A new analytical method to determine trace level concentrations of pharmaceuticals in influent wastewater: A tool to monitor human use patterns

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ABSTRACT

The occurrence of pharmaceuticals in influent wastewater samples (IWW) is a recurrent issue. The monitoring of their presence is not only valuable from an environmental point of view, but also as a tool to analyze patterns of human use by the so-called wastewater-based epidemiology. The development of an analytical method based on solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry to monitor the occurrence of a group of seventeen pharmaceuticals including the most representative for various therapeutic families in IWW samples is described in this work. The samples were collected during a monitoring week in six wastewater treatment plants located in different cities and towns across Spain. The developed method provides acceptable figures of merit with apparent recoveries in IWW ranging from 42% to 139%, and low matrix effect (in general lower than $\pm$ 30%), and method quantification limits (MQL) between 1 ng/L and 24 ng/L for all compounds, except atenolol (58 ng/L). All the studied pharmaceuticals were found in all samples with concentrations ranging from < MQL to 10,393 ng/L, being the highest concentrations for tramadol. The population normalized daily loads revealed that the use of pharmaceuticals follows, in general, a similar pattern in all cities monitored.

1. Introduction

The consumption of pharmaceuticals and drugs of abuse has increased worldwide due to changes in clinical practice, and the need to cope with age-related and chronic diseases [1]. As an example, the COVID-19 pandemic has had a major impact in mental health, which provoked a major consumption of anti-depressant compounds [1]. Because of the drug consumption rise, the discharge of drugs and their metabolites in wastewater has also increased, as well as their environmental occurrence, becoming an issue of global concern. Therefore, active research on the occurrence, fate and toxic effects of drugs in the environment has dramatically increased over the last years [2–5].

Studies conducted worldwide reported the occurrence of different drugs and their metabolites in aquatic systems. A review [2] that collects information of drug occurrence in 71 countries across all continents reported that 631 out of the 713 drugs investigated occurred in different environmental compartments, being urban wastewater the dominant

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emission pathway. Another study [6] monitored the presence of 43 pharmaceuticals in the effluent wastewater of six seniors' residences located in Spain, France and Portugal, and revealed that 14 out of 30 pharmaceuticals, at concentration levels of µg/L, were still present in effluent wastewaters discharged into surface waters. Another example [7] is the monitoring of 39 pharmaceuticals in two wastewater treatment plants (WWTPs) serving two cities in Catalonia (NE, Spain) which revealed the presence of all the studied pharmaceuticals at concentrations between <1 µg/L and 200 µg/L in influent wastewater (IWW).

Accurate quantification of pharmaceuticals and drugs in IWW is challenging, and hyphenated analytical techniques such as liquid chromatography followed by mass spectrometry are the usual approach to selectively and quantitively determine these compounds in wastewater samples [8–10]. In addition, an adequate sample (pre) treatment step is essential to isolate and concentrate the selected compounds as well as to reduce matrix interferences from those complex environmental samples. In most cases, after collection of samples, solid-phase extraction (SPE) is applied, since the different SPE sorbents available enable to cover the extraction of the highest number of compounds within a broad chemical properties [11]. Polymer-based sorbents with hydrophilic moieties so as to exploit both polar and reversed phase interactions are the most commonly used; nevertheless, mixed-mode ion-exchange materials have been also chosen to selectively extract families of basic or acidic compounds [12].

The development of robust and reliable analytical methods to determine drugs within a wide polarity range in environmental samples is required not only to monitor the occurrence of these substances in environmental compartments, but also to back calculate the amount of these substances consumed by the population living in the WWTP catchment area. The so-called wastewater-based epidemiology (WBE) is an analytical approach based on the determination of parents and human metabolic excretion products (biomarkers) in IWW to monitor the consumption patterns of xenobiotics (e.g., drugs) at the population level [13,14]. This is accomplished by converting biomarker concentrations in IWW to per capita mass load (in mg/day) estimates using daily wastewater flow rates and number of inhabitants in the catchment area, thereby resulting in population normalized daily loads (in mg/day 1000/inhabitants). Therefore, wastewater contains an invaluable information about the catchment population connected to the WWTP. In addition, WBE allows comparing results between different WWTPs and/or different temporary points [15,16]. So far, WBE has been progressively implemented to obtain information about lifestyles and dietary habits through consumption patterns, mainly of illicit drugs [17–20], but also covered other xenobiotics as pharmaceuticals [7,21–23], tobacco [22,24] or alcohol [22]. WBE has also been applied to study population exposure to contaminants, such as plasticizers [25] or pesticides [26], among others; and, during the pandemic period, it has been used to assess the presence of SARS-CoV-2 virus particles in order to monitor the spread of COVID disease in different regions [27,28].

