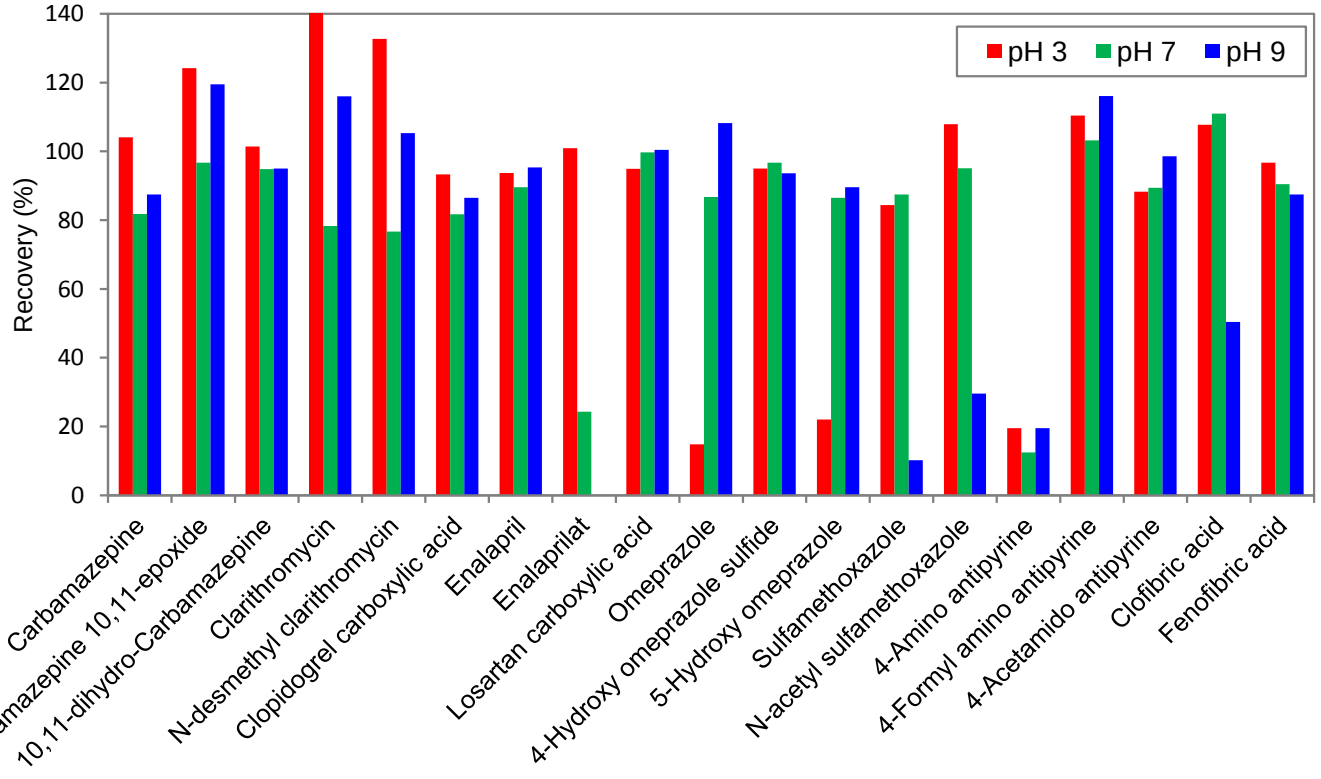

Figure 2 Recoveries obtained for 19 selected compounds after SPE with Oasis HLB (60 mg) at different sample pH values. Clopidogrel and losartan recoveries are not shown because their standards were not available at the laboratory when this test was carried out.

Formatat: Justificada, Interlineat: Doble, Tabulacions: 0 cm, Esquerra

Formatat: Tipus de lletra: 12 pt, No Negreta, anglès (EUA)

values (pH 3, 7 and 9) (see **Figure 2**). As a compromise, neutral pH was selected for sample extraction, as nearly all compounds showed satisfactory recoveries (between 70 and 120%), with the exception of 4-amino antipyrine (4-AA) and enalaprilat (recoveries around 40%).

Formatat: Normal, Sagnia: Primera línia: 0 cm, Interlineat: Doble

3.4 Method validation

The linearity of the method was satisfactory between 0.25 - 25 μg/L for all compounds. These values corresponded to 0.005-0.5 μg/L in the water sample, taking into account the 50-fold pre-concentration factor applied along the sample procedure. For validation purposes, each of the 12 water samples selected (6 SW and 6 EWW) were spiked at different concentration levels (0.02 μg/L in SW; 0.1 and 0.4μg/L in EWW). Experiments were performed by triplicate for each spiked sample. Recoveries were determined by comparing the concentration obtained after applying the recommended procedure with the nominal concentration of the spiked samples, performing quantification by standards calibration in solvent. Non- spiked samples, containing only the ILIS mix, were also processed to subtract the concentration of the target analyte when it was present in the sample used in the recovery experiments.

The method was tested at 0.02 μg/L in the surface water samples (recoveries shown in **Table 2**). A few compounds could not be properly validated in one of the samples tested (Jucar river) due to the high analyte concentration found in the "non-spiked" sample. Enalaprilat and clofibric acid could not be validated in some samples due to the low sensitivity observed for these compounds, which would have required higher spiking levels to be validated. With very rare exceptions, data were satisfactory (between 70% and 120%) for most of the compounds. In a few cases, recoveries varied significantly from one sample to another. This was the case of clarithromycin

Table 2. Method validation for surface water. Recovery (%) and relative standard deviation (RSD, %) for six different SW samples spiked at 0.02 ng/L. Each sample was analyzed in triplicate.

