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Identification and determination of emerging pollutants in sewage sludge driven by UPLC-QTOF-MS data mining

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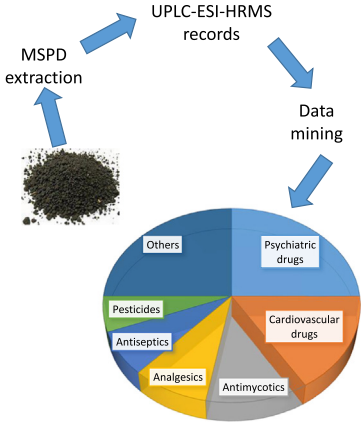
HIGHLIGHTS

- Screening of pollutants in sludge by matrix solid-phase dispersion and UPLC-QTOF-MS
- Assessment of the reliability of different acquisition modes with spiked samples
- Identification of more than 60 emerging pollutants in sewage sludge
- Median concentrations above 100 ng g^{-1} for 10 out of 37 quantified species

GRAPHICAL ABSTRACT



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ABSTRACT

Sludge from sewage treatment plants (STPs) is recognized as a sink of moderate to high lipophilic compounds resistant to biodegradation. Herein, we investigate the presence of emerging pollutants in sewage sludge combining the information provided by mass spectrometry detection, following ultra-performance liquid chromatography (UPLC), with the use of an accurate spectral database of pesticides and pharmaceuticals. In a first step, the performance of matrix solid-phase dispersion, as sample preparation technique, and two non-target data acquisition strategies (data dependent, DDA, and data independent analysis modes, DIA), used in combination with a UPLC quadrupole time-of-flight system, are assessed using a selection of deuterated compounds added either to freeze-dried sludge samples, or to sludge extracts. Possibilities and limitations of both modes are discussed. Following the DDA approach, a group of 68 micropollutants was identified in sludge from different STPs. Some of them are reported in this compartment for the first time. Finally, semi-quantitative concentration data are reported for a group of 37 pollutants in samples obtained from 16 STPs. Out of them, 10 pharmaceuticals, showing detection frequencies and median sludge residues above 50% and 100 ng g^{-1}, respectively; are highlighted as pollutants to be monitored in sludge in order to understand their behaviour during the wastewater treatment.

1. Introduction

A large number of pharmaceuticals and personal care products (PPCPs), pesticides, household cleaning products, and other substances employed in different industrial processes, are used in developed

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countries. Municipal wastewater is recognized as the most important source and route of entry of these chemicals into the aquatic environment, mainly due to their inefficient removal at sewage treatment plants (STPs) (Blum et al., 2018). Thus, the continuous release of these compounds in the aquatic media might constitute a potential risk to humans and ecosystems (Gavrilescu et al., 2015; Nika et al., 2020; Wu et al., 2019). Although the levels of pollutants in wastewater have been deeply studied during the last decade (Deblonde et al., 2011; Stuart et al., 2012), the potential accumulation of these compounds in sewage sludge remains mostly unexplored. Moreover, most efforts have been focused on the study of a preselected number of compounds, or family of compounds (Ivanová et al., 2018; Li et al., 2016; Martín-Pozo et al., 2019).

Sludge is a complex matrix, whose heterogeneous composition depends on the nature of the inputs at STPs (Hörsing et al., 2011). The presence of a large number of compounds in sludge extracts at different concentrations (from low ng g^{-1} to several μg g^{-1}), makes the study of these samples a real challenge (Martín-Pozo et al., 2019). Regarding the fact that this residue contains a wide variety of organic and inorganic substances (Svahn and Björklund, 2015), and that sludge from STPs is often re-used as agricultural fertilizer, and as raw material in compost (Christodoulou and Stamatelatou, 2016), the characterization of micropollutants existing in these samples, and the approach of new screening analysis strategies are highly recommended. Identification and quantification of micropollutants in sludge samples is also a basic requirement to obtain a global picture of their behaviour during municipal water treatments, comparing concentrations measured in the aqueous phase with those existing in sludge.

Some recent studies have summarized the analytical methodologies applied to the determination of emerging pollutants in sludge during the last years (Martín-Pozo et al., 2019; Pérez-Lemus et al., 2019). On the one hand, in target analysis, where the analytes are pre-selected, liquid chromatography coupled to triple quadrupole (QqQ) mass spectrometers, working in MRM mode, leads to high selectivity and extremely low quantification limits. However, this strategy covers only the set of pre-defined compounds and is totally blind to any other species present in the sample (Gago-Ferrero et al., 2015; Petrie et al., 2016). On the other hand, non-target screening analysis requires the use of high resolution/accurate mass spectrometry (HRMS), either based on the use of time-of-flight (TOF) or Orbitrap mass analyzers, where accurate spectral records in SCAN mode, throughout the entire chromatographic separation process, are obtained. Thus, the combination of HRMS with mild ionization sources, such as electrospray ionization (ESI), allows recording the accurate *m/z* ratios for pseudo-molecular ions (usually, $[M + H]^+$ or $[M\text{-}H]^-$) of any species existing in the sample, which survives the sample preparation and ionization steps (Krauss et al., 2010). This latent information offers the possibility of detecting new pollutants in environmental matrices and it results very attractive for the search of new pollutants in complex matrices such as sludge (Castro et al., 2016; Veenaas et al., 2018).

The most common operational modes of HRMS systems for non-target screening studies are termed as data dependent (DDA) and data independent (DIA) acquisition modes, (Ccanccapa-cartagena et al., 2019; Martínez-Bueno et al., 2019; Moschet et al., 2017). DDA combines accurate MS scan spectra with authentic product ion spectra for an automated selection of precursor ions isolated by the first MS analyzer (usually a quadrupole). DIA does not provides real MS/MS spectra; instead, pseudo-MS/MS scan spectra are recorded, applying different collision energies to the range of ions generated in the ionization source from those molecules with the same retention time in the LC column; then, all ions (primary features and their fragments) are directed to the accurate MS analyzer (i.e., TOF). In both cases, the mining of raw data is an intensive task, whose success depends on the existence of appropriate spectral databases of accurate MS and MS/MS spectra. Whatever the data acquisition mode, the quality of MS and MS/MS spectra (mass accuracy and purity of the isotopic profile for the pseudo-molecular ions and product ions) is a key issue to guarantee the proper identification of a database compound in the extract from a complex matrix, as it is the case of sludge samples, with a high percentage of organic matter, corresponding to natural and man-made chemicals.

