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Research article

High resolution mass spectrometry (HRMS) determination of drugs in wastewater and wastewater based epidemiology in Cadiz Bay (Spain)

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ABSTRACT

Multi-residue methods for the determination of the myriad of compounds of emerging concern (CECs) entering in the environment are key elements for further assessment on their distribution and fate. Here, we have developed an analytical protocol for the simultaneous analysis of 195 prescription, over-the-counter, and illicit drugs by using a combination of solid phase extraction (SPE) and determination by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). The method was applied to the analysis of influent sewage samples from 3 wastewater treatment plants (WWTPs) from Cadiz Bay (SW Spain), enabling the quantification of more than 100 pharmaceuticals, 19 of them at average concentrations higher than 1 μg L^{-1}, including caffeine (92 μg L^{-1}), paracetamol (72 μg L^{-1}), and ibuprofen (56 μg L^{-1}), as well as several illicit drugs (e.g., cocaine). Wastewater based epidemiology (WBE) was applied for 27 of the detected compounds to establish their consumption in the sampling area, which has been never attempted before. Caffeine, naproxen, and salicylic acid stood out because of their high consumption (638, 51, and 20 g $d^{-1}{\cdot}1000pop^{-1}$, respectively). Regarding illicit drugs, cocaine showed the highest frequency of detection and we estimated an average consumption of 3683 mg $d^{-1}{\cdot}1000pop^{-1}$ in Cadiz Bay. The combination of new HRMS methods, capable of discriminating thousands of chemicals, and WBE will allow for a more comprehensive characterization of chemical substances and their consumption in urban environments in the near future.

1. Introduction

Modern societies consume a wide variety of chemical products that reach wastewater on a daily basis. Among these substances are pharmaceuticals, illicit drugs, and food additives, among others, many of them contaminants of emerging concern (CECs) since they can potentially cause toxic effects in the receiving waters. The development of multi-residue methods plays a key role to improve our knowledge on the occurrence and concentrations of CECs in wastewater and impacted aquatic system. Application of such analytical methodologies is desirable for evaluation of different classes of chemicals in monitoring programs, early-warning systems and/or spatio-temporal variations (Boogaerts et al., 2021a; Mao et al., 2020; Senta et al., 2020).

For several decades now, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been the workhorse for the determination of different numbers of CECs at trace levels in the environment (Huizer et al., 2021). Triple quadrupole instruments offer enough sensitivity and selectivity to carry out this task in complicated matrices such as wastewater (Bade et al., 2017; Borden et al., 2020; Fontanals et al., 2017; Mastroianni et al., 2014). Previous isolation and preconcentration of analytes by solid phase extraction (SPE) or other extraction techniques is often performed to achieve lower detection limits and remove potential interferences (Causanilles et al., 2017b; Fontanals et al., 2017; González-Mariño et al., 2018; Mastroianni et al., 2017). New generation high-resolution mass spectrometry (HRMS) instruments that are more affordable as well as have higher sensitivity and mass resolution, allow for simultaneous screening of known and unknown CECs. Identification of such contaminants is also performed with high confidence as elemental composition can be determined and co-eluting isobaric compounds can be distinguished due to greater mass accuracy measurement (Lucci et al., 2017). The recent implementation of HRMS instruments in monitoring programs running simultaneously in full-scan and MS/MS modes allows for the unequivocal identification of hundreds or even thousands of target, suspect, and even non-target emerging contaminants (Arsand et al., 2018; Biswas et al., 2022; Chau et al., 2017; Gago-Ferrero et al., 2020), going beyond the limits of triple quadrupole instruments and enabling identification of new illicit drugs, pharmaceuticals, transformation and other CECs entering the water

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cycle (Huizer et al., 2021). This is critical as many PhACs are poorly removed (<60%) by conventional WWTPs (Baker and Kasprzyk-Hordern, 2013) and, hence, may cause significant adverse effects on receiving aquatic ecosystems (Evgenidou et al., 2015). Sewage-impacted surface waters are occasionally used for production of tap water, where some pharmaceuticals have been already detected (Rosa Boleda et al., 2011). Transfer of PhACs to soils and crops is also documented by application of WWTP sludge in agricultural fields (Evans et al., 2015).

Substances that are detected in urban sewage are mainly derived from different anthropogenic activities occurring in cities, including consumption of drinks, food, medicines, and illicit drugs, and later excretion by their inhabitants. Wastewater based epidemiology (WBE) is based on the premise that traces of these substances, whether in its original form or as some transformation product or metabolite after excretion, can be analysed and their concentrations used to estimate their consumption through back-calculation (Daughton, 2001). WBE has been mainly applied to determine consumption of illicit drugs or drugs of potential abuse (Bijlsma et al., 2021; Boogaerts et al., 2021b; Brandeburová et al., 2020; Croft et al., 2020; Hue et al., 2022; Jin et al., 2021; Liu et al., 2021; Rice et al., 2020), alcohol (Boogaerts et al., 2021b; Pocurull et al., 2020; Zheng et al., 2020), and tabaco (Boogaerts et al., 2021b; Gao et al., 2020; Pocurull et al., 2020) in different countries within the last decade. The consumption of illicit drugs have health consequences and may be also related to criminal activities (Hahn et al., 2021). WBE can, therefore, complement other strategies based on criminological or sociological indicators (Ort et al., 2014; Thomas et al., 2012). Because it is a non-invasive and cost-effective technique that can provide results almost in real-time, its application has grown rapidly over the last decade. Among them, the SCORE program of the European Union stands out for having been active for more than 10 years and analysing more than 100 cities (SCORE, n.d.). Other than for calculation of consumption of illicit drugs by populations served by wastewater treatment plants (WWTPs), WBE have been also used in more specific and/or limited contexts, such as festivals, vacations (Benaglia et al., 2020; Causanilles et al., 2017a; Centazzo et al., 2019; Foppe et al., 2018; Mackuľak et al., 2019) and sport events (Lemas et al., 2021; Montgomery et al., 2021).

More recently, WBE has been applied to estimate the consumption of a wide range of pharmaceutically active compounds (PhACs) (Escolà Casas et al., 2021), including antibiotics (Gao et al., 2022; Han et al., 2022; Zhang et al., 2019), antivirals (Yao et al., 2021), and drugs to treat erectile dysfunction (Shao et al., 2022). Although sales and production volumes of many PhACs can be known (e.g., drugs that are only dispensed under medical prescription), these data are often limited to consumption and, hence, patient compliance. By measuring the concentration of the analytes in urban sewage, WBE achieves to get a better understanding about the consumption habits and trends in different scenarios (Gent and Paul, 2021). Beyond drugs, the application of WBE is expanding towards other chemicals, including: mycotoxins (Berzina et al., 2022), pesticides (Rousis et al., 2020, 2021), bisphenol A and its analogues (Lopardo et al., 2019; Wang et al., 2020), phthalates (González-Mariño et al., 2017), artificial sweeteners (Li et al., 2020, 2021), and different pollutant mixtures (González-Mariño et al., 2017; Rousis et al., 2017). Exposure to pathogens and therefore prevalence of diseases such as COVID-19 can be also monitored following WBE principles (Johnson et al., 2022; Vitale et al., 2021).