In this study, a multiresidue SPE procedure followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) is proposed to determine seventeen pharmaceutical compounds in IWW samples from six Spanish WWTPs. The purpose of this study is, on one hand, to evaluate the occurrence of these pharmaceuticals in the IWW samples; and, on the other hand, to provide an analytical tool to estimate pharmaceutical use patterns across Spain as an approach to WBE. There are already several studies that demonstrated the occurrence of pharmaceuticals in different separate IWW samples in Spain [4,29–31]. However, to the best of our knowledge, there are no studies that monitored different WWTPs geographically distributed in Spain to provide a general picture of the pharmaceutical use in this country.

2. Experimental

2.1. Materials

The analytes (listed in Table 1 classified by therapeutic class and its structure is shown in Fig. S1) were purchased from Merck KGAa (Darmstadt, Germany) except codeine and morphine, which were purchased from Cerilliant (Round Rock, TX, USA). All standards were of the high purity available (>97%). Stock solutions of individual standards or the mixture thereof were prepared by diluting each compound in methanol (MeOH) or water/MeOH (4/1, v/v), respectively and stored at −20 °C in the dark. Ultrapure water was provided by a Synergy UV water purification system (Merck Millipore, Burlington, MA, USA) and HPLC grade MeOH, "MS grade" ACN and water were all supplied from Carlo Erba (Val de Reuil, France). Formic acid (HCOOH) (99.5% purity) and ammonium hydroxide (NH$_4$OH) (28 %) were acquired from Merck KGAa.

2.2. Sample collection

Composite (24 h) IWW samples were collected for one week during Spring or Autumn in six WWTPs (Table S1 for details) located across different regions and cities in Spain: two WWTPs that serve 30% of the total population of the metropolitan area of Madrid as models of a large-sized city with economy based on industry, services, administration and tourism; Tarragona and Reus WWTPs located in Catalonia, Northeast Spain, as WWTP models of medium-sized cities with industrial and tourism-based economy; and Palma de Mallorca and Las Palmas de Gran Canaria WWTPs located in the Balearic Islands, Mediterranean Sea, and Canary Islands in the Atlantic Ocean, respectively as models of WWTP in islands with tourism-based economy.

All samples were collected by automatic sampling devices. After collection, the samples were transported to the laboratory and stored in the dark at −20 °C. In total, 42 IWW samples were analyzed. Daily wastewater flow rate (m^3/day) used to calculate daily excretion loads and other details affecting the sampling or the quality of the wastewater samples were collected from the WWTPs facilities using a standardized questionnaire. Table S1 overviews the location and the main characteristics of the WWTPs included in this study.

2.3. Sample extraction

To select the optimum solid-phase extraction (SPE) protocol: Oasis HLB (150 mg/6 mL) and Oasis MCX (150 mg/6 mL) cartridges from Waters (Milford, MA, USA) were evaluated using standards in ultrapure water.

For the extraction of IWW, 100 mL of sample without pH adjustment were filtrated (Glass fiber followed by 0.45 µm Nylon filters) and loaded into Oasis HLB at a flow-rate of 4–6 mL/min previously conditioned with 6 mL of MeOH and 6 mL of ultrapure water. Then, the cartridges were rinsed with 5 mL of ultrapure water, dried, and the analytes were eluted with 5 mL of MeOH at a flow-rate of 2–4 mL/min. Extracts were evaporated to dryness using miVac Duo centrifuge evaporator (Genevac, Ipswich, UK) and reconstituted with 1 mL of MeOH:water (1/9, v/v). Reconstituted extracts were filtered with 0.22 µm PTFE syringe filters (Scharlab, Barcelona, Spain) directly to the analysis vial.

2.4. Liquid chromatography-tandem mass spectrometry

Sample extracts were analyzed using an Agilent model 1200 series liquid chromatograph coupled to 6460 QqQ mass spectrometer detector. The LC system was equipped with a degasser, a quaternary pump, an autosampler and a column compartment. A Luna Omega Polar C18 (150 mm × 3 mm, 5 µm) column with a precolumn (4 mm × 3 mm, 5 µm) from Phenomenex (Torrence, CA, USA) and thermostatized at 30 °C was used. The mobile phase consisted of (A) 0.1% HCOOH in water and (B)

Table 1
Retention time of the studied pharmaceuticals and the MS/MS parameters.