Compound	t_R(min)	Recovery (%)						Average recovery (%)	Average LOQ (ng/L)	LOQ range (ng/L)
		Sitjar reservoir	M[a] Cristina reservoir	Clot lake	Jucar river	Mijares river	Albufera lake			
Carbamazepine	8.32	72	59	85	90	99	56	77 (22)	0.6	0.3 – 0.7
Carbamazepine 10,11-epoxide	6.94	106	99	67	94	110	61	89 (23)	2.7	1.7 – 3.7
Carbamazepine 10,11-epoxide*		39	55	70	90	57	63	62 (27)		
10,11- Dihydro-10,11-dihydroxy carbamazepine	6.57	177	217	177	a	140	127	168 (21)	2.2	1.4 – 3.8
Clarithromycin	9.20	107	100	52	53	64	66	74 (32)	1.9	1.3 – 2.6
N-Desmethyl clarithromycin	9.24	101	88	69	69	73	53	75 (22)	2.5	0.6 – 4.4
Clopidogrel	11.23	50	40	52	39	38	36	43 (16)	0.4	0.2 – 0.7
Clopidogrel carboxylic acid	6.25	82	101	101	50	121	94	91 (26)	1.1	0.5 – 2.0
Enalapril	7.45	80	111	91	103	105	155	107 (24)	22.8	8.9 – 47.6
Enalaprilat	4.93	b	b	b	b	b	b	b	-	-
Enalaprilat*		b	b	b	b	b	b	b		
Losartan	9.19	105	109	104	101	128	131	113 (12)	1.3	0.6- 2.8
Losartan carboxylic acid	9.50	120	130	121	108	162	153	132 (16)	5.4	1.7 – 7.5
Omeprazole	7.99	161	237	167	207	176	345	215 (32)	2.4	2.2 – 2.8
Omeprazole*		102	68	70	87	103	93	87 (18)		
4-Hydroxy omeprazole sulfide	6.83	88	102	84	84	80	75	85 (11)	2.3	1.9 – 2.7
5-Hydroxy omeprazole	6.82	123	145	149	150	131	156	142 (9)	3.0	2.2 – 3.3
Sulfamethoxazole	4.79	98	77	112	106	112	70	96 (19)	7.7	4.0 – 24.8
N-Acetyl sulfamethoxazole	5.90	120	95	128	97	143	95	113 (18)	3.9	2.0 – 7.1
4-Acetamido antipyrine	4.09	87	116	114	a	122	66	101 (23)	3.0	0.9 – 6.6
4-Amino antipyrine	4.42	44	51	76	a	33	52	51 (31)	1.6	0.6 – 3.5
4-Formylamino antipyrine	3.99	95	136	111	a	142	103	117 (18)	2.7	1.3 – 5.5
Clofibric acid	9.28	108	90	83	62	b	b	86 (22)	24.5	17.1 – 30.6
Fenofibric acid	10.86	80	61	83	a	67	101	78 (20)	1.9	1.2 – 2.5

a: Not estimated due to the high analyte levels found in the "blank" sample; b: Not estimated due to the poor sensitivity.

* Recoveries calculated using their own ILIS

In bold the parent pharmaceuticals and with regular format, the metabolites.

Formatat: anglès (Regne Unit)

(individual recoveries between 52 - 107%). This variation might be explained by the distinct matrix effects that particularly affected to this compound and that varied notably from one water sample to another.

Clopidogrel and 4-amino antipyrine presented low recoveries (around 45%) in all samples. In order to know whether poor recoveries were due to matrix effects or to poor extraction in the SPE cartridge, for each individual SW sample a extract obtained after SPE was spiked and the analyte responses compared with standards in solvent at the same concentration (data not shown). Our results showed that low clopidogrel recoveries were due to matrix effects (signal suppression). On the contrary, no relevant matrix effects were observed for 4-amino antipyrine; consequently, its low recoveries were attributed to losses during the SPE process. On the other hand, four compounds showed recoveries above 120% (losartan carboxylic acid; 10,11-dihydro-10,11-dihydroxy carbamazepine; omeprazole; 5-hydroxy omeprazole) due to matrix signal enhancement. In the case of omeprazole, matrix effects could be corrected by using its own ILIS, obtaining satisfactory recoveries in all SW samples.

In total, three ILIS were used in this work and tested for matrix effects correction. In addition to the above mentioned omeprazole, two more compounds were corrected with their own ILIS (carbamazepine 10,11-epoxide and enalaprilat). It is interesting to notice that the ILIS carbamazepine 10,11-epoxide-d_{10} did not fully correct matrix effects for its own analyte. Similar situation has been reported for some labelled compounds with a high degree of deuterated atoms (Ripollés et al. 2012). It seems that the isotope-labelled compound and the analyte had different physico-chemical behaviour, leading to an (unexpected) unsatisfactory correction. A possible explanation could be related to different hydrolysis kinetics between labelled and unlabelled epoxide metabolites caused by the presence of deuteriums in the hydrolysis site.