In this work, our main objective was to develop a method for the simultaneous extraction and analysis of a wide range of prescription, over-the-counter, and illicit drugs in wastewater, and to apply it to calculate their loads and consumption in Cadiz Bay (SW Spain). Therefore, we have analysed 195 drugs in influent wastewater from three different WWTPs, covering a population of approximately 500 000 inhabitants. Such comprehensive characterization of drugs in sewage samples and the estimation of their potential load and consumption has been rarely attempted before and it has been possible by developing a new multi-residue method based on the combination of offline SPE followed by LC-HRMS analysis.

2. Experimental section

2.1. Material and reagents

Methanol (MeOH) and HPLC water were purchased from Scharlau (Barcelona, Spain); formic acid (99%), ammonium acetate (97%), ammonia (25%), and ammonium formate (97.8%) used as modifiers in mobile phases, were purchased either from Sigma Aldrich (Madrid, Spain) or Panreac (Barcelona, Spain). Analytical standards and internal standards (I.S.) (Table S1) were purchased from Merck (Darmstadt, Germany) and LCG standards. SPE cartridges (6 cc., 200 mg) were purchased from Waters (Milford, United States of America).

2.2. Sampling

Influent composite 24 h samples were taken daily during 1 week in June 2021 from 3 different WWTPs in Cadiz Bay serving different populations (number of inhabitants are taken from the Institute of Statistics and Cartography of Andalusia in year 2021): Jerez de la Frontera (JF, 212801 inhabitants) (Long = −6.12668750°, Lat = 36.64506250°), Cadiz and San Fernando (CA, 209111 inhabitants) (Long = −6.23906250°, Lat = 36.46093750°), and Puerto Real (PR, 41171 inhabitants) (Long = −6.23556250°, Lat = 36.52493750°). Aliquots (500 mL) of each 24 h composite sample were transferred polyethylene bottles and frozen at −20 °C until analysis. Relevant information on the samples is provided in Table S2, including Biological Demand of Oxygen (BDO_5), water flow, equivalent inhabitants, and pH.

2.3. Extraction and determination of target compounds

Optimisation of the SPE was based on a previous work by our group (María Baena-Nogueras et al., 2015). Briefly, 100 mL aliquots of HPLC water at either neutral or acid pH (pH = 3) were spiked with 1 μg L^{-1} of a stock solution containing >350 analytes (Table S1). These aliquots were extracted using 4 different cartridges (Oasis HLB, Oasis Prime, Oasis MCX, and Bond Elut C18), which were previously conditioned with 5 mL of MeOH and 5 mL of HPLC water. Target compounds were eluted with 10 mL of MeOH (except for MCX cartridges, which were eluted with 5 mL of MeOH + 5 mL of MeOH with 5%NH_4OH) after a cleaning step using 5 mL of HPLC water. The eluate was dried using a gentle stream of nitrogen in a thermostatic bath and reconstituted in 1 mL of MeOH:H_2O 50:50 (v/v). Before analysis, samples were filtered through 0.22 μm using PTFE filters. The same optimisation procedure was repeated again using 100 mL aliquots of real wastewater spiked at the same concentration level than HPLC water. Blank concentrations were subtracted in both experiments in order to calculate SPE recoveries. Wastewater samples collected from the 3 different WWTPs over 1-week period were extracted following the SPE protocol once optimised.

SPE extracts were injected on a Bruker Impact II q-ToF-MS equipped with an electrospray ionisation source (ESI) and coupled to an Elute UPLC module with autosampler. Samples were analysed separately in negative and positive ionisation modes. Target compounds were separated using an Acquity UPLC BEH C18 Column (1.7 μm particle size, 2.1 mm × 150 mm) from Waters. Mobile phase in negative ionisation mode consisted in two solvents (A = H_2O and B = MeOH) containing 5 mM ammonia and 5 mM ammonium acetate. Mobile phase in positive ionisation mode was similar but adding 0.1% formic acid and 5 mM ammonium formate instead. The same gradient was used in both ionisation modes, starting with 95% A and moving to 0% A during 9 min. These conditions (0% A) remained for 3 additional minutes and then changed to initial conditions in 0.5 min (95%A). Re-equilibration time was 1 min before the next injection.

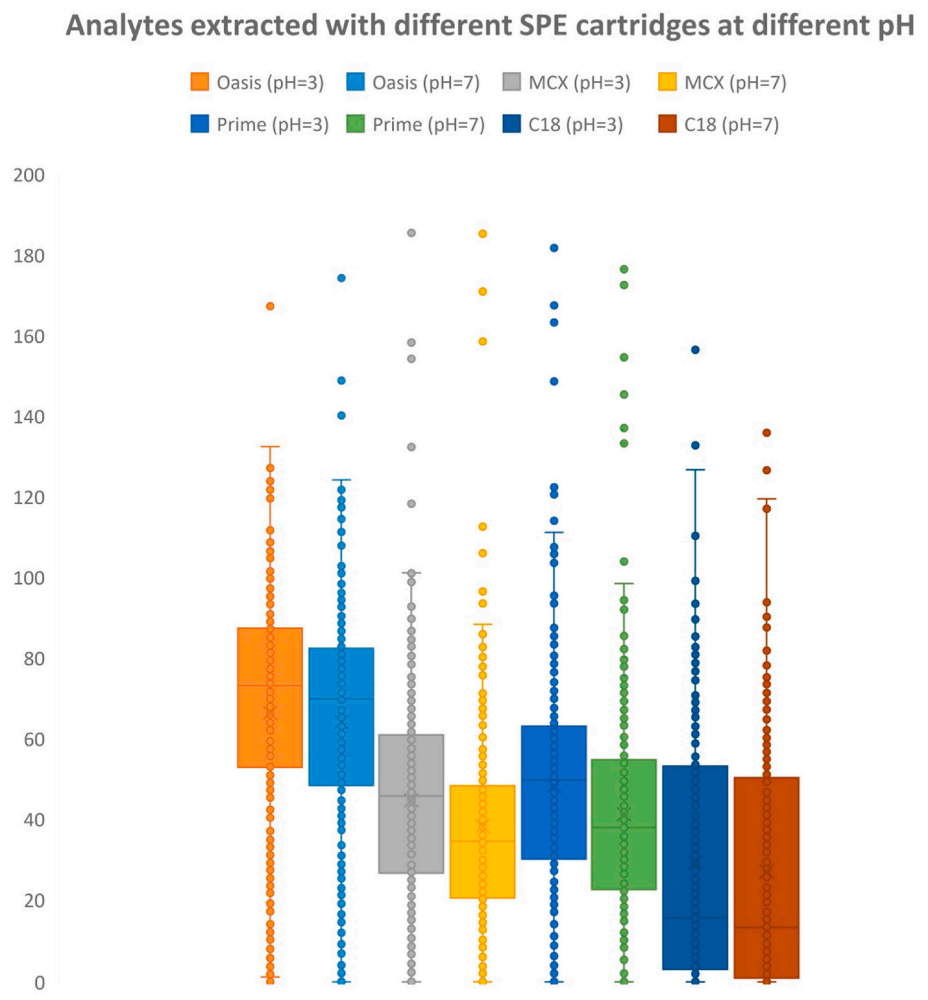

Fig. 1. Number of analytes extracted with different SPE cartridges and recovery efficiencies.