In this research, the range of possibilities offered by QTOF-MS systems, for the non-target screening of unknown species, are investigated for the particular case of sewage sludge samples. To that aim, matrix-solid phase dispersion (MSPD) was first applied to the non-selective extraction of organic compounds existing in sludge samples. Extracts were processed by UPLC-QTOF-MS using reversed-phase separation conditions. Thereafter, acquisition and data mining strategies, based on DDA and DIA analysis, were compared for the identification of a selection of deuterated compounds spiked to sludge at different concentration levels. After choosing the data acquisition mode, the presence of emerging pollutants was screened in different sludge samples. Additionally, semi-quantitative concentration data, estimated using matrix-matched standards prepared with spiked extracts obtained from a pool of sludge samples, are provided for a selection of 37 compounds.

2. Material and methods

2.1. Material, solvents and standards

Polypropylene syringes (12 mL volume) and polyethylene frits for MSPD extraction (20 μm, 15 mL) were supplied by International Sorbent Technology (Mid Glamorgan, UK). The dispersant sorbent (C_{18}-bonded silica) was purchased from Agilent Technologies (Santa Clara, CA, USA). Diatomaceous earth, used as co-sorbent, was obtained from Sigma-Aldrich (Milwaukee, WI, USA).

Methanol (MeOH), HPLC-grade, and formic acid (FA) were purchased from Merck (Darmstadt, Germany). Ultrapure water (18.2 mΩ cm^{-1}) was obtained from a Milli-Q system by Millipore (Billerica, MA, USA).

Standards of tentatively identified compounds in the extracts from sludge samples, were acquired from Sigma-Aldrich and TCI Europe (Zwijndrecht, Belgium). Deuterated compounds, either used as surrogate standards (SS) through sample preparation, or as model species during assessment of data acquisition approaches: clotrimazole-d_5 (CTZ-d_5), amiodarone-d_4 (AMI-d_4), irbesartan-d_4 (IRB-d_4), miconazole-d_5 (MCZ-d_5) and itraconazole-d_5 (ITZ-d_5) were supplied by Toronto Research Chemicals (North York, ON, Canada), whereas imazalil-d_5 (IMZ-d_5) and sertraline-d_3 (SER-d_3) were purchased from Sigma-Aldrich. Individual stock solutions of each compound were prepared in MeOH. Further dilutions were made in the same solvent.

2.2. Samples and sample preparation

Grab sludge samples were obtained from municipal STPs located in Galicia (Northwest of Spain) during 2018. Fig. S1 shows the geographic situation of STPs. After reception, samples were lyophilized and stored in glass vessels at 4 °C. Sample preparation was based on a generic MSPD extraction procedure (Celano et al., 2014). In brief, a fraction of 0.5 g of lyophilized sludge, spiked with 1 μg g^{-1} of the mixture of SSs, was dispersed using 2 g of C_{18} in a glass mortar, for 5 min. The blend was loaded into a polypropylene syringe containing a polyethylene frit and 1 g of diatomaceous earth. A second frit was placed on the top to compact the cartridge. Compounds were extracted passing MeOH through the MSPD cartridge, collecting an extract volume of 10 mL. This extract was filtered with a PTFE filter, 0.2 μm pore size, and injected in the UPLC-QTOF-MS system without any additional treatment.

Spiked samples, employed to evaluate the efficiency of the MSPD extraction, were prepared by addition of methanolic mixtures of 42 selected drugs to a pool of freeze-dried sludge samples (addition level 1 μg g^{-1}). Procedural blanks (without any sludge in the MSPD packed syringe) were prepared every 5 samples in order to investigate possible

contamination problems during the extraction protocol. In preliminary sample preparation experiments, the yield of MSPD extraction and the performance of data acquisition and data mining strategies were evaluated using sludge samples spiked with the mixture of deuterated compounds reported in section 2.1.

2.3. Equipment and software

The screening and determination of emerging pollutants residues in sludge extracts were performed in a UPLC-QTOF-MS system. The chromatograph was an Agilent 1290 Series (Wilmington, DE, USA), consisting of a binary high-pressure mixing pump, oven and autosampler. The QTOF was an Agilent 6550 model, equipped with a Dual-Spray ESI source, which used nitrogen (99.999%) for nebulization (30 psi) and drying gas (200 °C, 12 L min^{-1}). Nitrogen was also employed as sheath gas (12 L min^{-1}, 350 °C). Capillary, nozzle and fragmentor voltages were set at 3500, 0 and 170 V, respectively. Regarding the QTOF hybrid analyzer, it worked in 2 GHz Extended Dynamic Range resolution mode (mass resolution 10,000 at *m/z* value of 118.0862). Considering that molecules of most of the prescribed drugs contain basic moieties, the ESI source was operated in the positive mode (ESI+) throughout the current study. Recalibration of the mass axis was continuously performed considering ions with *m/z* values of 121.0509 (purine) and 922.0098 (hexakis(1H, 1H, 3H-tetrafluoropropoxy) phosphazine).

LC separations were performed in a Zorbax Eclipse Plus C_{18} Rapid Resolution HD column (2.1 × 50 mm, 1.8 μm) acquired from Agilent Technologies. The analytical column was connected to a C_{18} 2.1 mm i.d. Security Guard™ ultra-cartridge supplied by Phenomenex (Torrance, CA, USA). Mobile phases were ultrapure water (A) and MeOH (B), both containing 0.1% FA, at a constant flow rate of 0.4 mL min^{-1}. The gradient was programmed as follow: 0–0.5 min, 20% B; 6–7 min, 100% B; 7.1–10 min, 20% B. Column and pre-column were maintained at 40 °C. The injected volume for solvent standards and sample extracts was 1 μL.