Regarding EWW, notably matrix effects are normally expected. In previous works (Gracia-Lor et al. 2011 and 2012) the use of ILIS was the alternative chosen to compensate for

Formatat: Normal, Justificada, Sagnia: Esquerra: 0,5 cm, Primera línia: 0 cm, Interlineat: Doble

matrix effects in pharmaceutical analysis in water. When the analyte ILIS is not available, the use of an analogue might be satisfactory, although commonly it can not ensure appropriate correction for all analyte/water sample combinations (Gracia-Lor et al. 2012). In the present work, the availability of only three ILIS made the correction of matrix effects problematic. Sample dilution might be a good alternative, also simple and fast, if sufficient sensitivity is achieved by the analytical method. After testing different dilutions of sample with HPLC-grade water, a 4-fold dilution was found to be adequate for accurate quantification, also maintaining a satisfactory sensitivity. Spiking levels tested in the non-diluted effluent wastewaters from different WWTPs were 0.1 and 0.4 µg/L (i.e. 0.025 and 0.1 µg/L in the 4-diluted samples). In general, recoveries and precision were satisfactory for most compounds at both fortification levels (**Table 3**). Two metabolites of dipyrone (4-acetamido antipyrine and 4-formyl antipyrine) could not be validated in some samples due to the high concentrations found in the non-spiked samples. Low recoveries were obtained for clofibric acid, fenofibric acid and clopidogrel at both levels due to signal suppression that could not be compensated by sample dilution (x 4). On the contrary, some analytes (e.g. losartan carboxylic acid, N-acetyl sulfamethoxazole, 5-hydroxy omeprazole or enalapril) yielded values above 120% in several samples. This behaviour was also observed in SW, as previously commented.

Enalaprilat could not be validated at the lowest level assayed (0.1 µg/L) due to its low sensitivity. At the highest concentration (0.4 µg/L), the average recovery was 66%, which could be improved to 108% by correction of matrix effects thanks to the availability of ILIS enalaprilat-d_5. As occurred in SW, the use of ILIS carbamazepine 10,11-epoxide-d_{10} did not fully correct matrix effects for its own analyte leading to recoveries of 45% and 74% at the low and high spiking concentrations, respectively.

It is worth to notice the case of omeprazole, which could not be validated in EWW probably due to its low stability. In order to evaluate the stability of the omeprazole standard, an

Formatat: Sagnia: Primera línia: 1,25 cm

~~individual new solution of this pharmaceutical was prepared, and injected in the LC-MS/MS instrument every~~

Table 3. Method validation for effluent wastewater. Recovery (%) and relative standard deviation (RSD%) for six different EWW samples spiked at 0.1 and 0.4 µg/L. Each sample was analyzed in triplicate.

Compound	Recovery (%) at 0.1 µg/L						Average	Recovery (%) at 0.4 µg/L						Average	Average LOQ (ng/L)	LOQ range (ng/L)
	EWW1	EWW2	EWW3	EWW4	EWW5	EWW6		EWW1	EWW2	EWW3	EWW4	EWW5	EWW6			
Carbamazepine	118	90	112	114	89	113	106 (12)	125	115	87	111	100	110	108 (12)	17.3	7.5 – 31.3
Carbamazepine 10,11-epoxide	107	119	116	107	107	118	112 (5)	137	137	96	144	137	131	130 (13)	36.1	21.6 – 56.3
Carbamazepine 10,11-epoxide*	35	57	41	42	41	53	45 (19)	80	78	58	79	79	71	74 (12)		
10,11- Dihydro-10,11-dihydroxy carbamazepine	96	97	111	a	70	88	92 (16)	126	118	91	134	137	142	125 (19)	144.9	71.1 – 287.1
Clarithromycin	121	139	115	119	135	127	126 (8)	104	95	62	98	111	67	90 (22)	13.5	3.5 – 24.7
N-Desmethyl clarithromycin	97	107	89	102	106	92	99 (8)	74	71	57	72	90	57	70 (17)	13.8	4.6 – 19.4
Clopidogrel	29	31	32	27	39	41	33 (17)	53	55	53	61	46	53	53 (9)	1.8	0.7 – 2.7
Clopidogrel carboxylic acid	99	114	136	96	102	119	111 (14)	113	124	111	108	99	91	108 (11)	15.7	10.1 – 21.8
Enalapril	138	153	136	149	141	156	146 (6)	158	149	146	163	107	171	149 (15)	132.7	85.0 -179.7
Enalaprilat	b	b	b	b	b	b	b	89	68	70	70	59	42	66 (23)	513	348 - 693
Enalaprilat*	b	b	b	b	b	b	b	105	117	80	106	106	132	108 (16)		
Losartan	96	109	92	96	111	124	105 (12)	116	111	68	109	103	103	102 (17)	5.9	3.2 – 13.0
Losartan carboxylic acid	131	153	182	183	149	207	168 (17)	197	166	113	165	159	132	155 (19)	44.5	25.2 – 77.5
Omeprazole	c	c	c	c	c	c	c	c	c	c	c	c	c	c	-	-
Omeprazole*	c	c	c	c	c	c	c	c	c	c	c	c	c	c		
4-Hydroxy omeprazole sulfide	94	123	91	107	114	81	102 (16)	104	120	94	105	121	97	107 (11)	37.2	19.6 – 60.1
5-Hydroxy omeprazole	103	132	145	132	108	117	123 (13)	137	139	124	137	129	123	131 (5)	33.9	9.5 – 60.1
Sulfamethoxazole	71	59	134	100	108	115	98 (29)	92	110	74	97	103	100	96 (13)	64.3	35.1 – 77.9
N-Acetyl sulfamethoxazole	73	143	112	112	154	140	123 (24)	112	129	119	115	128	118	120 (6)	56.9	32.4 – 80.8
4-Acetamidoantipyrine	a	a	a	a	a	83	83	a	a	a	a	a	106	106	60.3	60.3
4-Aminoantipyrine	55	64	64	49	a	a	58 (13)	47	48	88	59	a	59	60 (28)	130.8	54.8 – 310.1
4-Formylaminoantipyrine	149	a	a	118	a	a	134 (16)	216	222	a	109	179	a	182 (29)	129.5	84.9 –189.3
Clofibric acid	51	51	30	51	46	40	45 (19)	51	52	41	29	29	26	38 (30)	106.8	78.7 – 160.0
Fenofibric acid	47	65	41	58	65	67	57 (19)	65	65	52	44	59	59	58 (14)	5.9	4.7 – 8.5

a: Not estimated due to the high analyte levels found in the "blank" sample.; b: Not estimated due to the poor sensitivity; c: Not estimated due to the standard degradation.