Determination of target compounds was done using TASQ 1.4 software of Bruker. Identification of target compounds was achieved by considering their retention time (RT) and exact mass of the molecular ion. Quantification was performed using a calibration curve (from 0.1 μg L^{-1} to 100 μg L^{-1}). Method quantification limits (MQL) (Table S3) were calculated as half of the concentration of the lowest point of the calibration curve in which an analyte was detected divided by the preconcentration factor achieved after SPE. Matrix effect (M.E.) was calculated using I.S. (Table S4). All wastewater samples were spiked after SPE to a concentration of 50 μg L^{-1} using an I.S. mixture, as well as the calibration curve. M.E. was measured as the relationship of the signal in the calibration curve and the signal in the wastewater samples. A positive value of M.E. indicates the percentage of reduction of the MS signal; on the contrary, a negative value of M.E. indicates an increase of the signal.

2.4. Calculations

Table S5 contains the different formulae applied to calculate drug consumption according to different authors and target compounds. For a more accurate calculation, instead of using the official census data (section 2.2), populations were calculated through BDO_5 using the following formula:

$$Population = \frac{F\left(\frac{m^3}{d}\right) * BDO_5\left(\frac{mg}{L}\right)}{60\left(\frac{g}{\frac{pop}{d}}\right)} \quad (1)$$

where F is the daily flow ($m^3 \cdot d^{-1}$) entering the WWTP, BDO_5 is the biological demand of oxygen in 5 days and pop is the population.

The wastewater flows (F) treated by each of the WWTPs during the sampling week were: 41986 ± 11 m^3 d^{-1} (JF), 32048 ± 13 m^3 d^{-1} (CA), and 6759 ± 12 m^3 d^{-1} (PR). Equivalent inhabitants according to the formula presented above were: 297240 ± 29 (JF), 188711 ± 45 (CA), and 32037 ± 25 (PR).

In order to perform the WBE, it is necessary to establish first the loads of the analytes into the WWTP. Loads were calculated by the following equation:

$$Load\left(\frac{\frac{mg}{d}}{1000pop}\right) = \frac{C\left(\frac{ng}{L}\right) * F\left(\frac{m^3}{d}\right)}{Population} \quad (2)$$

where C is the concentration measured for every analyte and F is the daily flow ($m^3 \cdot d^{-1}$) entering the WWTP.

Then, in order to calculate the consumption, the load of the WWTP for each analyte was multiplied by different factors (% excretion, % of stability, molecular weight relationship between the parent compound and the metabolite, etc.), or a correction factor that involves several of the previous mentioned parameters. Therefore, consumption was calculated according to the following equations:

$$Consumption\left(\frac{\frac{mg}{d}}{1000pop}\right) = \frac{C\left(\frac{ng}{L}\right) * F\left(\frac{m^3}{d}\right)}{Population} * Correction\ factor \quad (3)$$

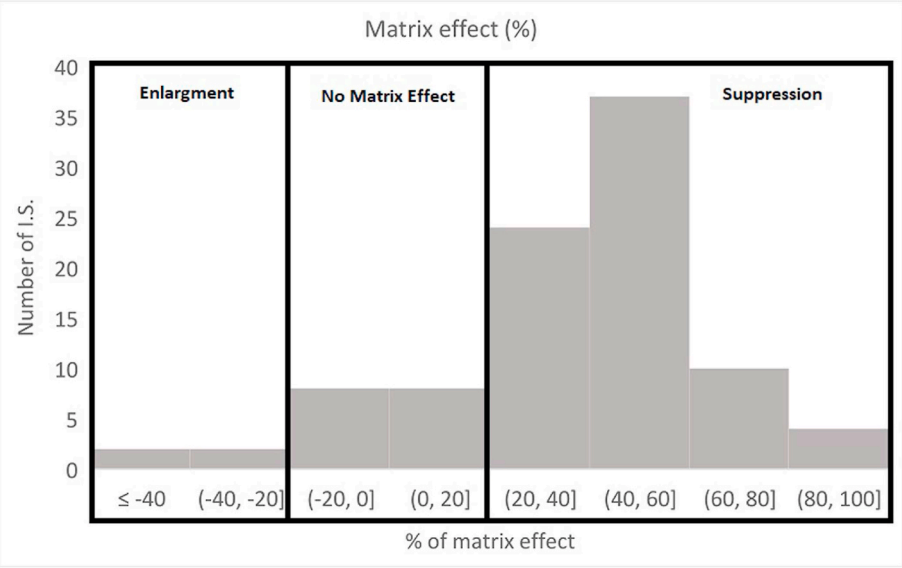

Fig. 2. Matrix effect (%) in wastewater samples determined by the use of I.S.

or:

$$Consumption\left(\frac{\frac{mg}{d}}{1000pop}\right)=\frac{C\left(\frac{ng}{L}\right)*F\left(\frac{m^3}{d}\right)}{Population}*\left(\frac{100}{Excretation}\right)*\left(\frac{MW_{par}}{MW_{met}}\right)*\left(\frac{100}{100+Stability}\right) \quad (4)$$

where C is the mean concentration for each analyte, F is the daily flow, pop is the population and MW is the molecular weight of the parent compound (par) or its metabolite (met).

Statistical analysis of the data was performed using Statgraphics 19. Analysis of the variance (ANOVA) followed by Tukey HSD multi-range test was used to discern whether there were or not differences among different samples sites (JF, CA, and PR) and times (weekdays vs. weekends) at p-value = 0.05.