Therapeutic class	Compound Name	Rt (min)	Precursor Ion (*m/z*)*	Product Ion (*m/z*)*	Cone Volt. (V)	Col. Ener. (eV)	q/Q
Nervous system Opioids	**Morphine**	6.73	**286.0**	**201.0** 165.0	100	25 50	0.87
Cardiovascular syst. B-Blockers	**Atenolol**	9.97	**267.2**	**190.1** 145.0	125	20 28	0.61
Respiratory syst. Cough suppressants	**Codeine**	11.43	**300.0**	**215.0** 199.0	150	30 30	0.93
Nervous system Opioids	**Tramadol**	15.20	**264.0**	**58.2**	100	24	–
Cardiovascular syst. Peripheral vasodilat.	**Pentoxifylline**	16.02	**279.0**	**181.0** 138.0	100	12 28	0.32
Nervous system Antidepressant	**Venlafaxine**	16.99	**278.0**	**260.3** 58.2	100	10 16	0.23
Alimentary track Drugs for peptic ulcer	**Omeprazole**	17.50	**330.0**	**182.0** 120.0	100	20 20	0.16
Nervous system Psych analeptics	**Trazodone**	17.73	**372.0**	**176.0** 148.0	100	24 40	0.70
Anti-infective Antibacterial	**Sulfamethoxazole**	17.91	**254.0**	**156.0** 108.0	100	15 15	0.47
Nervous system Antipsychotic	**Quetiapine**	17.94	**384.0**	**253.0** 221.0	100	20 40	0.50
Nervous system Antiepileptics	**Carbamazepine**	21.15	**237.1**	**194.1** 179.1	130	16 35	0.14
Nervous system Anxiolytics	**Oxazepam**	21.69	**287.0**	**269.0** 241.0	130	10 20	0.68
Nervous system Addictive disorders	**Methadone**	21.82	**310.0**	**265.0** 105.0	130	10 28	0.47
Cardiovascular syst. Renin-angiotensin	**Losartan**	22.57	**423.0**	**405.0** 207.0	130	10 10	0.61
Nervous system Anxiolytics	**Diazepam**	25.05	**285.0**	**222.0** 193.0	125	30 30	0.48
Cardiovascular syst. Lipid modif. agents	**Bezafibrate**	25.43	**362.2**	**316.2** 276.2	125	10 10	0.43
Muscul-skeletal sys. Anti-inflammatory	**Diclofenac**	28.56	**296.0**	**250.0** 215.0	100	10 15	0.56

* Bold denotes the quantification transition.

0.1% HCOOH in ACN. Gradient elution started at 5% B, which was maintained for 3 min, increasing to 75% B in 25 min and then to 100% B in 2 min, held for 1 min and returned to the initial conditions in 2 min, which were maintained during 5 min for column equilibration. The flow-rate was set at 0.4 mL/min and the injection volume was 10 µL.

Electrospray ionization (ESI), working in the positive mode was used as interface. The source parameters were optimized and the compromised values are as follows: gas temperature, 320 °C; gas flow, 12 L/min; nebulizer pressure, 45 psi; and capillary voltage, 3000 V. Cone voltages (50 to 200 V) and collision energy (CE) (0 to 60 eV) were optimized for each compound to obtain precursor ions and two product ions with the optimum values listed in Table 1. The acquisition was performed under dynamic multiple reaction monitoring (dMRM) mode using the most abundant precursor/product ion transition as quantifier (Q) and the second most abundant transition as qualifier (q). Data were collected using MassHunter software from Agilent Technologies. For confirmation purposes, both the ratio of these transitions (q/Q) considering a tolerance level of ±30% relative standard deviation (% RSD) and the retention time (considering ±0.1 min) were assessed following SANCO guidelines [32].

2.5. Quality assurance

Calibration was performed by injecting in triplicate eight standard solutions at concentrations ranging from the individual instrumental limit of quantification (IQL) to 1000 µg/L. IQL is the lowest concentration in the calibration curve and that accomplishes a signal-to-noise (S/N) ratio of 10, whereas the instrumental limit of detection (IDL) accomplishes the S/N ratio of 3.

The recoveries (%R) were calculated as the ratio of concentrations (obtained by interpolation of the signal in the calibration curve) measured in the extract when the analytes were spiked in ultrapure water before SPE and the theoretical concentration. The apparent recoveries (%R$_{app}$) were used in IWW samples and include losses during the extraction process and the signal suppression or enhancement due to matrix effects in the ESI source. When dealing with IWW, the natural occurrence of the studied compounds was subtracted.

Matrix effects were assessed using the following equation:

$$\%ME = (C_{POST-EXTR}/C_{STD} \times 100) - 100 \qquad (1)$$

Where C$_{POST-EXTR}$ is the concentration obtained from the sample extract that was spiked after the SPE (after subtracting the concentration of the naturally present compounds), and C$_{STD}$ is the spiked concentration in the standard solution. All concentrations are obtained after interpolation of the signal in the calibration curve. A negative value indicates signal suppression, while a positive value indicates signal enhancement.

The method detection limits (MDLs) and method quantification limits (MQLs) in IWW were estimated from the IDL or IQL by applying % R$_{app}$ and the SPE concentration factor.

Repeatability and reproducibility were assessed as the percentage of RSD of five replicated samples spiked at 1000 ng/L that were analyzed the same day or in different days, respectively.