* Recoveries calculated using their own ILIS

In bold the parent pharmaceuticals and with regular format, the metabolites.

individual new solution of this pharmaceutical was prepared, and injected in the LC-MS/MS instrument every week. As can be seen in **Figure 3a**, the omeprazole signal notably decreased along the time, while in parallel the concentration of 4-hydroxy omeprazole sulfide, which was not included in the standard solution, increased (**Figure 3b**). Thus, it seems clear that omeprazole was unstable in aqueous solution and was transformed to 4-hydroxy omeprazole sulfide. The degradation of omeprazole to the sulfide derivative in HPLC-grade water and kept in dark has been previously reported in literature (DellaGreca et al. 2006). In our study, the transformation started to be more evident after 14 days of storage in the cooler (-4 ºC, darkness). Thus, to avoid a wrong quantification for omeprazole and 4-hydroxy omeprazole sulfide when analyzing real samples, standards should be renewed weekly.

LOQs were estimated for every water sample tested (i.e., 6 SW and 6 EWW). For SW, average LOQs ranged from 0.4 to 7.7 ng/L (**Table 2**). The two exceptions were clofibric acid and enalapril, which presented low sensitivity, with the result of higher LOQs (around 25 ng/L in SW). With the exception of enalaprilat, average LOQs for EWW varied from 1.8 to 145 ng/L: for 3 compounds LOQs were < 6 ng/L, and for another 10 analytes they were lower than 60 ng/L (**Table 3**). As can be seen, in several cases the LOQs varied notably from one sample to other. This highlights once more that matrix effects can be rather different from one sample to another. Therefore, giving an LOQ value estimated just from a given sample, as reported in most of papers, might not be realistic. Consequently, LOQ reported should be taken with precaution, as the situation may be rather different when applying the method to a set of real-world samples. Concerning IDLs, they ranged from 0.3 to 10 pg, except for clofibric acid, the only compound that was measured in negative ionization mode (**Table 1**).