3. Results and discussion

3.1. Determination of pharmaceuticals and illicit drugs

Fig. 1 shows extraction recovery percentages (n = 3) for all target compounds extracted from spiked HPLC water using four different SPE cartridges under neutral and acidic pH. Milli-Q water was spiked with more than 350 analytes (Table S1) at 1 μg L^{-1}, with 75 of them analysed under both positive and negative ionisation modes. Overall, the best results were obtained using Oasis HLB at pH = 3, extracting 57% of the analytes with recoveries ranging from 70 to 120% (235 analytes), so this setting was selected for following analysis. Under neutral conditions, the number of analytes extracted with the same cartridge under the same range of recovery (between 70 and 120%) decreased by 19%. The same pattern was observed for Oasis Prime HLB and MCX, for which 18% and 133% more drugs were efficiently extracted when decreasing pH from 7 to 3, respectively. On the opposite, C18 cartridges extracted 10% more analytes at pH = 7 than at pH = 3 (Fig. 1) within the optimal recovery interval (70–120%).

For some specific compound classes such as amphetamines and other drugs with high pK_a values, the best results were achieved using cationic exchange SPE cartridges (Oasis MCX) at pH = 3, including: cathine (pK_a = 9.19), methamphetamine (pK_a = 9.87) and amphetamine (pK_a = 9.9), ephedrine (pK_a = 9.65), epinephrine (pK_a = 8.69), phenylephrine (pK_a = 8.97), norephedrine (pK_a = 9.05), oseltamivir (pK_a = 7.7), hydromorphone (pK_a = 8.92), stiripentol, pregabalin (pK_a = 10.6), cimaterol, prenalterol, sotalol (pK_a = 9.76), and 1-benzylpiperazin. These compounds are positively charged at low pH, so they are easily extractable with MCX cartridges. Oasis HLB Prime, which is a new iteration of the more widely used Oasis HLB but aimed to remove more interferences (i.e, salts, proteins and lipids), did not provide better extraction recoveries than the latter, except for atenolol-desisopropyl, hydromorphone, oseltamivir, alfuzosin, 4-hydroxy-3-methoxymethamphetamine (HMMA), simvastatin, vildagliptin, trimetazidine, and sotalol. C18 cartridges, less suitable for retention of highly polar contaminants, offered the poorest efficiency for most analytes, except for canagliflozin (log K_{ow} = 4.05), furosemide (log K_{ow} = 2.03), metoprolol (log K_{ow} = 1.88), simvastatin (log K_{ow} = 4.68) and zoplicone.

On a second stage, real influent wastewater was acidified (pH = 3), spiked with target compounds at 1 μg L^{-1} and extracted (n = 5) using Oasis HLB cartridges. The number of analytes detected decreased due to reduction of their MS signal as a combination of matrix effect and/or competition of the organic matter in wastewater with the SPE sorbent active sites. To determine the effect of both, SPE recoveries were recalculated to account for losses due to matrix effect for those analytes for which we had I.S. (Table S1). After this, extraction recoveries increased and were similar to those previously calculated in HPLC water in most cases. For instance, atorvastatin had a recovery of 42% in spiked HPLC water and 31% in wastewater, however, after I.S. correction, its recovery increased to 40%. Overall, the matrix effect was determined to be an average reduction of 34% of the MS signal in influent wastewater (Fig. 2, Table S4).

Finally, those compounds with extraction recoveries lower than 30% and residual standard deviation (RSD) higher than 20%, were discarded from the method. Nevertheless, recoveries higher than 50% and lower than 120% were obtained for 195 compounds in the influent wastewater (68% of all the target compounds), which we considered good enough for the purpose of the work. MQL ranged from 0.4 to 142 ng L^{-1}, depending on the target compound, showing mean values of 8 ng L^{-1} and 27 ng L^{-1} in ESI+ and ESI-, respectively.

3.2. Occurrence of target compounds in wastewater: concentrations and loads

Table 1 shows the concentrations of target compounds detected in influent samples from the 3 WWTPs (JF, PR, and CA) monitored in Cadiz

Table 1
Frequency of detection, lowest, mean, and highest concentration of target compounds ($ng \cdot L^{-1}$) detected in the influent of the WWTPs.

Compound	Class	Lowest detected concentration	Mean concentration	Highest concentration	Frequency of detection (%)
4-acetamidoantipyrine (n-acetyl-4-aminoantipyrin)	Analgesics	8427	18707	27079	100
Flufenamic acid		265	536	737	95
Niflumic acid		9	12	14	14
Paracetamol		33563	72715	115493	95
Phenazone (antipyrine)		17	90	722	86
Salicylic acid		3985	16524	55086	71
Azithromycin	Antibiotics	1957	5321	8701	62
Etodolac		16	26	33	14
N4-acetylsulfapyridine		16	35	61	29
N-desmethyl clarithromycin		3	20	26	19
Rifaximin		94	262	774	67
Sulfamethoxazole		266	646	1036	90
Trimethoprim		124	173	289	67
Amitriptyline	Antidepressants	7	20	38	29
Bupropion		11	12	13	10
Hydroxy-bupropion		14	25	37	33
Maprotiline		21	29	37	48
Sertraline		11	12	12	10
Venlafaxine		43	253	511	90
Venlafaxine-N,N-didesmethyl		349	705	1185	90
Venlafaxine-N,O-didesmethyl		70	162	324	90
Venlafaxine-N-desmethyl		789	1394	2278	95
Venlafaxine-O-desmethyl		310	628	976	86
Carbamazepine	Antiepileptics	37	66	85	90
Carbamazepine-10,11-epoxide		11	45	70	76
Gabapentin		3314	7622	11706	86
Lamotrigin		165	230	347	86
Lidocaine		127	237	321	90
Phenytoin		180	267	387	86
Topiramate		258	660	1025	95
5-fluoro cystosine	Antifungals	175	368	531	86
Climbazole		43	103	203	90
Fluconazole		64	141	235	24
Ketoconazol		94	154	227	52
Doxylamine	Antihistamines	8	32	46	48
Fexofenadine		6	41	72	76
Pheniramine		19	22	25	10
Aliskiren	Antihypertensives	116	154	213	86
Candesartan		186	293	448	38
Chlorthalidone		64	176	311	48
Eprosartan		416	655	1101	86
Irbesartan		140	368	680	90
Losartan		280	1893	4359	62
Telmisartan		163	836	1704	90
Valsartan		2902	7694	12737	76
Verapamil		17	255	431	81
Amisulpride	Antipsychotics	41	117	204	90
Clozapine		24	219	356	67
Sulpiride		175	656	1100	90
Atenolol	Beta blockers	361	767	1320	67
Bisoprolol		3680	4463	5658	29
Metoprolol		10	16	27	81
Propranolol		6	18	33	29
Sotalol		23	40	47	19
R-deprenyl n-oxide	Illicit drugs	12	13	13	10
Cannabidiol (CBD)		50	166	224	14
Delta9-Tetrahydrocannabinol (THC)		40	144	298	24
Methadone		12	36	52	81
2C-E (2,5-Dimethoxy-4-ethylphenethylamine)		7887	18920	27541	100
Benzoylecgonine		2963	6465	11517	95
Cocaethylene		18	23	28	10
Cocaine		414	881	1645	90
Atorvastatin	Lipid regulators	167	332	494	86
Bezafibrate		24	57	107	90
Rosuvastatin		226	382	719	86
4′-Hydroxy diclofenac	Non-steroidal anti-inflammatories	171	368	555	67
Diclofenac	(NSAI)	420	702	970	76
Ibuprofen		21869	56763	104227	33
Ketoprofen		447	1086	1479	95
Meclofenamic acid		86	209	285	38
Naproxen		9814	19815	31340	100