MassHunter software package (Agilent Technologies) was used to control the acquisition parameters of the UPLC-QTOF-MS instrument, in the different operational modes, as well as for processing the obtained data. *MassHunter Qualitative* software (version B.08.00), in combination with a *PCDL* library (*ForTox* PCDL database from Agilent Technologies, containing above 9000 compounds), was applied during data mining from raw LC-MS records acquired following DDA or DIA modes. The *ForTox* database was completed with the product ion spectra of isotopically labelled species involved in the current research. Spectra were acquired at collision energies of 10, 20 and 40 eV, from $[M + H]^+$ ions of deuterated compounds, except in case of CTZ-d_5, (product ion spectra acquired from $[M\text{-}C_3H_3N_2]^+$).

2.4. UPLC-HRMS screening of emerging pollutants

2.4.1. DIA analysis

DIA (also known as *all ions*, or MS^E acquisition mode) spectra were recorded in the range of *m/z* values from 50 to 1000, at a rate of 4 spectra s^{-1}. In addition to MS spectra, three acquisition functions, with different collision energies (10, 20 and 40 eV), were used with the aim to obtain as much information as possible for compounds ionized in the ESI source. Data mining was developed using a target/suspect screening workflow, *Find by formula* tool (with fragment confirmation), which uses the input formulae and product ion spectra from *ForTox* spectral database to search for the presence of pseudo-molecular ions (i.e. $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$) and possible fragment ions, showing same retention time, from low (MS) and high energy (10, 20 or 40 eV) acquisition functions, respectively. A minimum of one fragment ion was used for a tentative identification of a compound from the *ForTox* database, Fig. S2. The maximum difference allowed for *m/z* ratios of ions observed in experimental spectra (recorded at low and high collision energies) versus those compiled in the spectral database was 20 ppm.

2.4.2. DDA analysis

In the *DDA* acquisition mode, spectra were recorded in the range of *m/z* values from 50 to 1000. Selection of precursor ions for MS/MS experiments was triggered attending to their intensities. A maximum of 5 precursors were simultaneously considered for isolation in the Q MS analyzer and CID fragmentation. Exclusion of a given precursor was activated after 3 MS/MS spectra (collision energies of 10, 20 and 40 eV) were recorded. Selection of same ion was re-activated after 0.5 min. The acquisition frequencies in MS and MS/MS modes were 4 and 8 Hz, respectively. So, the duty cycle of the DDA operational mode was 2.13 s, versus 1 s for DIA. The DDA acquisition algorithm was applied in the iterative mode during four consecutive injections of the same sample, thus, precursors previously selected for MS/MS fragmentation are excluded in further injections, within the same retention time window. This way allowed selecting up to 20 different precursor ions, within the same retention time window (30 s), through the four consecutive injections of the same sample. *Find by autoMS/MS* function, also available in *MassHunter Qualitative Analysis Workflows B.08.00* software, was used to mine spectral information from DDA records. Tentative identifications are based on normalized spectral matches above 40% (using both reversed and forward comparison modes) between the experimental product ion spectra and those contained in the *ForTox* PCDL, at least at one of the three tested collision energies. Forward search compares experimental spectra with those existing in the *ForTox* PCDL, for the same collision energy. The reversed mode checks whether fragment ions in the library spectrum of a given compound are present in the experimental record. Data mining workflows following both approaches are shown in Fig. S2.

2.5. Characterization of the determination method and estimation of environmental concentrations

The performance of the analytical methodology, including sample preparation and determination steps, was assessed for a selection of 42 compounds, from different chemical families, confirmed in sludge samples during screening studies. To this end, same chromatographic conditions as those employed during screening studies were maintained, whilst the QTOF instrument was operated in the target MS/MS mode (a retention time window of 0.5 min was set for each compound, selecting the *m/z* of its precursor ion and a fixed CE). The response for the precursor ion, obtained from MS channel, was used for quantification purposes, whilst product ion scan spectra were employed for identity confirmation. The maximum allowed errors for retention time, quantification and confirmation ions were 0.1 min, 10 ppm and 20 ppm, respectively.

For this selection of compounds, the *extraction efficiency (EE)* of MSPD was calculated as the ratio between the responses (peak areas without SS correction) measured for a pool of spiked sludge samples (1 μg g^{-1}, referred to freeze-dried sludge) and the extracts from non-spiked fractions of the same matrix fortified after finishing sample preparation, multiplied by 100. *Matrix effects (MEs)* were evaluated as the difference between the responses obtained for spiked and non-spiked extracts from the pooled sludge matrix, divided by the response for a solvent-based standard of the same concentration (50 ng mL^{-1}). MEs close to 100% indicate the absence of changes between ionization yield for sludge extracts and standard solutions (Matuszewski et al., 2003).

Concentrations existing in sludge samples were calculated using matrix-matched standards, prepared by addition of increasing concentrations of selected species (4–100 ng mL^{-1}, $n = 5$ levels) to sludge extracts. Responses obtained for each compound (peak areas corrected with that for the selected SS) were plotted versus the addition level and fitted to a linear model. Semi-quantitative estimation of concentrations was calculated considering the final extract volume (10 mL) and

the sample amount (0.5 g), after assessing that MSPD provided extraction yields in the range from 70 to 120% Concentrations are reported for 37 out of 42 identified species, after excluding those poorly recovered by MSPD, presenting very high signal suppression effects, or considered as natural origin compounds.

2.6. Quality assurance and quality control measurements

Different QA/QC proceedings were implemented during the sample preparation and analysis, in order to reduce the number of false positives and false negatives. For this purpose, the glass material was cleaned with methanol and acetone and baked at 200 °C before its use. In addition, one procedural blank of MSPD extraction (without sample) was performed per batch of 5 samples, either during screening or quantification experiments. All the samples were fortified with a mixture of labelled-SSs and analysed in triplicate during assessment of the analytical method performance. Semi-quantitative concentration data were derived from duplicate analysis (extraction and determination steps) of each sludge sample. A mixture of solvent-based standards was injected at the beginning and at the end of the sequence, in order to evaluate the variations in the signals and also, the injection of several solvents, to detect possible carry over contamination effects between samples.

3. Results and discussion

3.1. Preliminary experiments

Without retention time information, the likelihood to identify a given pollutant from its accurate spectrum in the extract from a complex matrix (as it is the case of freeze-dried sludge samples) depends on combination of factors, which are related to the employed sample preparation conditions, the data acquisition mode, the data mining strategy and, obviously, the spectral database of candidates. As regards sample preparation, it is obvious that any compound not recovered from the sludge matrix will remain undetected. However, hard extraction conditions might result in too rich extracts, which might lead a decrease of the efficiency of compounds ionization at the ESI source.