Formatat: Sagnia: Esquerra: 0,75 cm, Primera línia: 0 cm

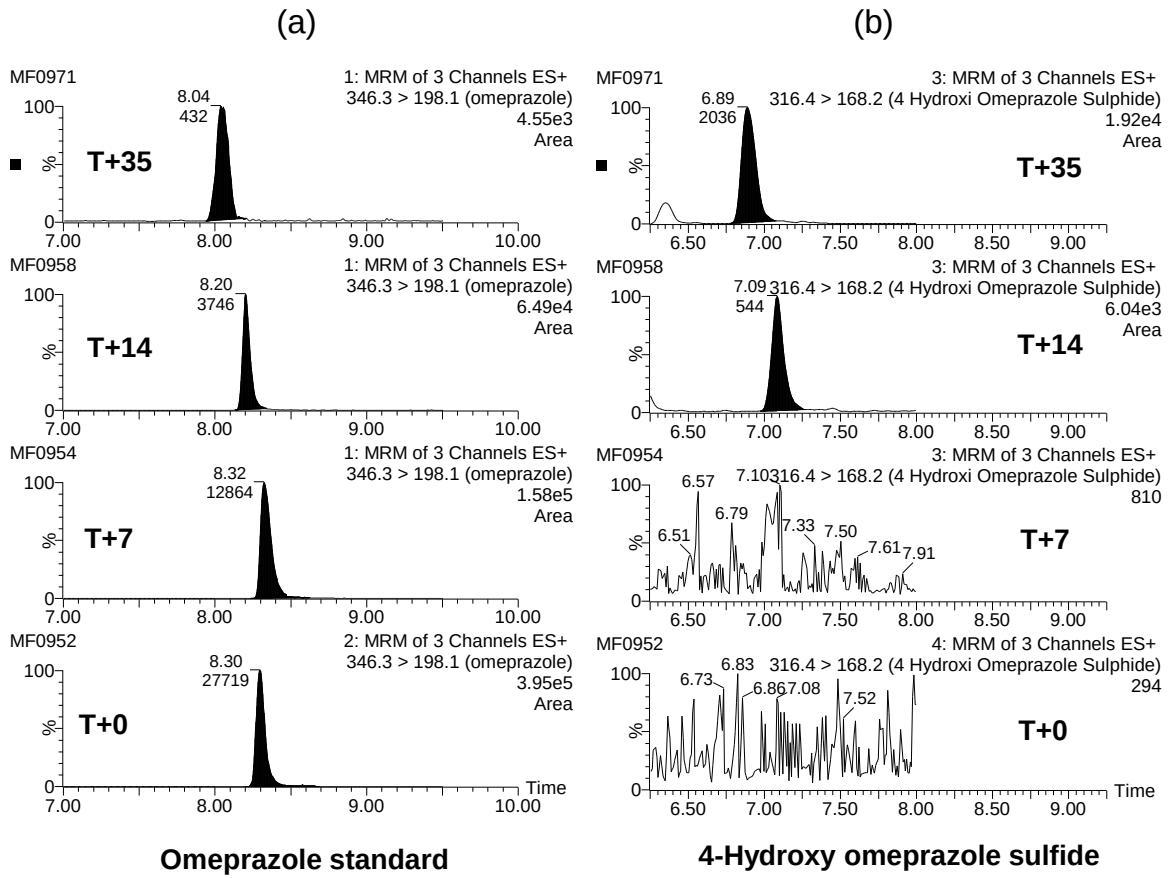

Figure 3 LC-MS/MS chromatograms for (a) omeprazole reference standard (100 µg/L) injected in different days after preparation of the standard solution (b) 4-hydroxy omeprazole sulfide formed by degradation of omeprazole.

3.5 Application to water samples

The developed methodology was applied to the analysis of 12 SW samples collected at selected sites from the Spanish Mediterranean area of Valencia and 12 EWW samples from different WWTPs of this area.

In every sequence of sample analysis, the calibration curve was injected twice, at the beginning and the end of the sample batch. Moreover, quality control samples (QCs) were included in every sample sequence. QCs consisted on SW or EWW samples spiked at the LOQ level. They were prepared randomly selecting one of the water samples analyzed within the batch, following the same analytical procedure than for the samples. In the case that the sample used for QC preparation contained any of the compounds analyzed, the concentration calculated in the sample was subtracted from that calculated in the spiked sample.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q_1 and q_2) transitions. As three transitions were acquired, two intensity ion-ratios could be used for confirmation of the identity. The finding was considered as positive when the experimental ion-ratios were within the tolerance range (European Union Decision 2002/657/EC) and the retention time of the compound in the sample within ±2.5% the retention time of the reference standard.

All parent compounds, except omeprazole, were detected in the surface water samples at least once (**Table 4**). Regarding metabolites, only three of them (4-amino antipyrine, clofibric acid and enalapilat) were never found. The highest concentrations corresponded to the dipyrone metabolites 4-acetamido antipyrine and 4-formylamino antipyrine (0.89 and 0.87 µg/L, respectively). Dipyrone is a pro-drug widely used as antipyretic. Its main metabolites have been previously studied in several works, and high concentrations have been reported (Martínez Bueno et al. 2007; Rosal et al. 2010).

Table 4. Summary of the compounds detected in the analysis of surface and effluent wastewater samples

Compounds	SW (n = 12)				EWW (n = 12)			
	Min Level (µg/L)	Max Level (µg/L)	Median (µg/L)	% positive findings	Min Level (µg/L)	Max Level (µg/L)	Median (µg/L)	% positive findings
Carbamazepine	0.002	0.026	0.009	58	0.43	2.76	1.01	100
Carbamazepine 10,11-epoxide	< LOQ	< LOQ	< LOQ	17	n.d.	n.d.	n.d.	0
10,11- Dihydro-10,11-dihydroxy carbamazepine	0.004	0.374	0.048	92	0.55	6.85	1.76	100
Clarithromycin	< LOQ	0.012	0.004	67	0.04	0.39	0.09	67
N-Desmethyl clarithromycin	< LOQ	0.004	0.004	50	0.04	0.15	0.09	100
Clopidogrel	< LOQ	0.002	0.002	100	0.006	0.022	0.008	100
Clopidogrel carboxylic acid	0.002	0.012	0.003	67	0.09	0.61	0.20	100
Enalapril	< LOQ	< LOQ	< LOQ	8	n.d.	n.d.	n.d.	0
Enalaprilat	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	0
Losartan	0.003	0.099	0.004	67	0.03	0.37	0.29	100
Losartan carboxylic acid	<LOQ	0.116	0.009	67	0.01	1.56	0.49	100
Omeprazole	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	0
4-Hydroxy omeprazole sulphide	<LOQ	0.028	< LOQ	33	0.06	0.29	0.11	100
5-Hydroxy omeprazole	0.004	0.004	0.004	8	0.12	0.25	0.22	58
Sulfamethoxazole	< LOQ	< LOQ	< LOQ	8	0.05	1.86	0.11	100
N-Acetyl sulfamethoxazole	< LOQ	< LOQ	< LOQ	33	0.03	0.96	0.09	100
4-Acetamido antipyrine	0.002	0.894	0.068	75	0.49	7.91	3.20	100
4-Amino antipyrine	n.d.	n.d.	n.d.	0	0.53	7.98	0.85	25
4-Formylamino antipyrine	0.037	0.871	0.079	67	0.98	5.76	3.23	100
Clofibric acid	n.d.	nd	n.d.	0	n.d.	n.d.	n.d.	0
Fenofibric acid	0.008	0.182	0.020	33	0.01	0.36	0.23	100

n.d.: Not detected, i.e., none transition was observed

<LOQ: Both quantification and confirmation transitions were observed but the concentration level was lower than the LOQ shown in Tables 2-3)