(*continued on next page*)

Table 1 (*continued*)

Compound	Class	Lowest detected concentration	Mean concentration	Highest concentration	Frequency of detection (%)
2-ethylidene-1-5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	Opiates	5	37	68	71
Codeine		36	80	135	71
Hydrocodone		21	23	26	10
Morphine		14	39	81	29
N-bisdesmethyl tramadol		10	11	11	10
N-desmethyltramadol		60	244	495	81
Noroxycodone		24	39	54	10
Oxycodone		90	104	121	71
Tapentadol		804	1297	1923	100
Tramadol		533	907	1352	90
Prilocaine	Anesthetics	2	47	92	71
Flecainide	Antiarrhythmics	359	557	756	86
Cyclophosphamide	Anticancer drugs	3	5	7	33
Amantadine	Anticonvulsants	12	28	41	29
Oxcarbazepine		39	71	100	81
Canagliflozin	Antidiabetics	47	106	151	48
Metformin		32	290	1205	81
Lopinavir	Antiretrovirals	19	36	58	19
Ritonavir		16	28	39	10
Lorazepam	Anxiolytics	32	134	200	62
Diltiazem	Calcium channel blockers	18	31	39	43
Benproperine	Cough suppressants	12	24	31	48
Dextrorphan		5	26	43	43
Furosemide	Diuretics	303	708	985	90
Corticosterone	Glucocorticoids	751	1036	1321	10
Dopamine	Neurotransmitters	6775	10739	15673	90
Genistein	Phytoestrogens	148	336	595	57
Citalopram	Selective serotonin reuptake inhibitors (SSRI)	15	141	281	86
Progesterone	Steroids	90	120	143	29
Caffeine	Stimulants	48725	92779	154906	100

Bay during 1 week every day. From the 195 target compounds covered by the analytical method, more than 100 were detected at least once in untreated sewage samples and 64 drugs were detected in more than 50% of the samples (Fig. 3). Among the compounds with high frequency of detection are illicit drugs like cocaine and its metabolite benzoylecgonine, which were detected in more than 90% of the samples, and 2C-E (2,5-Dimethoxy-4-ethylphenethylamine), which was detected in all samples. Some opiates were also detected with high frequency such as tapentadol and tramadol. The stimulant caffeine, the non-steroidal anti-inflammatory naproxen or the metabolite from the non-steroidal anti-inflammatory metamizole (4-acetamidoantipyrin) were also detected in all samples. Other compounds that stood out due to their high frequency of detection are the antidepressant venlafaxine and its metabolites, and several antiepileptics and antihypertensives (Fig. 3).

Caffeine, the analgesics paracetamol and 4-acetamidoantipyrine, the non-steroidal anti-inflammatory naproxen, and the illicit drug 2C-E were detected at very high frequency and have the highest mean concentrations values (>18.500 ng L^{-1}) (Fig. 3, Table 1). This is not surprising since caffeine, ibuprofen, naproxen, and 4-acetamidoantipyrine are often among the target pharmaceuticals detected at the highest concentrations ($1 \times 10^5 - 1 \times 10^6$ ng L^{-1}) in developed countries due to their widespread consumption (Huidobro-López et al., 2022). In a previous work by our group performed in JE WWTP in 2014 using a more limited analytical method, concentrations of diclofenac (0.7 μg L^{-1}), salicylic acid (16.9 μg L^{-1}), sulfamethoxazole (0.6 μg L^{-1}), and trimethoprim (0.3 μg L^{-1}) (Biel-Maeso et al., 2018) were very similar to those reported here (0.7 μg L^{-1}, 16.5 μg L^{-1}, 0.7 μg L^{-1} and 0.2 μg L^{-1}, respectively). Other analytes such caffeine (54.7 μg L^{-1}), ibuprofen (28.6 μg L^{-1}), and naproxen (8.47 μg L^{-1}), however, were detected at lower levels in our previous work in comparison to current data (92.8 μg L^{-1}, 56.8 μg L^{-1} and 19.8 μg L^{-1}, respectively). Such discrepancies can be attributed to different sampling periods and/or changes in the consumption of specific drugs over the last decade. In any case, the values reported in Cadiz Bay for specific pharmaceuticals and illicit drugs are within the same order of magnitude than those observed in other Spanish cities. For instance, a previous study conducted in Gran Canaria reported 18.2 μg L^{-1} of ibuprofen, 8.6 μg L^{-1} of naproxen, 4.8 μg L^{-1} of metamizole (parent drug of 4-acetamidoantipyrine), and 49.1 μg L^{-1} of caffeine (Afonso-Olivares et al., 2017), whereas up to 35.1 μg L^{-1} of salicylic acid were measured in Valencia (Gracia-Lor et al., 2012). Similar results are observed in other Mediterranean countries such as Greece, where mean concentrations of 41 μg L^{-1} for caffeine, 8.5 μg L^{-1} for ibuprofen, 14 μg L^{-1} for paracetamol, 7.5 μg L^{-1} for salicylic acid, and 16.6 μg L^{-1} for valsartan have been recently reported in sewage (Papageorgiou et al., 2019). On the other hand, several compounds were detected in our study at relatively low concentrations (<100 ng L^{-1}) but with high frequency, like the beta-blocker metoprolol, the illicit drug methadone and its metabolite 2-ethylidene-1-5-dimethyl-3,3-diphenylpyrrolidine (EDDP), the lipid regulator bezafibrate, and the antiepileptic carbamazepine. These compounds should not be dismissed due to their low concentrations, as their continuous input could potentially lead to environmental effects if no proper removal is achieved during sewage treatment (Wu et al., 2023).