Sample preparation conditions considered in the current study were sludge extraction under mild conditions (room temperature and atmospheric pressure) typical of the MSPD technique. No clean-up sorbent was placed into the MSPD syringe to prevent potential losses of sludge pollutants due to a too strong interaction with this sorbent, and MeOH was employed as non-selective elution solvent. These conditions are similar to those considered for extraction of organophosphate compounds, with large differences among their polarities, from sludge (Celano et al., 2014). In these preliminary experiments, the selection of deuterated compounds reported in section 2.1 was either added to sludge, or to MSPD extracts, in order to assess the feasibility to recover these compounds from the spiked sludge and the magnitude of MEs, respectively. The native analogues of these compounds are moderately to low polar species, used as pesticides or pharmaceuticals, which have been previously determined in sludge following targeted methods (Casado et al., 2015; Castro et al., 2016). The efficiency of MSPD extraction, corresponding to three different sludge samples spiked at 1 μg g^{-1}, varied between 69% to 100%, with associated standard deviations between 1 and 10%, Table 1. The assessment of MEs for spiked sludge extracts (10 mL volume) pointed out to small changes in the efficiency of compounds ionization compared to that observed for methanol-based standards of the same concentration (50 ng mL^{-1}), Table 1.

The efficiency of DDA and DIA strategies to discover above compounds in sludge extracts was carried out following the scheme depicted in Fig. S2. Extracts from 3 different sludge samples, spiked at two levels (50 and 10 ng mL^{-1}, equivalent to sludge concentrations of 1000 and 200 ng g^{-1}, respectively) were used. Table 2 summarizes the number of positive identifications for each compound at both addition levels. Globally, DDA performed slightly better than DIA. In the first case, false negatives were normally associated with a complex chemical environment in the vicinity of the retention time of a given compound, as it was the case of ITZ-d_5. Under these conditions, despite performing 4 injections of each sample, the $[M + H]^+$ of this compound was not within the set of 20 most intense molecular features co-eluting at same retention time; thus, it was not selected for MS/MS fragmentation. Sometimes, DDA launched MS/MS experiments from the adduct of ITZ-d_5 with sodium, which showed a higher intensity than that of the $[M + H]^+$ ion. In this case, the experimental spectrum will not match with that included in the PCDL library (recorded for the $[M + H]^+$ ion), so the compound remains unidentified, which means a false negative, Fig. S3; unless in a further iteration the same parent ion as that used in the PCDL database is selected by the DDA algorithm for fragmentation. A possibility to increase the number of ions submitted MS/MS experiments is to raise the acquisition frequency in both MS and auto MS/MS modes. Unfortunately, the highest the acquisition frequency, the lower the number of transients accumulated per spectrum, which negatively affects the sensitivity of TOF MS analyzers, Fig. S4. Thus, this alternative is unsuitable to detect trace compounds.

The DIA acquisition mode does not present the above commented limitations. Usually, the isotopic pattern for the pseudo-molecular ions of a given compound can be recognized even in a complex chemical environment as that existing at the retention time of ITZ-d_5. However, for ITZ-d_5 at the lowest addition level, a false negative was again reported systematically, Table 2. As illustrated in Fig. S5, responses for major product ions in the PCDL spectra of this compound (PCDL spectra were obtained from the $[M + H]^+$ ion) remained undetected in the high energy (40 eV) EIC chromatograms. DIA also failed to detect the presence of CTZ-d_5 in sludge extracts spiked at 10 ng mL^{-1}. CTZ illustrates the behaviour of labile molecules, which are fragmented in the ESI source. Thus, the pseudo-molecular ion ($[M + H]^+$) represents a minor feature compared to the major fragment corresponding, in this case, to the loss of the imidazole ring ($[M\text{-}C_3H_3N_2]^+$). So, the *Find by Formula* algorithm fails to identify a molecular ion for the formula of CTZ-d_5

Table 1
Retention times, quantification ions, MSPD extraction efficiency (EE) and matrix effect (ME) for the selection of deuterated compounds. Average data for three different sludge samples processed in duplicate.

Compound	Family	Retention time (min)	Quantification ion	EE (%) ± SD	ME (%) ± SD
IMZ-d_5	Pesticide	3.59	302.0870	95 ± 2	102 ± 2
CTZ-d_5	Antimycotic	3.94	282.1090	96 ± 3	101 ± 1
IRB-d_4	ARA II[a]	4.21	433.2648	93 ± 4	95 ± 2
SER-d_3	Antidepressant	4.71	309.0990	69 ± 10	101 ± 3
MCZ-d_5	Antimycotic	4.72	422.0247	100 ± 4	96 ± 2
AMI-d_4	Antiarrhythmic	5.18	650.0585	87 ± 5	95 ± 1
ITZ-d_5	Antimycotic	5.59	710.2780	76 ± 1	96 ± 4

[a] ARA II, angiotensin II receptor antagonist.

Table 2
Efficiency of DDA and DIA modes for detection of selected compounds in spiked extracts from 3 sludge samples.

Compound	DDA		DIA	
	[a]50 ng mL^{-1}	[a]10 ng mL^{-1}	[a]50 ng mL^{-1}	[a]10 ng mL^{-1}
IMZ-d_5	2	3	3	3
CTZ-d_5	2	3	3	0
IRB-d_4	3	3	3	3
SER-d_3	3	3	2	3
MCZ-d_5	3	3	3	2
AMI-d_4	3	3	3	3
ITZ-d_5	2	0	2	0
Percentage of positive identifications	86%	86%	90%	67%

included in the PCDL. On the basis of the results summarized in Table 1, the DDA mode was selected for the screening of sludge extracts for compounds compiled in the *ForTox* PCDL.