Median concentrations for most of the compounds were lower than 0.01 µg/L. The metabolite 10,11-dihydro-10,11-dihydroxy carbamazepine was however present at higher concentrations. This compound was found in 92% of the samples in contrast to the other carbamazepine metabolite studied in this work (carbamazepine 10,11-epoxide), which was only present in 17% of the samples. This is in accordance with the metabolism of carbamazepine. This pharmaceutical, used for the treatment of epilepsy, schizophrenia and bipolar disorder, undergoes extensive metabolism by cytochrome P450 system in the liver. Carbamazepine seems not to be efficiently removed in WWTPs, which may explain its frequent detection in environmental samples (Radjenovic et al. 2009; Langford and Thomas 2011). Also in agreement with our findings is the fact that the most relevant metabolite 10,11-dihydro-10,11-dihydroxy carbamazepine was widely detected in waters, and to a lesser extent the metabolite carbamazepine 10,11-epoxide (Miao and Metcalfe 2003). It is worth to notice that most of papers dealing with the presence of carbamazepine and metabolites in the aquatic environment only take into account carbamazepine 10,11-epoxide (Langford and Thomas 2011; Valcárcel et al. 2011; López-Serna et al. 2012a; Jelic et al. 2012), despite that higher concentrations are commonly found for 10,11-dihydro-10,11-dihydroxy carbamazepine (Miao and Metcalfe 2003). This might be due to the fact that the epoxide is the active metabolite of carbamazepine (Miao and Metcalfe 2003).

In relation to effluent wastewater samples, 13 out of 21 compounds were detected in 100% of the samples, which illustrates the ubiquity of these compounds in the wastewaters. All parent compounds, except omeprazole and enalapril, were frequently detected. In the case of omeprazole, despite being one of the most consumed pharmaceuticals in Spain, it was not detected in any of the samples. On the contrary, the two omeprazole metabolites included in this study were detected, and one of them (4-hydroxy omeprazole sulfide) was found in all the samples analyzed (**Table 4**). This is in agreement with our previous research on omeprazole

357 metabolism and on its transformation products in water Boix et al. 2013a and 2013b. Enalapril
358 and enalaprilat (its active metabolite) were not detected in any of the water samples. Both
359 compounds are frequently found in influent wastewater (Gracia-Lor et al. 2010; Tarcomnicu et
360 al. 2011) but they are much less ubiquitous in EWW.

361 Sulfamethoxazole and its N-acetyl derivate were found in all EWW samples at similar
362 average concentration level. Despite the derivative does not have pharmacological activity, it
363 however presents ecotoxicity (López-Serna et al. 2012a).

364 The highest concentrations were by far for dipyrone metabolites, with maximum
365 concentrations around 8 μg/L, for both 4-acetamido antipyrine and 4-amino antipyrine, while 4-
366 formylamino antipyrine reached a maximum value around 6 μg/L. 4-amino antipyrine was was
367 present in 25% of the samples while the other two were found in all the effluent wastewaters
368 analyzed.

369 Similarly, the concentrations of the pharmaceuticals losartan, carbamazepine and
370 especially for clopidogrel were lower than for their metabolites. As occurred in surface waters,
371 high levels of 10,11-dihydro-10,11-dihydroxy carbamazepine were found due to the extensive
372 metabolism of carbamazepine.

373 Regarding the pro-drugs, only fenofibric acid was detected while clofibric acid was not
374 found in any sample. The latter might be explained by a lower consumption of this
375 pharmaceutical this area (it is not within the list of the most consumed pharmaceuticals in Spain)
376 and/or to some kind of transformation/degradation in the sewer system or in the aquatic
377 environment, all these facts making making its detection in water samples unlikely.

378 Illustrative UHPLC-MS/MS chromatograms are shown in **Figure 4** for a EWW sample
379 that was positive for up to 16 compounds, with concentrations varying from 0.02 μg/L
380 (clopidogrel) to 7.98 μg/L (4-amino antipyrine).