Fig. 4A shows concentrations of different classes of target compounds in influent samples from the three WWTPs analysed in this work. In general terms, concentrations were within the same order of magnitude for most analytes, being fairly similar for antiepileptics, antihypertensives, and lipid regulators, among others. Concentrations of antibiotics measured in CA were, however, 50% lower than those in PR and JF, and the same pattern was observed for antihistamines, antipsychotics, non-steroidal anti-inflammatories, and opiates. On the contrary, levels of beta-blockers were 5 times higher in CA than in PR and JF. More in detail, we have found that the concentrations of specific compounds were quite similar in JF and PR but significantly different (p-

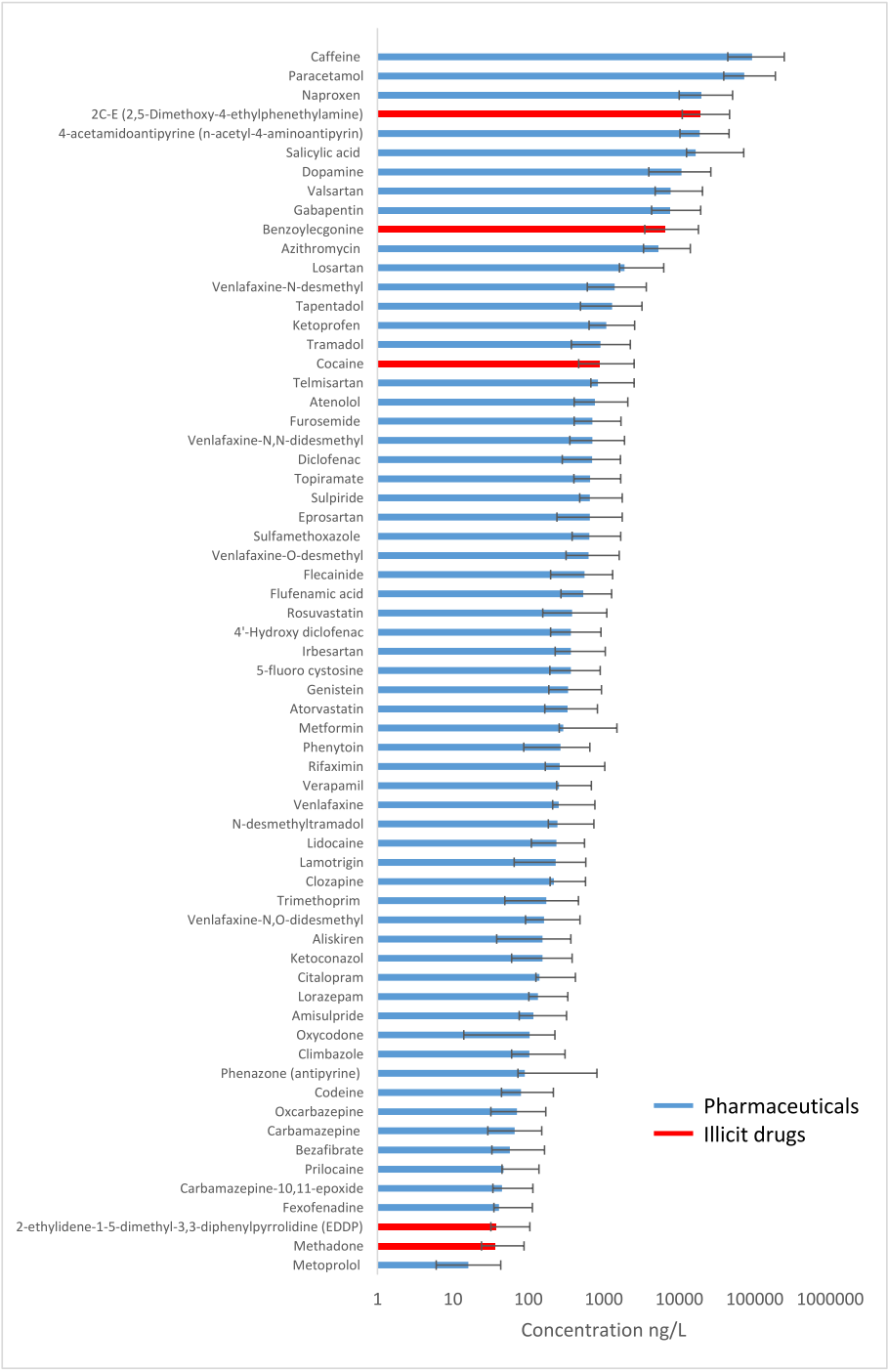

Fig. 3. Mean concentration (ng L^{-1}) of compounds detected in more than 50% of the samples.

value <0.05) in CA. For instance, EDDP was detected with a mean concentration of 57 and 36 ng L^{-1} in PR and JF, respectively, and much lower (7 ng L^{-1}) in CA. The same trend was found for several drugs: azithromycin (antibiotic), carbamazepine-10,11-epoxide (antiepileptic), citalopram (selective serotonin reuptake inhibitor), clozapine (antipsychotic), doxylamine (antihistamine), fexofenadine (antihistamine), methadone (illicit drug) and verapamil (antihypertensive), all of them detected at similar concentrations in JF and PR, and at lower concentrations in CA. In addition, some drugs were measured in PR and JF and were not detected in CA, like benproperine (cough suppressant), dextrorphan (cough suppressant), diltiazem (calcium channel blockers) and maprotiline (antidepressant). On the other hand, there were a few compounds that were either only detected in CA or had a significantly higher concentrations in CA compared to PR and JF: amantadine (anticonvulsant), bisoprolol (beta blockers), cannabidiol (illicit drug) and ritonavir (antiretroviral). Variations may be due to various reasons. Some compounds are not reported in some WWTPs because their concentration was below the MQL or their RSD was high (diltiazem, amantadine). Differences in pharmaceutical consumption patterns for specific diseases among different populations cannot be ruled out. For example, the use of cough suppressants (benproperine or dextrorphan) in PR and JF is higher than in CA, where the antiretroviral ritonavir