2.6. Calculation of population normalized daily load

Pharmaceutical concentrations (ng/L) measured in the 24 h composite IWW samples were multiplied by their corresponding wastewater flow rates (m^3/day) (Table S1) to calculate the daily mass loads (µg/day) Eq. (2). The loads were then normalized by dividing them by the

estimated population of the area served by each WWTP that sourced the samples Eq. (3) to end with the population normalized daily load (PNDL) (mg/day 1000/inhabitants).

$$Daily\ load\ (\mu g/day) = concentration\ (\mu g/L) \times wastewater\ daily\ flow - rate\ (L/day) \tag{2}$$

$$PNDL\ (mg/day\ 1000/inhabitants) = daily\ load \times 10^{-3} \times (1000/n^{\circ}\ inhabitants) \tag{3}$$

3. Results and discussion

3.1. LC-MS/MS optimization and validation

Initially, the triple quadrupole detection conditions were optimized by direct infusion of individual standard solutions at 1 mg/L in ultrapure water/MeOH (1/1, v/v) using this solvent containing 0.1% HCOOH as carrier at 0.4 mL/min. Table 1 summarizes the precursor ion and the most intense product ions for each compound obtained under the optimum cone voltage (CV) (tested from 50 to 200 V) and collision energies (CE) (from 0 to 60 eV). For tramadol, only one product ion could be recorded due to its poor fragmentation. The ions selected for the target compounds have been already described elsewhere [30,33,34].The ESI conditions were adopted as a compromise (details in section 2.4) to get the highest analytical signal when a mixture of all compounds at 1 mg/L was infused. All compounds yielded more intense signals in ESI$^+$ using the protonated molecule [M + H]$^+$ as a precursor ion, since all the studied compounds, except diclofenac, are basic compounds (see structure in Fig. S1). The dynamic MRM acquisition mode, which maximizes the dwell time for each transition, was employed for monitoring the two transitions per compound at the analyte retention time so that the S/N ratio and the reproducibility of the method is enhanced as compared to the MRM mode. In fact, dynamic MRM has been widely adopted in multiresidue analysis.

As for the LC optimization, Luna Omega Polar C18 (150 mm × 3 mm, 5 μm) column from Phenomenex was used with mobile phase combining aqueous solution and ACN or MeOH as organic solvent with or without the addition of 0.1% HCOOH. Luna Omega Polar column is suitable for the separation of highly polar compounds and enables enhanced retention when using the mobile phase gradient described in section 2.4 (starting isocratically at 5% of ACN for 3 min). Under these conditions, the first eluting compounds (morphine and atenolol) eluted after 6.5 min. The late eluting of the most polar compounds when working in reversed-phase LC is advisable to minimize matrix effects in the ESI source. For the mobile phase, a combination of water/ACN with the addition of 0.1% HCOOH under the gradient described in section 2.4 was selected since it provided better peak performance and S/N ratio of the studied analytes. Fig. S2 shows an extracted ion chromatogram using the optimized separation conditions, where it can be observed that all compounds were separated in <29 min. Moreover, it should be mentioned that the addition of an acidic modifier favors the ionization of the basic compounds.

3.2. Sample preparation

One of the aims of this work was to optimize a sample extraction procedure able to extract the maximum number of target compounds. For this, SPE using either Oasis HLB or Oasis MCX was considered. These types of sorbents (or Strata-X analogues) have been widely used in multi-residue methods encompassing similar analytes [21,33,35,36].

Different experimental variables were assayed to improve the

extraction recoveries when percolating 100 mL of ultrapure water spiked at 1000 ng/L with the mixture of the analytes across the SPE sorbents. Among them, the sample pH was tested at pH 3 and neutral conditions (without pH adjustment); for the washing step, 5 mL of water or 5 mL of MeOH were considered; and the elution was performed with 5 mL of MeOH (Oasis HLB) or 5 mL of 5% NH$_4$OH in MeOH (Oasis MCX) according to recommended protocols [37]. As for the elution optimization, increasing the elution volume did not yield any improvement; therefore, the initial elution conditions were fixed. As per the sample pH, pH 3 was required for Oasis MCX sorbent in order to foster cation-exchange interactions with the majority of compounds as most compounds are basic drugs (structure in Fig. S1). Samples adjusted at pH 3 or without pH adjustment did not entail any differences in recoveries for Oasis HLB; thus, natural pH was selected in order to minimize sample manipulation. The volume of sample was fixed at 100 mL in all cases, which is the maximum volume of IWW usually percolated with 150 mg/ 6cc cartridges. As for the washing step, MeOH-based clean-up was only applied to mixed-mode cation-exchange sorbent (Oasis MCX) and 5 mL of water were applied to Oasis HLB. With these washing protocols, no substantial losses (up to 10%) of the analytes (evaluated as percentage of analyte lost in the washing step) were encountered during the washing step in any of the cartridges.

Fig. 1 shows the recoveries obtained in ultrapure water under the optimum conditions for the sorbents evaluated. The recoveries obtained for Oasis HLB were very similar to those obtained with Oasis MCX for most compounds, with the exception of omeprazole, losartan and methadone which yielded better recoveries with Oasis HLB.