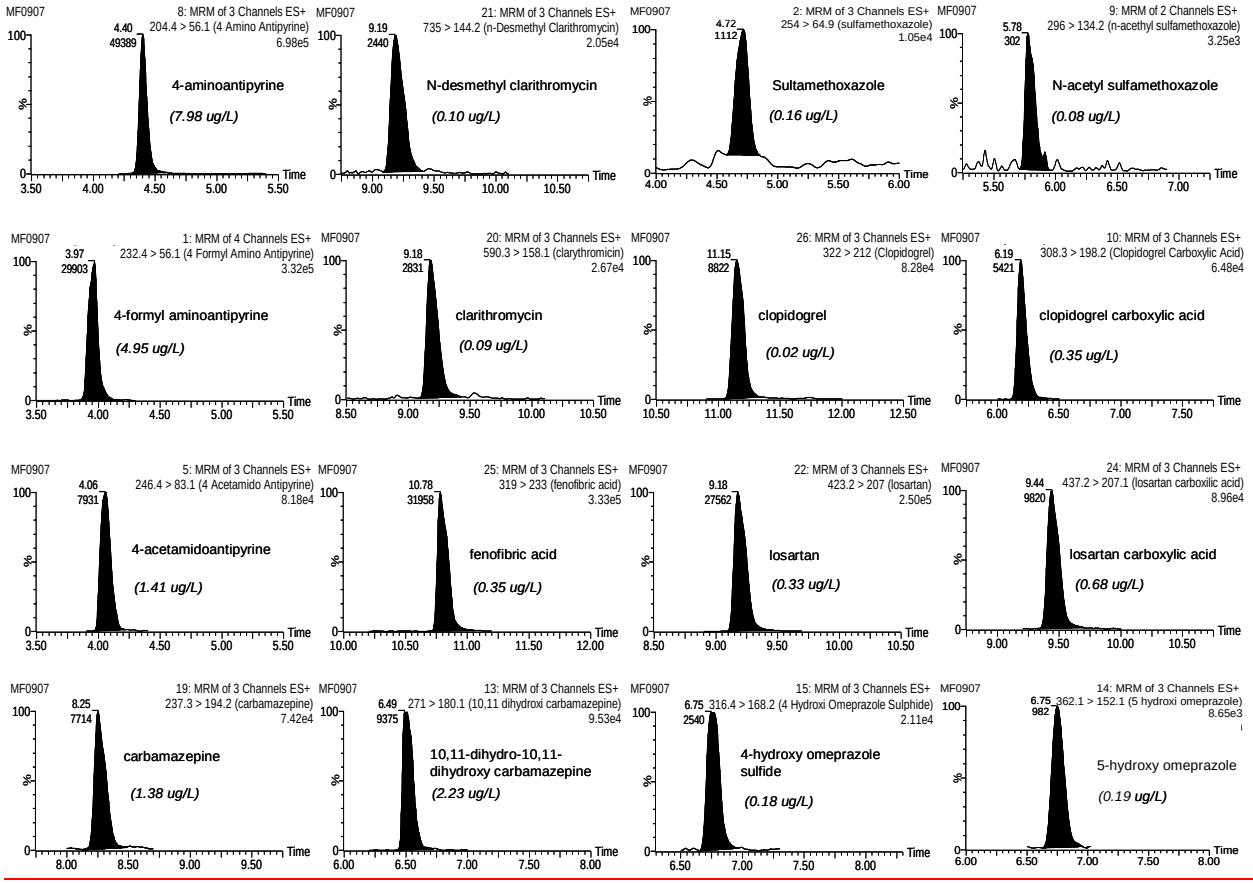

Figure 4

LC–MS/MS chromatograms (Q transition) for an effluent wastewater that was positive to 16 compounds (5 pharmaceuticals and 11 metabolites).

Formatat: Esquerra: 2,5 cm, Dreta: 2,5 cm, Superior: 2,51 cm, Inferior: 1,5 cm, Amplada: 29,7 cm, Alçada: 21 cm

Formatat: anglès (Regne Unit)

Formatat: Esquerra: 1,5 cm, Dreta: 2,51 cm

4. CONCLUSIONS

The analytical method developed in this work, based on the use of UHPLC-MS/MS with triple quadrupole analyzer, has allowed the simultaneous determination of 21 analytes, including seven parent pharmaceuticals and 14 major metabolites, in surface water and urban effluent wastewater samples. In order to have a more realistic overview on the method performance and its applicability to different water samples, the method was validated in twelve different matrices (six surface water and six effluent wastewater samples) at several concentration levels. In absence of most of analyte ILIS, a 4-dilution of the effluent wastewater samples was found a rapid, simple and rather efficient approach to minimize signal suppression or enhancement due to matrix effects in this complex matrix. Confirmation of the analyte identity was guaranteed by acquiring 3 SRM transitions and evaluating their Q/q ratios and retention time.

The application of this method to water samples showed that 6 out of 14 metabolites were present in 50% of the surface samples analyzed. Regarding effluent wastewater, 9 out of 14 metabolites were detected in 100% of the samples. Interestingly, metabolite concentrations were on the same order or even higher than those of the parent compound. This illustrates the importance of including metabolites and transformation products in the methods applied for water analysis in order to have a wider and realistic knowledge on the occurrence and fate of pharmaceuticals in the aquatic environment.

Acknowledgments

This work has been supported by the Spanish Ministry of Education and Science (Project CTQ-2009-12347). The authors also acknowledge the financial support of Generalitat Valenciana (research group of excellence PROMETEO/2009/054). E. Gracia-Lor is very grateful to University Jaume I for her pre-doctoral grant. The authors acknowledge the Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for using the TQD triple quadrupole mass spectrometer.

References

Batt AL, Kostich MS, Lazorchak JM (2008) Analysis of ecologically relevant pharmaceuticals in wastewater and surface water using selective solid-phase extraction and UPLC-MS/MS. Anal Chem 80: 5021-5030.

Boix C, Ibáñez M, Zamora T, Sancho JV, Niessen WMA, Hernández F (2013a). Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry. Degradation experiments. J Mass Spectrom.

Boix C, Ibáñez M, Zamora T, Sancho JV, Niessen WMA, Hernández F (2013b). Identification of new omeprazole metabolites in wastewater and surface. Sci Total Environ.

DellaGreca M, Iesce MR, Previtera L, Rubino M, Temussi F, Brigante M (2006) Degradation of lansoprazole and omeprazole in the aquatic environment. Chemosphere 63: 1087-1093.

Ferrer I, Thurman EM (2012) Analysis of 100 pharmaceuticals and their degradates in water samples by liquid chromatography/quadrupole time-of-flight mass spectrometry. J Chromatogr A 1259:148-157

Gómez MJ, Sirtori C, Mezcua M, Fernández-Alba AR, Agüera A (2008) Photodegradation study of three dipyrone metabolites in various water systems: Identification and toxicity of their photodegradation products. Water Res 42: 2698-2706.

Gómez-Ramos MM, Pérez-Parada A, García-Reyes JF, Fernández-Alba AR, Agüera AJ (2011) Use of an accurate-mass database for the systematic identification of transformation products of organic contaminants in wastewater effluents. J Chromatogr A 1218: 8002-8012.

González Alonso S, Catalá M, Romo Maroto R, Rodríguez Gil JL, Gil de Miguel A, Valcárcel Y (2010) Pollution of psychoactive pharmaceuticals in the Rivers of Madrid metropolitan área (Spain). Environ Int 36: 195-201.