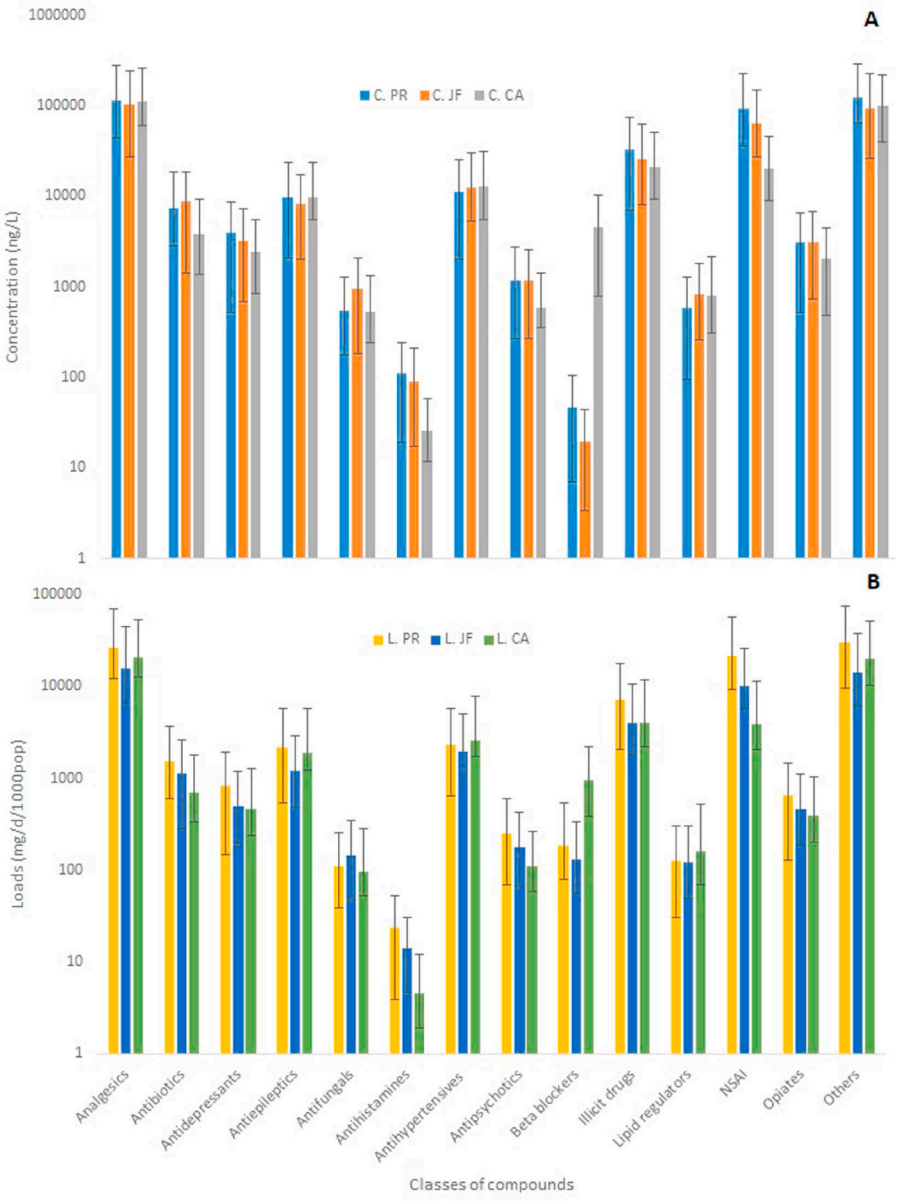

Fig. 4. Concentrations (ng/L) (A) and loads ($mg{\cdot}d^{-1}{\cdot}1000pop^{-1}$) (B) of different classes of target compounds in WWTPs from Cadiz Bay.

seems to have an increased use in the latter. Other compounds such as maprotiline (antidepressant) or bisoprolol (beta-blockers) are only measured at specific WWTPs.

Fig. 4B shows loads of different classes of drugs entering Cadiz Bay WWTPs. Determination of mass loads requires additional information on the number of inhabitants and the flow entering the WWTP, and allows for more reliable conclusions when comparing different sites rather than using concentration data only (Yao et al., 2021). In comparison to the data presented in Fig. 4A, we observed how differences among JE, PR, and CA were severely reduced when using loads instead of concentrations, which indicates a relatively homogenous consumption of drugs in the Cadiz Bay area. Nevertheless, there were still significantly higher inputs of antipsychotics (p-value = 0.0018) and NSAI (p-value = 0.0443) in PR compared to CA. Statistical differences were also found between PR, CA, and JF for illicit drugs (p-value = 0.0089) and other pharmaceuticals (p-value = 0.0088), with higher loads entering PR. On the contrary, beta-blockers loads are considerably higher in CA (p-value = 0.0003) compared to JF.

3.3. *Consumption of selected pharmaceuticals and illicit drugs in Cadiz Bay*

Table 2 shows average consumption values for those target compounds for which information on their excretions rate and/or stability in sewage was available. Table S5 summarizes several correction factors used by different authors in order to calculate pharmaceutical and illicit drug consumption from measured concentrations. These factors may differ widely depending on specific authors and/or compounds. For instance, the most commonly used excretion rates for benzoylecgonine are between 29% (Hue et al., 2022; Liu et al., 2021; Rice et al., 2020) and 35% (Croft et al., 2020; Lai et al., 2011), whereas for atenolol it can be either 92.5% (Escolà Casas et al., 2021) or 37% (Lai et al., 2011). Similarly, trimethoprim excretion rate can be differ significantly depending on the source, from 46% (Escolà Casas et al., 2021) to 85% (Gao et al., 2022). In some occasions, metabolites are preferred instead of the parent compound as biomarkers to assess consumption due to their higher stability in wastewater (Senta et al., 2020). This could explain the differences in consumption of cocaine in Cadiz Bay reported

Table 2
Mean consumption (mg·d^{-1}·1000pop^{-1}) of target analytes in Cadiz Bay.

Compounds	Puerto Real	Jerez de la Frontera	Cádiz
Atenolol	192 ± 123	128 ± 46	112 ± 44
Azithromycin	2151 ± 968	1710 ± 342	1055 ± 222
Bezafibrate	20 ± 5	10 ± 3	37 ± 14
Caffeine	908660 ± 299924	405463 ± 170294	602048 ± 198675
Carbamazepine	98 ± 32	92 ± 24	117 ± 48
Citalopram	191 ± 63	130 ± 39	23 ± 5
Cocaine	4419 ± 1458	1931 ± 637	2285 ± 1028
Cocaine (using benzoylecgonine)	6043 ± 906	2381 ± 1048	2624 ± 945
Codeine	49 ± 11	51 ± 18	39 ± 17
Furosemide	232 ± 86	166 ± 46	198 ± 103
Gabapentin	2345 ± 1032	1264 ± 379	2240 ± 1299
Irbesartan	60 ± 22	113 ± 31	79 ± 31
Lorazepam	23 ± 7	35 ± 12	21 ± 12
Losartan	627 ± 245	585 ± 450	567 ± 618
Metformin	39 ± 19	11 ± 5	161 ± 113
Methadone	33 ± 7	24 ± 6	14 ± 2
Methadone (using EDDP)	86 ± 22	39 ± 11	7 ± 2
Metoprolol	31 ± 10	16 ± 5	34 ± 12
Naproxen	70613 ± 22596	35247 ± 13746	48255 ± 20267
Oxycodone	188 ± 23	135 ± 32	184 ± 31
Salicylic acid	40764 ± 28942	12983 ± 7240	7326 ± 3443
Sulfamethoxazole	1398 ± 559	1167 ± 362	1010 ± 717
Tramadol	620 ± 143	590 ± 142	511 ± 169
Trimethoprim	97 ± 18	47 ± 11	68 ± 24
Valsartan	1627 ± 456	1161 ± 488	2258 ± 1061
Venlafaxine	2017 ± 444	767 ± 192	526 ± 195
Venlafaxine (using venlafaxine-O-desmethyl)	394 ± 32	442 ± 133	373 ± 160

in Table 2, which is 28% higher when using benzoylecgonine (cocaine metabolite) rather than cocaine itself. Similarly, consumption of methadone estimated through its metabolite EDDP was 85% higher than when the parent compound was considered for calculations. On the contrary, consumption of venlafaxine through the metabolite venlafaxine-O-desmethyl is estimated to be lower than when using the parent compound concentration in sewage for calculation, which could be attribute to dumping of the pharmaceutical to the sewers without consumption. Another parameters that may affect the determination of the consumption are the stability of the analytes (Croft et al., 2020; Lin et al., 2021) and sorption to the particulate matter (Yao et al., 2021). In this work, for those compounds for which several correction factors or excretion rates were available, consumption has been calculated using those that fit best for later comparison of our results with previous works.