3.2. DDA screening of pollutants in sludge

Non-target screening was carried out on MSPD extracts from 10 different sludge samples. Experimental MS/MS spectra, obtained using DDA acquisition mode, were compared to those existing in *ForTox* PCDL library. As commented in section 2.4, the minimum normalized scores (0—100) for a tentative identification were set at 40, considering forward and reversed search modes. As an example, Fig. 1 summarizes the graphical outputs obtained during identification of the antidepressant trazodone in a non-spiked sample. Fig. 1A shows the chromatogram corresponding to the ion submitted to MS/MS fragmentation (extraction window 20 ppm). Points where product ion spectra were recorded are represented by the line (spectra are just recorded at the beginning the end and the apex of the line, at retention times of 2.657, 2.661 and 2.659 min, respectively) in Fig. 1B. The MS/MS spectra obtained in these points, at three different collision energies, are depicted in Fig. 1C. In the three cases, reverse and forward scores between these spectra and those of trazodone in the *ForTox* PCDL stayed above 90 and 80 (0–100 scale); so, the compound is considered as positively identified. Empirical formulae for fragment ions compatible with the empirical formula of trazodone (those highlighted in green in the plot) are also calculated by the data mining software. Finally, a detail of the MS spectrum at the retention time of trazodone is also shown, Fig. 1D. In this case, the cluster of signals corresponding to the $[M + H]^+$ ion of this chlorinated species is evident, and the normalized score corresponding to the fitting between the calculated and the experimental MS spectrum of trazodone stayed around 95. However, during mining of DDA records of sludge extracts, the fitting between experimental and theoretical MS spectra was not considered; thus, identifications were derived from matches between experimental MS/MS spectra and those compiled in the *ForTox* database.

After excluding compounds noticed in procedural blanks, those displaying a poor chromatographic shape (broad and/or tailing peaks), and species showing similar scores at several retention times through the same chromatogram, 68 compounds were identified in the set of processed sludge samples. Table 3 compiles the list of compounds, including their names, empirical formulae, CAS numbers, retention times and log D value at pH 7. The number of positive samples for each compound is also shown. The identification level was assigned accordingly to the scale proposed by Schymanski and co-workers (Schymanski et al., 2014). Thus, code 1 corresponds to compounds whose identity has been confirmed against authentic standards.

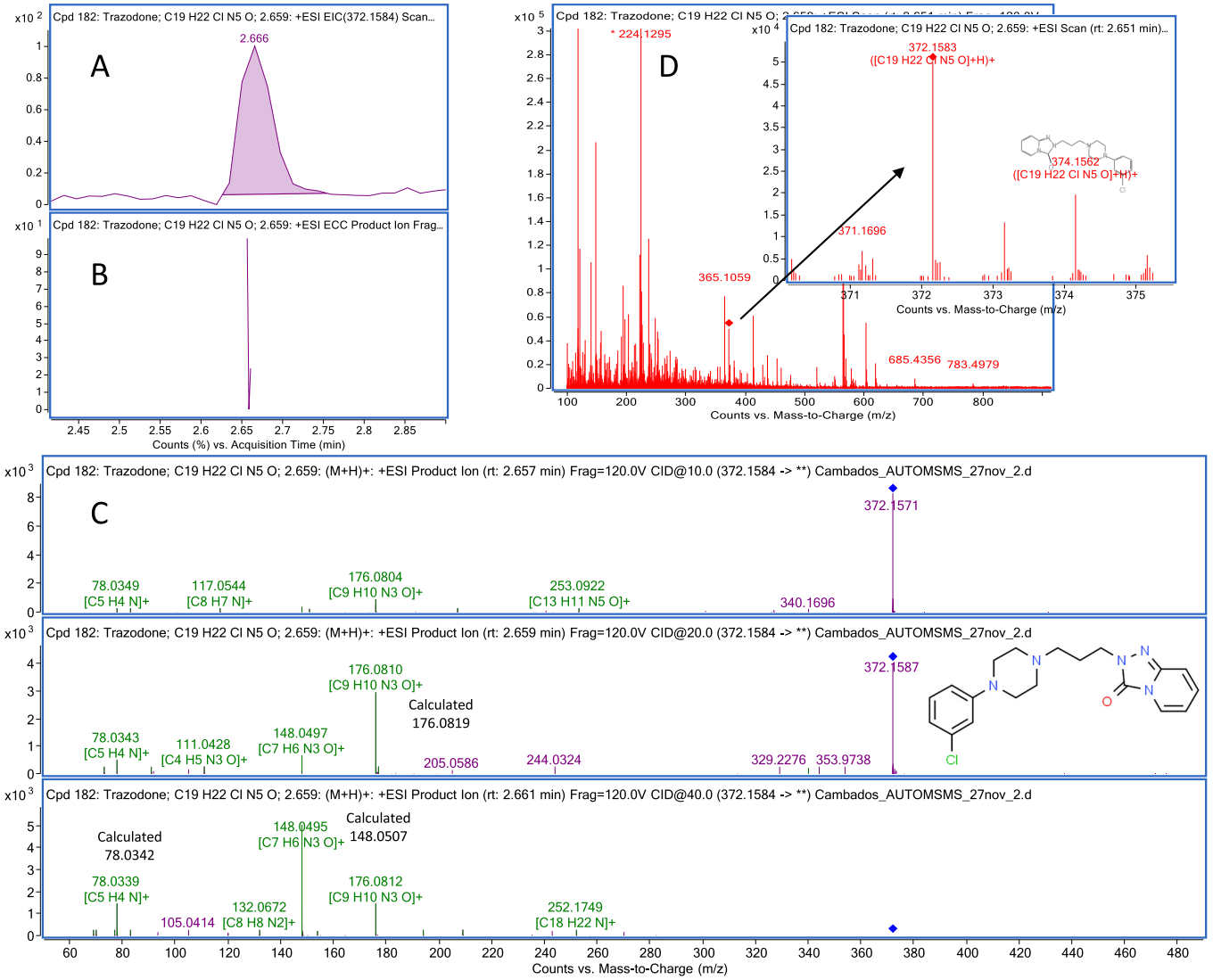

Fig. 1. Graphical information obtained from DDA data during the identification of trazodone in a non-spiked sludge sample.

Table 3
Summary of compounds identified in the extracts from 10 sludge samples using the DDA mode.