Next, %R$_{app}$ and matrix effects were evaluated with Oasis HLB and Oasis MCX when 100 mL of IWW samples spiked at 1000 ng/L with the analyte mixture were percolated through both sorbents. A washing step consisting of 5 mL of water (Oasis HLB) or 5 mL of MeOH (Oasis MCX) was applied. Finally, the analytes were eluted with 5 mL of MeOH (Oasis HLB) or 5 mL of 5% NH$_4$OH in MeOH (Oasis MCX). To account for the native presence of these compounds in the analyzed IWW, signals obtained in non-spiked samples were subtracted from signals found in spiked samples. Although the %R$_{app}$ and matrix effect evaluation was planned at two concentration levels, finally this was not possible since the native concentration in IWW was higher than the lowest addition level. Table 2 shows the results for both sorbents. As expected, recovery values in complex samples were lower than those in ultrapure water. The %R$_{app}$ obtained for Oasis HLB were higher than those of Oasis MCX since some of the compounds (viz., pentoxifylline, quetiapine, omeprazole, carbamazepine and diclofenac) were lost during the washing step applied in Oasis MCX resulting in poor recoveries (from 2% to 18%). In fact, some of these lost compounds bear acidic or neutral properties (Fig. S1), therefore they were eluted with MeOH. In addition, %R$_{app}$ of Oasis MCX ranged from 15 to 80 % for the rest of compounds, whereas the %R$_{app}$ for Oasis HLB ranged from 39 to 139 %.

Regarding matrix effect, the experimental values obtained using both sorbents are acceptable taking into account the high complexity of the samples with values in general ±30% for most of the compounds. In detail, the matrix effect encountered for Oasis MCX when applying a washing step was slightly lower than the one yielded by Oasis HLB because of the less effective washing step (5 mL of water). For instance, the matrix effect of oxazepam is 33% with Oasis HLB and negligible with Oasis MCX; however, this compound is only 28% recovered with Oasis MCX, whereas the %R$_{app}$ is 117% with Oasis HLB. When the same experiments were conducted with Oasis MCX without applying the washing step, the %R$_{app}$ only slightly improved and the % matrix effect
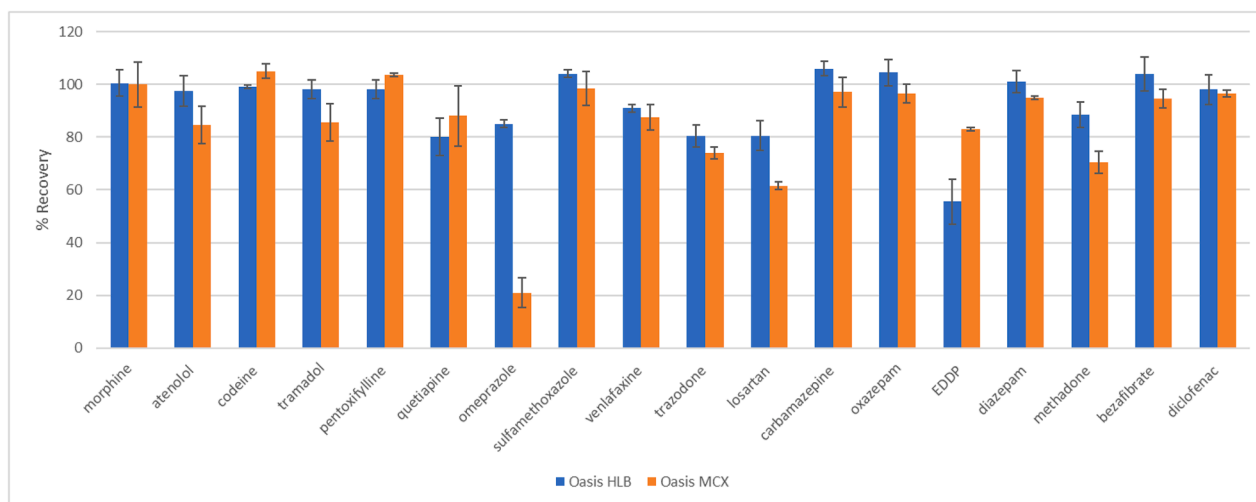
Fig. 1. % Recoveries obtained in ultrapure water using the selected experimental SPE conditions for each sorbent assayed.

Table 2
Percentage of apparent recovery (% R_{app}) and percentage of matrix effect (%ME) of the studied compounds in IWW samples using Oasis HLB and Oasis MCX as SPE sorbents.

	Oasis HLB		Oasis MCX	
	% R_{app}	%ME	%R_{app}	%ME
Morphine	87	−3	62	−31
Atenolol	87	−14	63	−15
Codeine	57	−26	61	−27
Tramadol	46	−39	58	−51
Pentoxifylline	122	29	5	12
Quetiapine	39	−36	2	−21
Omeprazole	42	−43	2	−19
Sulfamethoxazole	62	5	15	12
Venlafaxine	43	−42	58	−31
Trazodone	54	−26	28	−25
Losartan	101	5	68	12
Carbamazepine	97	6	18	−15
Oxazepam	117	33	28	0
Diazepam	68	−19	80	−1
Methadone	100	26	72	17
Bezafibrate	126	26	77	−19
Diclofenac	139	38	4	−4

% RSD (n = 3) < 7%.

was significantly higher (values between −50% to +42%).