Table 2 shows that the four most consumed compounds were the same in the 3 cities under study in Cadiz Bay: the stimulant caffeine, followed by the non-steroidal anti-inflammatory naproxen, salicylic acid, and the illicit drug cocaine. No significant differences (p-value <0.05) were observed when comparing consumption data between weekdays (Monday - Thursday) and weekends (Friday - Sunday), except for the antibiotic azithromycin. However, almost half of the drugs of Table 2 showed significant differences among the studied populations, with most of the analytes being less consumed in JF than in CA and/or PR. For instance, 3 drugs that were consumed in greater quantities in PR compared to CA and JF were: methadone (illicit drug), cocaine (illicit drug), and salicylic acid (analgesic). Citalopram (SSRI) was statistically less consumed in CA than in PR and JF (p-value <0.05), whereas bezafibrate (lipid regulator) showed the opposite trend. The consumption of metformin (antidiabetic) and metropolol (beta-blocker) was significantly higher in CA than in JF, and caffeine (stimulant), naproxen (NSAI) and trimethoprim (antibiotic), were more consumed in PR than in JF. Only irbesartan (antihypertensive) showed higher usage in JF than in PR. Such differences may be partly explained by different consumption habits due to a combination of changes in population dynamics over the last decades (e.g., almost 25% of CA population is currently over 65 years, where this number is <20% in PR and JF, according to the National Institute of Statistics) and differences in income among populations. Comparison of our results against prescription data could be used to corroborate this hypothesis for some specific drugs (Escolà Casas et al., 2021), but such task is out of the scope of this work as this information is not currently available.

Finally, a comparison of our data with those obtained in other cities from the centre and north of Spain yields similar results regarding the consumption of illicit drugs (for which most WBE studies have been conducted). Estimated consumption of cocaine (through benzoylecgonine analysis) was in the range of 900–2800 mg d^{-1}·1000pop^{-1} in the northern half of Spain (Bijlsma et al., 2021), while in Cadiz Bay is between 2381 and 2624 mg d^{-1}·1000pop^{-1} (JF and CA, respectively). Nevertheless, a higher value is observed in PR, 6043 mg d^{-1}·1000pop^{-1} on average (Table S5). Other countries such as United Kingdom and United States show similar or slightly higher cocaine consumption than in Spain (3490 and 864–3830 mg d^{-1}·1000pop^{-1}, respectively) (Croft et al., 2020; Rice et al., 2020). Higher discrepancies are found when comparing methadone consumption, with numbers between 92 and 1410 mg d^{-1}·1000pop^{-1} in the United States (Croft et al., 2020) (Table S5), while our data show much lower values (44 mg d^{-1}·1000pop^{-1}). Regarding pharmaceuticals, citalopram consumption was estimated to be between 165 and 382 mg d^{-1}·1000pop^{-1} in Italy (Riva et al., 2020), similarly to our observations in PR (191 mg d^{-1}·1000pop^{-1}) and JF (130 mg d^{-1}·1000pop^{-1}). Consumption of losartan, however, seems to be higher in that country (1627–2486 mg d^{-1}·1000pop^{-1}) than in Cadiz Bay (567–627 mg d^{-1}·1000pop^{-1}) (Table S5). Carbamazepine (615–1580 mg d^{-1}·1000pop^{-1}), morphine (99–312 mg d^{-1}·1000pop^{-1}) and oxycodone (14–1859 mg d^{-1}·1000pop^{-1}) are also prescribed in higher numbers in United States (Croft et al., 2020; Gushgari et al., 2019) than in our sampling area (92–117 mg d^{-1}·1000pop^{-1}, 5 and 14 mg d^{-1}·1000pop^{-1}, and 135–188 mg d^{-1}·1000pop^{-1}, respectively). Available data from China show similar consumption of antibiotics such as sulfamethoxazole or trimethoprim (3–1715 and 1–72 mg d^{-1}·1000pop^{-1}, respectively) (Gao et al., 2022) than in Spain according to our study (1010–1398 and 47–97 mg d^{-1}·1000pop^{-1}, respectively).

4. Conclusions

In this work, we have optimised a multi-residue method capable of determining almost 200 pharmaceuticals and illicit drugs in sewage, therefore providing a more comprehensive picture of the loads of these substances reaching WWTPs in comparison with previous studies. Analysis of influent samples from southern Spain was achieved for the first time, revealing the occurrence of more than 100 drugs, 64 of them showing a frequency detection greater than 50%. Differences in the concentrations of several classes of compounds were observed among different WWTPs, but they were minimized after normalisation by calculating their loads, which implies relatively similar consumption patterns among cities within the same area of study. In this regards, statistical differences for several classes (antipsychotics, beta-blockers, illicit drugs, NSAI, etc.) were found mainly between PR ad CA. Consumption of selected substances (n = 27) could be also estimated using WBE, which, to the date, has been mostly applied to illicit drugs only. Currently, the main limitation when trying to apply WBE to calculate the consumption of a wider spectrum of chemicals is related to the lack of data regarding their percentage of excretion, stability in wastewater, and sorption capacity. Normalisation of such data among different studies is also needed to enable direct comparison among different datasets, as well as further research to identify new metabolites suitable to be used as biomarkers, for which HRMS can be a very valuable tool. Additionally, the use of HRMS can be expanded in future studies to the

determination of drug transformation products during wastewater treatment, providing a better picture on how parent compounds are eliminated before their discharge into the receiving waters and arising new questions on the fate and ecotoxicity of their transformation products.

Credit author statement

Sergio Santana-Viera: Data Formal analysis; Investigation; Conceptualization; Methodology; Formal analysis; Writing – original draft. **Pablo A. Lara-Martin**: Conceptualization; Supervision; Methodology; Writing – review & editing; Project administration. **Eduardo González-Mazo**: Supervision; Writing – review & editing. All authors read and approved the final version of the manuscript.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.118000.

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