Compound	Formula	CAS number	Retention time (min)	Application	Number of positive samples	Level of identification	LogD (pH 7)
Octodrine	C8H19N	543–82-8	1.04	Stimulant	5	2	−0.27
Amisulpride	C17H27N3O4S	71675–85-9	1.16	Antipsycotic	6	1	−0.7
O-Desmethyltramadol	C15H23NO2	148262–77-5	1.18	Analgesic	3	2	n.a.
Thiabendazole	C10H7N3S	148–79-8	1.47	Pesticide	4	1	2.47
Levofloxacin	C18H20FN3O4	100986–85-4	1.48	Antibiotic	2	2	−0.2
Lidocaine	C14H22N2O	137–58-6	1.54	Analgesic	3	2	1.36
Norharman	C11H8N2	244–63-3	1.58	Alkaloid	10	1	2.03
Myosmine	C9H10N2	532–12-7	1.64	Alkaloid	10	2	−2
O-Desmethylvenlafaxine	C16H25NO2	142761–12-4	1.90	Antidepressant	2	1	−0.37
Tramadol	C16H25NO2	27203–92-5	1.92	Analgesic	6	1	−0.6
Harman	C12H10N2	486–84-0	1.94	Alkaloid	6	1	1.48
Mirtazapine	C17H19N3	85650–52-8	1.95	Antidepressant	6	1	−0.05
Dextrorphan	C17H23NO	125–73-5	2.03	Antitussive	6	2	1.33
Lamotrigine	C9H7Cl2N5	84057–84-1	2.17	Anticonvulsant	8	1	1.23
Tapentadol	C14H23NO	175591–23-8	2.28	Analgesic	10	2	0.66
8-Hydroxyquinoline	C9H7NO	148–24-3	2.45	Antiseptic	10	2	0.82
Trazodone	C19H22ClN5O	19794–93-5	2.61	Antidepressant	9	1	2.41
Berberine	C20H18NO4	2086–83-1	2.78	Alkaloid	7	1	n.a.
Azithromycin	C38H72N2O12	83905–01-5	2.81	Antibiotic	4	1	−0.06
Domperidone	C22H24ClN5O2	57808–66-9	2.82	Antiemetic	1	2	2.55
Venlafaxine	C17H27NO2	93413–69-5	2.84	Antidepressant	7	1	0.39
Clozapine	C18H19ClN4	5786-21-0	2.94	Antipsycotic	6	1	3.23
Propranolol	C16H21NO2	525–66-6	3.01	Antihypertensive	5	1	0.45
Citalopram	C20H21FN2O	59729–33-8	3.06	Antidepressant	10	1	1.02
Flecainide	C17H20F6N2O3	54143–55-4	3.14	Antihypertensive	9	1	0.72
Norcitalopram	C19H19FN2O	62498–67-3	3.15	Antidepressant	7	1	−0.14
5-Hydroxypropafenone	C21H27NO4	86384–10-3	3.19	Antiarrhythmic	4	2	0.86
Raloxifene	C28H27NO4S	84449–90-1	3.21	Antitumorall	7	2	3.05
Haloperidol	C21H23ClFNO2	52–86-8	3.34	Antipsycotic	2	1	2.58
Carvedilol	C24H26N2O4	72956–09-3	3.41	Antihypertensive	9	1	2.69
Benzydamine	C19H23N3O	642–72-8	3.47	Analgesic	4	1	1.29
Pimozide	C28H29F2N3O	2062-78-4	3.49	Antipsycotic	5	2	3.99
Imazalil	C14H14Cl2N2O	35554–44-0	3.58	Pesticide	6	1	3.37
Propafenone	C21H27NO3	54063–53-5	3.67	Antiarrhythmic	6	1	1.02
Cyclobenzaprine	C20H21N	303–53-7	3.71	Ansiolitic	4	2	4.08
Levomethadone/methadone	C21H27NO	125–58-6	3.78	Analgesic	6	2	1.92
Amitriptyline	C20H23N	50–48-6	3.82	Antidepressant	9	1	2.28
Chlorhexidine	C22H30N10Cl2	55–56-1	3.83	Antiseptic	2	1	1.58
Ketoconazole	C26H28Cl2N4O4	65277–42-1	3.84	Antimycotic	9	1	3.8
Nortriptyline	C19H21N	72–69-5	3.86	Antidepressant	2	2	1.22
Clotrimazole	C22H17ClN2	23593–75-1	3.93	Antimycotic	8	1	4.87
Cloperastine	C20H24ClNO	3703-76-2	3.96	Antitussive	7	1	2.9
Terbutryn	C10H19N5S	886–50-0	4.02	Pesticide	8	1	3.38
Fenticonazole	C24H20Cl2N2OS	72479–26-6	4.04	Antimycotic	3	1	4.56
Amorolfine	C21H35NO	78613–35-1	4.05	Antimycotic	6	2	5.14
Sertraline	C17H17Cl2N	79617–96-2	4.05	Antidepressant	2	1	2.7
HU-331	C21H28O3	137252–25-6	4.10	Antitumoral	4	2	2.93
Telmisartan	C33H30N4O2	144701–48-4	4.14	Antihypertensive	10	1	3.65
Cinnarizine	C26H28N2	298–57-7	4.18	Vasodilator	5	1	4.69
Irbesartan	C25H28N6O	138402–11-6	4.21	Antihypertensive	8	1	3.31
Clomipramine	C19H23ClN2	303–49-1	4.22	Antidepressant	5	1	2.6
Tioconazole	C16H13Cl3N2OS	65899–73-2	4.34	Antimycotic	3	1	4.11
Cyprodinil	C14H15N3	121552–61-2	4.37	Pesticide	3	1	3.01
Dronedarone	C31H44N2O5S	141626–36-0	4.56	Antihypertensive	5	1	5.6
Octamylamine	C13H29N	502–59-0	4.74	Anticonvulsant	4	2	1.73
Miconazole	C18H14Cl4N2O	22916–47-8	4.75	Antimycotic	2	1	4.81
Sertaconazole	C20H15Cl3N2OS	99592–32-2	4.80	Antimycotic	8	1	5.6
Isoconazole	C18H14Cl4N2O	27523–40-6	4.80	Antimycotic	5	2	4.72
Tamoxifen	C26H29NO	10540–29-1	4.83	Antitumoral	4	2	3.45
Benzododecinium	C21H38N	10328–35-5	4.98	Antiseptic	6	2	n. a.
Amiodarone	C25H29I2NO3	1951-25-3	5.15	Antihypertensive	4	1	5.51
N-Desethylamiodarone	C23H25I2NO3	83409–32-9	5.16	Antihypertensive	3	1	5.34
Desmethylclomipramine	C18H21ClN2	303–48-0	5.49	Antidepressant	3	2	1.67
Ethyl hexadecyl dimethyl ammonium	C20H44N	3006-10-8	5.66	Antiseptic	2	2	n.a.
Hexetidine	C21H45N3	141–94-6	5.67	Antiseptic	1	2	6.07
Fenofibrate	C20H21ClO4	49562–28-9	5.77	Antihypertensive	1	2	n.a.
9-Octadecenamide	C18H35NO	3322-62-1	6.37	Ansiolitic	5	2	6.88
Stearamide	C18H37NO	124–26-5	6.59	Plasticizer	7	2	7.29