For all of this, Oasis HLB was selected because (i) it provides the best %R_{app} in IWW that ranged from 39% to 139 % for compounds with a wide range of physicochemical properties, and (ii) it uses a simple and generic protocol that does not require the sample pH adjustment. The development of a generic sample treatment protocol is of great interest in laboratories dealing with numerous pollutants, because the same SPE extracts might be injected in another target or even non-target analysis protocol. A similar conclusion was reached out by previous researchers [8,38] that selected Oasis HLB over Oasis MCX.

3.3. Method validation

Instrumental linearity was investigated using an eight-point calibration curve ranging from individual IQLs to 1000 μg/L, except for omeprazole, venlafaxine and diazepam whose upper concentration was set at 500 μg/L and codeine that was 250 μg/L. The linearity was good with $R^2 > 0.99$ for all compounds. IQLs ranged from 0.1 μg/L to 1 μg/L, except for atenolol (5 μg/L) and diclofenac (2 μg/L), whereas the IDLs were between 0.02 and 0.5 μg/L (except atenolol 2 μg/L). Details on validation parameters are compiled in Table S2.

The %R_{app} values (Table 2) ranged from 39% for quetiapine to 139% for diclofenac. These values are in the line with %R_{app} obtained using similar analytical approaches [9,21,33,38]. For instance, sulfamethoxazole attained similar recoveries in IWW (65% [9,21] compared to 62% in the present study) when this compound was quantified using an isotopically labelled internal standard analogue such as carbamazepine-d10 [9] or sulfamethoxazole-d4 [21]. Nonetheless, in another study [33] the recovery for the same compound decreased to 32% when it was also quantified by the internal standard method. Another compound that presented diversity of recoveries in the literature is losartan that was quantitatively recovered (%R_{app} 101%) in the present study. However, in previous studies, reported recoveries of losartan ranged from 40% [35] to 214% [9], in spite of using internal standard analogue approaches.

As for the matrix effect (values in Table 2), some of the compounds suffered ion suppression with values ranging from −3 to −43, whereas some others were affected with ion enhancement (5 to 38%). These values are again in the line with other studies in which similar compounds and samples were analyzed [30,35]. As an example, codeine matrix effect was −26% in our study, vs −20% in the study reported by Carmona et al [30]. In general, both %R_{app} and % matrix effect were acceptable and comparable to those obtained in studies that use similar conditions. In addition, the %R_{app} and % matrix effect among the different IWW analyzed is similar.

As all the compounds were present in IWW, matrix-matched calibration curves were not used. Instead, external calibration curves applying the %R_{app} and SPE concentration factor were adopted for quantification. MDLs and MQLs were at the low ng/L for all compounds (Table S2), in detail, MQLs were between 1 and 24 ng/L for all compounds, except atenolol (58 ng/L) due to its higher IQL; and MDLs ranged from 0.2 ng/L to 12 ng/L with the exception of atenolol (23 ng/L). These limits are in line or even lower than other similar studies where some of these compounds were determined in IWW samples [21,38,39]. For instance, recently, Halwatura et al [39] reported limits between 0.25 ng/L to 50 ng/L when a group of 60 compounds were determined from IWW using SPE followed by LC-Q-Orbitrap. In detail, the MQLs for sulfamethoxazole and venlafaxine were 18 ng/L and 9.8 ng/L respectively, which is similar or a bit larger to the ones of the present study (16 ng/L and 2 ng/L, respectively).

The precision in form of %RSD (n = 5) at the 1000 ng/L level in terms of repeatability (intra-day) and reproducibility (inter-day) ranged from 4.3% to 6.7% and 6.1% to 14.1%, respectively (see Table S2).

Table 3

Concentration range and mean concentration in ng/L of the studied analytes in the IWW of the different WWTPs.

	Concentration in ng/L											
	Madrid 1		Madrid 2		Tarragona		Reus		Palma de Mallorca		Las Palmas de Gran Canaria	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Morphine	133–148	143	75–168	134	159–220	192	62–100	79	505–635	563	235–513	405
Atenolol	1477–1587	1540	1024–1915	1582	1504–1702	1585	1415–2026	1646	1113–1454	1253	688–1224	1015
Codeine	327–348	344	249–374	329	155–193	176	174–292	241	399–485	435	195–372	309
Tramadol	2029–2444	2188	2402–3535	3137	3024–3877	3444	1582–3843	3032	8039–10393	9050	4147–5975	5280
Pentoxifylline	41–46	43	33–49	42	48–70	56	167–311	252	279–359	318	222–317	284
Quetiapine	337–357	349	127–256	195	91–514	323	122–427	313	341–378	359	243–266	252
Omeprazole	40–48	45	68–108	92	<MQL-974	582	5623–8491	6801	2114–2484	2349	1561–2013	1779
Sulfamethoxazole	1958–1973	1964	1171–2854	2087	586–726	663	1322–2029	1784	945–1162	1036	1121–1667	1445
Venlafaxine	1094–1215	1141	607–908	795	1225–1242	1231	642–1007	847	1129–1362	1226	481–754	659
Trazodone	335–383	360	31–133	88	52–168	99	65–137	89	137–196	170	179–427	327
Losartan	960–978	970	721–1464	1164	1025–1211	1131	923–1393	1106	1389–1806	1571	392–1072	730
Carbamazepine	79–82	81	45–72	63	76–93	87	78–384	190	99–146	117	51–145	104
Oxazepam	159–172	164	85–152	122	153–189	172	208–378	293	340–401	368	375–555	478
Diazepam	19–42	27	17–44	26	19–33	24	9–35	22	<MQL	<MQL	23–35	28
Methadone	13–33	20	23–37	30	23–63	45	30–37	34	<MQL	<MQL	43–61	55
Bezafibrate	99–108	103	85–160	133	78–97	86	10–60	29	334–462	390	239–523	380
Diclofenac	786–804	797	537–1042	801	713–859	813	5043–7203	6367	854–1094	963	876–1666	1345