Gracia-Lor E, Sancho JV, Hernández F (2010) Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra-high pressure liquid chromatography-tandem mass spectrometry. J Chromatogr A 1217:622-632

Gracia-Lor E, Sancho JV, Hernández F (2011) Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. J Chromatogr A 1218: 2264-2275.

Gracia-Lor E, Martínez M, Sancho JV, Peñuela G, Hernández F (2012) Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high performance liquid-chromatography-tandem mass spectrometry. Talanta 99: 1011-1023.

Gros M, Rodríguez-Mozaz S, Barceló, D (2012) Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J Chromatogr A 1248:104-121.

Hernández, F, Ibáñez M, Gracia-Lor E, Sancho, JV (2011) Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater. J Sep Sci 34: 3517-3526.

Ibáñez M, Gracia-Lor E, Sancho JV, Hernández F (2012) Importance of MS selectivity and chromatographic separation in LC-MS/MS-based methods when investigating pharmaceutical metabolites in water. Dipyrone as a case of study. J Mass Spectrom 47: 1040-1046

Jelic A, Fatone F, Di Fabio S, Petrovic M, Cecchi F, Barceló D (2012) Tracing pharmaceuticals in the municipal plant for integrated wastewater and organic solid waste treatment. Sci Total Environ 433: 352-361.

Jjemba PK (2006) Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. Ecotoxicol Environ Saf 63: 113-130.

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2007) Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra performance liquid chromatography-positive electrospray ionisation tandem mass spectrometry. J Chromatogr A 1161: 132-145.

Khetan S, Collins TJ (2007) Human pharmaceuticals in the aquatic environment: A challenge to green chemisty. Chem Rev 107: 2319-2364.

Kovalova L, Siegrist H, Singer H, Wittmer A, McArdell CS (2012) Hospital wastewater treatment by membrane bioreactor: performance and efficiency for organic micropollutant elimination. Environ Sci Technol 46: 1536-1545.

Langford K, Thomas KV (2011) Input of selected human pharmaceutical metabolites into the Norwegian aquatic environment. J Environ Monit 13: 416-421.

López-Serna R, Petrovic M, Barceló D (2012a) Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain). Sci Total Environ 440: 280-289

López-Serna R, Petrovic M, Barceló D (2012b) Direct analysis of pharmaceuticals, their metabolites and transformation products in environmental waters using on-line TurboFlow™ chromatography-liquid chromatography-tandem mass spectrometry. J Chromatogr A 1252: 115-129.

Martínez Bueno MJ, Agüera A, Gómez MJ, Hernando MD, García-Reyes JF, Fernández-Alba AR (2007) Application of liquid chromatography/quadrupole-linear ion trap mass spectrometry and time-of-flight mass spectrometry to the determination o pharmaceuticals and related contaminants in wastewater. Anal Chem 79: 9372-9384

Miao X S, Metcalfe CD (2003) Determination of carbamazepine and its metabolites in aqueous samples using liquid chromatography - Electrospray tandem mass spectrometry. Anal Chem 75: 3731-3738.

Mompelat S, Le Bot B, Thomas O (2009) Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. Environ Int 35: 803-814.

Radjenovic J, Petrovic M, Barceló D (2009) Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res 43: 831-841.

Ripollés C, Sancho JV, López FJ, Hernández F (2012) Liquid chromatography coupled to tandem mass spectrometry for the residue determination of ethylenethiourea (ETU) and propylenethiourea (PTU) in water. J Chromatogr A 1243: 53-61.

Rosal R, Rodríguez A, Perdigón-Melón A, Petre A, García-Calvo E, Gómez MJ, Agüera A, Fernández-Alba AR (2010) Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Res 44: 578 – 588.

Tarcomnicu I, van Nuijs ALN, Simons W, Bervoets L, Blust R, Jorens PG, Neels H,Covaci A (2011) Simultaneous determination of 15 top-prescribed pharmaceuticals and their metabolites in influent wastewater by reversed-phase liquid chromatography coupled to tandem mass spectrometry. Talanta 83: 795-803.

Valcárcel Y, González Alonso S, Rodríguez-Gil JL, Gil A, Catalá M (2011) Detection of pharmaceutically active compounds in rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84: 1336-1348.

Wille K, De Brabander HF, De Wulf E, Van Caeter P, Janssen CR,Vanhaecke L (2012) Coupled chromatographic and mass-spectrometric techniques for the analysis of emerging pollutants in the aquatic environment. TrAC -Trends Anal Chem35: 87-108.

~~FIGURE CAPTIONS~~

~~Figure 1.~~ ~~Structures of the selected compounds.~~

~~Figure 2.~~ ~~Recoveries obtained for 19 selected compounds after SPE with Oasis HLB (60 mg) at different sample pH values. Clopidogrel and losartan recoveries are not shown because their standards were not available at the laboratory when this test was carried out.~~

~~Figure 3.~~ ~~LC-MS/MS chromatograms for (a) omeprazole reference standard (100 µg/L) injected in different days after preparation of the standard solution (b) 4-hydroxy omeprazole sulfide formed by degradation of omeprazole.~~

~~Figure 4.~~ ~~LC-MS/MS chromatograms (Q transition) for an effluent wastewater that was positive to 16 compounds (5 pharmaceuticals and 11 metabolites).~~

Formatat: Sagnia: Esquerra: 0,75 cm

Formatat: Sagnia: Esquerra: 0,75 cm, Tabulacions: 0 cm, Esquerra

Formatat: Justificada, Sagnia: Esquerra: 0,75 cm, Primera línia: 0 cm, Tabulacions: 0 cm, Esquerra