n.a., not available.

Compounds with code 2, matched the empirical formula and *m/z* ratios of product ions with those existing in the *ForTox* PCDL; however, their identity was not confirmed against standards.

Compounds in Table 3 are sorted attending to their increasing retention time. Even some polar species, considering their Log D value and weak retention in the reversed-phase column (i.e., the antipsychotic

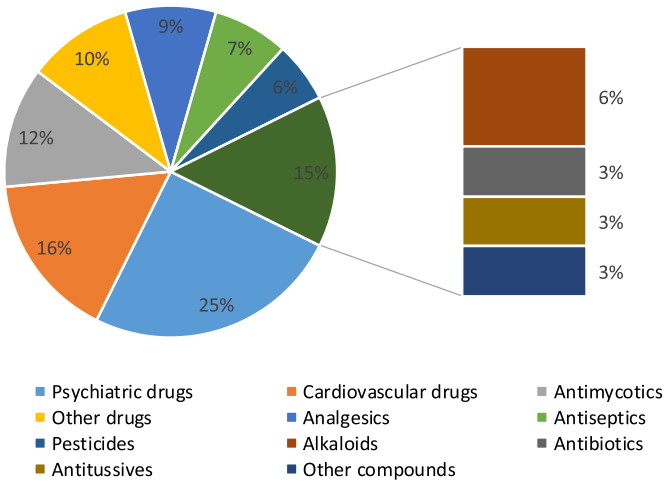

Fig. 2. Summary of the different families of chemicals identified in sludge, with the normalized number of compounds per family.

drug amisulpride) were detected in sludge extracts. On the other hand, the list of compounds with retention times longer than 5 min was limited. Between 5 and 7 min, chromatograms presented a high complexity (Fig. S3A); so, only those compounds from the PCDL database showing high intensity responses have a chance to be identified in this region. Attending to their uses, most compounds belong to different classes of pharmaceuticals, alkaloids of natural sources (such as harman and norharman), some pesticides, and active ingredients (i.e., antiseptics) of personal care compounds and household products. As shown in Fig. 2, above 50% of the identified species belong to three different groups of drugs employed in chronic treatments: psychiatric drugs (including some of their metabolites), cardiovascular and antimycotic pharmaceuticals. Several compounds listed in Table 3 have already been reported in the literature (Peysson and Vulliet, 2013) and also deeply studied in sludge, such as the antibiotics (Östman et al., 2017), the antimycotics (Castro et al., 2016; Lindberg et al., 2010) and several cardiovascular drugs (Castro et al., 2018; Montes et al., 2015). It is worth to mention that, several compounds included in the 3rd revision of the EU watch list of water pollutants (i.e., imazalil, clotrimazole, miconazole, venlafaxine and its *O*-demethylated form) were identified in sludge using the non-target DDA mode. Other species included in Table 3, such as levomethadone/methadone, amiodarone, lamotrigine, raloxifene, cyclobenzaprine, trazodone and chlorhexidine are included in the list of high production volume pharmaceuticals (Howard and Muir, 2011). To the best of our knowledge, pharmaceuticals as carvedilol, cinnarizine, cyclobenzaprine, dextorphan, octamylamine and raloxifene; and the fungicides amorolfine and hexetidine, are reported in sludge for the first time.

3.3. Characterization of the analytical methodology

The performance of sample preparation and determination conditions were evaluated for those compounds in Table 3, whose identity was confirmed with standards (attending to their availability and budget limitations), in terms of EEs and MEs. Out of 42 identified compounds, chlorhexidine and 8-hydroxyquinoline showed poor extraction yield (c.a. 25%), and low ionization efficiency in MSPD extracts (signal suppression around 80%), respectively. Data for the rest of compounds are compiled in Table 4. In addition to EEs and MEs, determination coefficients (R^2) obtained for spiked sludge extracts (concentration range 0–100 ng mL^{-1}, $n = 5$ levels), instrumental and procedural limits of quantification (LOQs) are provided. Instrumental LOQs are defined as the lowest concentration for a solvent-based standard providing a signal to noise ratio of 10 for the quantification ion. Procedural LOQs were calculated from instrumental LOQs multiplied by EEs and MEs, when they remained below 80%. Overall, the EEs varied from 79 to 123%, with relative standard deviations below 17%. MEs ranged between 51 and 135% with SD below 12%, depending on the compound. The obtained LOQs varied from 0.3 ng g^{-1} to 45 ng g^{-1} for amitriptyline and haloperidol, respectively.