3.4. Occurrence of studied compounds in influent wastewater

Table 3 shows the concentration ranges of the target compounds in the different WWTPs considered in this study. All the analytes were quantified above MQLs in all the samples analyzed, with the exception of omeprazole, diazepam and methadone that were found at levels below their MQLs in some samples or in all instances (diazepam and methadone in samples from Palma de Mallorca WWTP). The compounds quantified at the highest concentrations in all WWTPs were atenolol (1015–1646 ng/L), tramadol (2188–9050 ng/L), sulfamethoxazole (663–1964 ng/L) and diclofenac (797–6367 ng/L). Thus, the higher MQLs encountered for atenolol and diclofenac were not an issue, because the levels found in samples largely exceeded MQLs. Similar levels of concentration in IWWs for atenolol and diclofenac were previously reported [21,30]. For instance, atenolol and diclofenac were found at levels up to 2280 and 3910 ng/L, respectively, in a IWW sample from a WWTP in the Canary Islands [29]. Diclofenac, tramadol and losartan were reported among the pharmaceuticals occurring at the highest levels in effluent wastewater samples from ten WWTP located in NE coastal villages in Spain [31]. In fact, diclofenac concentration (6367 ng/L) in Reus WWTP was higher than the values found in the remaining WWTPs (797–1345 ng/L). The same holds true for omeprazole values (5623–8491 ng/L) in Reus WWTP that were higher than those found in the other WWTP considered. Nevertheless, except for those compounds, the concentrations of the rest of pharmaceuticals are very much alike in all WWTPs.

No trends were observed for the inter-day concentration variation in each WWTP, with the exception of codeine, methadone and tramadol that showed an increased concentration during Saturday-Monday period attributed to an increased drug consumption. This finding is in good agreement with similar studies worldwide [18,38,40].

3.5. Normalized mass loads of drugs

Population normalized daily load (PNDL) (calculated with the data in Table S1) for the studied compounds is presented in Table 4. Atenolol, tramadol, sulfamethoxazole, venlafaxine, losartan and diclofenac showed the highest PNDLs in all locations, which is in line with the prescription data across Spain [6,7,41,42]. For instance, atenolol and losartan are among the most administrated drugs to deal with cardiovascular diseases in Catalonia, Spain [41].

All pharmaceuticals monitored followed a similar PNDL pattern across locations. It should be highlighted the case of omeprazole (already discussed in section 3.4), whose values are very disparate. The

Table 4

Population normalized daily load for the studied compounds per WWTP (mg/day 1000/inhabitants).

	Population normalized daily load (mg/day·1000/inhabitants)											
	Madrid 1		Madrid 2		Tarragona		Reus		Palma de Mallorca		Las Palmas de Gran Canaria	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Morphine	28–31	30	7–15	12	34–47	41	11–17	13	70–88	80	19–42	33
Atenolol	312–335	325	93–174	144	319–361	340	240–315	279	154–201	179	55–99	83
Codeine	69–75	73	23–34	30	33–42	38	33–45	40	55–67	62	16–30	25
Tramadol	428–516	462	219–322	285	641–822	739	297–624	506	1115–1435	1290	329–485	429
Pentoxifylline	9–10	9	3–4	4	10–15	12	31–48	42	39–50	45	18–26	23
Quetiapine	71–75	74	12–23	18	19–109	69	23–66	52	47–57	35	19–22	20
Omeprazole	8–10	10	6–10	8	<MQL-206	123	955–1596	1176	320–344	334	124–163	144
Sulfamethoxazole	413–416	415	107–260	190	124–154	142	248–345	301	131–160	148	89–138	117
Venlafaxine	231–256	241	55–83	72	260–273	264	121–156	143	157–188	175	38–62	54
Trazodone	71–81	76	3–12	8	11–36	21	10–23	15	21–27	24	14–35	27
Losartan	203–206	205	66–133	106	218–266	243	170–216	187	193–249	224	31–89	60
Carbamazepine	17–17	17	4–7	6	16–20	19	15–60	31	14–20	17	4–12	8
Oxazepam	33–36	35	8–14	11	34–40	37	39–59	49	47–55	53	30–45	39
Diazepam	4–9	6	2–4	2	4–7	5	2–5	4	<MQL	<MQL	2–3	2
Methadone	3–7	4	2–3	3	5–13	10	5–7	6	<MQL	<MQL	3–5	4
Bezafibrate	21–23	22	8–15	12	17–20	18	2–9	5	46–64	56	19–42	31
Diclofenac	166–170	168	49–95	73	151–189	174	948–1165	1077	118–151	137	69–135	109

values in the two WWTP located in Madrid were 6–10 mg/day 1000/inhabitants; whereas higher values were obtained for Palma de Mallorca (320–344) and Las Palmas de Gran Canaria (124–163) and unusual high values were found in Reus (955–1596). The low values reported in Madrid were expected even when omeprazole is a highly prescribed drug because it is almost metabolized in urine (77%) [43], so its occurrence in sewage samples is scarce, and only in some instances omeprazole is found in sewage samples [43,44], attributed to the direct disposal from household waste. In addition, as seen in previous section, abnormally high PNDL values of diclofenac are reported in Reus WWTP (1077 mg/day 1000/inhabitants) compared to the remaining WWTPs (73–174 mg/day·1000/inhabitants).

Comparing across locations in an attempt to cover different lifestyles depending on the city, the PNDLs in all WWTPs were found to be rather similar, and thus, it is difficult to attribute a pharmaceutical release pattern as a function of the type of city or its lifestyle. This is not surprising taking into account that pharmaceuticals are regularly prescribed with similar doses and rates along the different cities in Spain. In a recent study [28] performed in 10 different cities in UK, the authors also observed a constant PNDL across cities (or WWTP) for the different pharmaceuticals monitored that included non-steroidal inflammatory drugs and cardiovascular drugs. The only exception was the high PNDL level of ibuprofen that was attributed to direct disposal into the sanitation system.

In the case of those pharmaceuticals that are also used as drugs of abuse, such as opioids including morphine, codeine, methadone or tramadol, the PNLD is expected to differ across cities as the consumption of some drugs of abuse has been attributed to different urban lifestyles [38]. This trend can be observed for tramadol (Table 4) that shows different PNDL values depending on the WWTP. The rest of opioids studied presented similar values regardless of the location. A similar conclusion was drawn in a study [34] that compared PNDL of different opioids in six different WWTPs in Belgium. Apart from this observation, the authors claimed that the monitoring was only performed for one week and consumption rates might be different to the rest of the year. This assumption also holds true for the present study.

In general, the data on PNDLs found here is comparable with other WBE studies in literature [7,40,45,46]. For instance, similar patterns were observed for atenolol, losartan, carbamazepine and venlafaxine in Belgium [47]; atenolol and carbamazepine in Australia [45]; or tramadol, venlafaxine or diazepam in UK [46]. In this sense, in a study [46] performed in London in which >60 illicit drugs and pharmaceuticals were monitored, the authors found similar profiles for diazepam and oxazepam (two antidepressant drugs) than those found in the present study. They found levels below or near MQLs for diazepam, whereas the levels of oxazepam ranged from 23 to 30 mg/day 1000/inhabitants (except in the case of one WWTP that was from 8 to 14 mg/day 1000/inhabitants). The PNDL values in the present study for oxazepam are between 33 and 55 mg/day 1000/inhabitants, while diazepam levels varied from < MQL to 9 mg/day 1000/inhabitants. It should be borne in mind that oxazepam is one of the most important diazepam metabolites as well as a prescribed antidepressant.

4. Conclusions

A multiresidue method based on SPE followed by LC-MS/MS was successfully developed for the determination of a group of relevant pharmaceuticals in IWW. From the two different sorbents compared (Oasis HLB and Oasis MCX), Oasis HLB was selected and all compounds showed suitable apparent recoveries and low matrix effect, which proved the method to be selective.

The method was successfully validated and applied to the analysis of 42 IWW samples from six different WWTP in Spain that revealed the occurrence of all studied pharmaceuticals at ng/L or μg/L concentration levels.

The calculation of the population normalized daily load of each pharmaceutical in each WWTP provided a comparative dataset for pharmaceutical use patterns across different cities in Spain.

CRediT authorship contribution statement

Núria Fontanals: Investigation, Validation, Writing – original draft. **Eva Pocurull:** Investigation, Writing – original draft, Supervision. **Rosa Montes:** Conceptualization, Methodology, Writing – review & editing. **Iria González-Mariño:** Conceptualization, Methodology, Writing – review & editing. **Sergio Santana-Viera:** Conceptualization, Methodology. **Manuel Miró:** Writing – review & editing, Funding acquisition. **Andreu Rico:** Conceptualization, Methodology. **Sara Rodríguez-Mozaz:** Conceptualization, Methodology, Writing – review & editing. **Francesc Borrull:** Methodology, Supervision, Funding acquisition. **José Benito Quintana:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Rosa Maria Marcé:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109131.

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