3.4. Semi-quantitative determination of emerging pollutants

Compound residues were estimated in a set of samples obtained from 16 STPs, Fig. S1. Samples were processed in four batches with a procedural blank per sample. Concentrations referred to freeze dried sludge, assuming identical MEs for every sample, are shown as supplementary information, Table S1. Table S1 compiles also their detection frequencies, median, minimum and maximum concentrations levels. Table S1 includes those compounds for which method performance was previously assessed (Table 4), excluding 3 species which might have a natural origin (harman, norharman and berberine). Thus, 20 out of the 37 compounds in this table were found in at least the 80% of the studied samples, with median concentrations between 5 and up to 2000 ng g^{-1}. Fig. 3 shows the sum of their concentrations in each sample, the number of compounds above LOQs and the highest concentration species. The total residue for the set of 37 compounds in sludge varied from 1.4 to more than 15 μg g^{-1}, being telmisartan the pollutant displaying the highest concentrations in 12 out of 16 sludge samples. From data summarized in Table S1, 13 species presented median concentrations above 100 ng g^{-1}, and 10 of these micropollutants were noticed in more than 50% of the processed samples. Fig. 4 presents the normalized contribution of each of these compounds to the total residue found in each sample, being all of them employed as pharmaceuticals. *O*-desmethylvenlafaxine is not only an authorized antidepressant, but also one of the main metabolites of venlafaxine. The median residues of the *O*-desmethylated species were higher than those found for venlafaxine, which agrees with their relative levels in raw wastewater (González-Mariño et al., 2018). Moreover, except for sample code 8, the sum of concentrations for compounds compiled in Fig. 4 represents more than 50% of total residues measured in these samples (Table S1). The odd pattern observed for sludge code 8 is due to the presence of a very high residue of cinnarizine (above 8000 ng g^{-1} compared with a median value of 40 ng g^{-1} in the set of processed samples). Among others, cinnarizine is used in anti-motion sickness preparations; thus, an accidental or intentional direct disposal of the compound in the net of sewers cannot be excluded. It is worth noting that compounds with the highest concentrations, such as the antidepressants, sertraline and *O*-desmethylvenlafaxine; the antimycotics ketoconazole, miconazole and clotrimazole; and the antihypertensive, telmisartan are highly consumed drugs in Spain, with 9 daily diary doses (DDD) per 1000 inhabitants for sertraline in year 2014; 0.3 DDD for antimycotics and 3.4 DDD for telmisartan in 2018 ("Agencia española del medicamento y productos sanitarios (AEMPS),", n.d.). In addition to their high consumption, the Log D of these substances, at neutral pH, points out to very low polarity (4.2–6.13), thus the absorption in the sludge is highly favoured, which, combined with a limited biodegradation, explains the high concentrations in this type of samples. In case of species found at relevant concentrations in the water phase of STPs, as it is the case of sertraline, *O*-desmethylvenlafaxine, cloperastine and amitriptyline (Skees et al., 2018), their partial accumulation in sludge need to be kept in mind to calculate their removal efficiencies during wastewater treatment.

4. Conclusions

The combination of soft extraction conditions employed in MSPD with UPLC-ESI-QTOF-MS permitted the non-target identification of more than 60 micropollutants in sludge from municipal STPs. During preliminary method development, it has been demonstrated that both

Table 4
Analytical features of the determination method for a mixture of model compounds.

Compound	SS	$[M + H]^+$	CE (eV)	Product ion	Linearity (R^2, 0–100 ng mL-1)	EE (%) ± SD	ME (%) ± SD	Instrumental LOQs (ng mL^{-1})	Procedural LOQs (ng g^{-1})
O-Desmethyl venlafaxine	SER-d_3	264.1958	20	58.0650	0.9999	112 ± 6	111 ± 6	0.7	14.5
Mirtazapine	SER-d_3	266.1652	20	195.0924; 72.0810	0.9926	112 ± 3	83 ± 3	0.1	1.8
Venlafaxine	SER-d_3	278.2115	20	58.0651; 121.0648	0.9967	110 ± 3	89 ± 1	0.1	2.3
Sertraline	SER-d_3	306.0811	20	158.9765; 275.0389	0.9987	98 ± 2	114 ± 1	0.8	15.0
Norcitalopram	SER-d_3	311.1554	20	262.1035; 109.0448	0.9979	106 ± 5	114 ± 1	0.7	14.3
Citalopram	SER-d_3	325.1711	20	262.1026; 109.0448	0.9988	104 ± 5	100 ± 4	0.2	4.5
Clozapine	SER-d_3	327.1371	20	270.0792	0.9941	107 ± 6	101 ± 1	0.3	6.8
Amisulpride	SER-d_3	370.1795	20	242.0487	0.9988	98 ± 6	94 ± 3	0.2	3.1
Trazodone	SER-d_3	372.1586	20	176.0823	0.9984	123 ± 6	88 ± 4	0.2	3.1
Thiabendazole	IMZ-d_5	202.0433	40	175.0324; 131.0604	0.9959	116 ± 10	83 ± 5	0.5	10.9
Cyprodinil	IMZ-d_5	226.1339	40	93.0573	0.9961	80 ± 9	74 ± 8	0.5	13.5
Terbutryn	IMZ-d_5	242.1434	20	186.0808	0.9926	111 ± 8	90 ± 3	1.1	22.1
Clotrimazole	CTZ-d_5	277.0785	20	242.1090; 165.0698	0.9843	108 ± 8	73 ± 4	0.1	3.1
Imazalil	IMZ-d_5	297.0556	20	255.0086; 158.9763	0.9942	120 ± 9	86 ± 8	0.2	3.9
Tioconazole	CTZ-d_5	386.9887	20	130.9719	0.9998	86 ± 11	51 ± 7	0.2	7.5
Miconazole	MCZ-d_5	416.9900	20	158.8746	0.9988	96 ± 6	78 ± 5	0.4	11.4
Sertaconazole	CTZ-d_5	437.0043	20	180.9873	0.9868	99 ± 2	74 ± 1	0.4	10.8
Fenticonazole	CTZ-d_5	455.0746	20	199.0576	0.9966	115 ± 11	80 ± 7	0.6	16.6
Ketoconazole	CTZ-d_5	531.156	40	489.1455; 82.0625	0.9996	112 ± 11	78 ± 1	0.2	6.9
Norharman	IRB-d_4	169.0760	40	115.0543	0.9870	118 ± 11	85 ± 4	0.02	0.5
Harman	IRB-d_4	183.0917	40	115.0544	0.9940	113 ± 9	77 ± 3	0.02	0.6
Propranolol	IRB-d_4	260.1645	20	183.0804; 116.1069	0.9967	113 ± 12	96 ± 5	0.31	6.1
Cloperastine	IRB-d_4	330.1619	20	201.0465; 166.0777	0.9976	114 ± 10	84 ± 4	0.32	6.3
Flecainide	IRB-d_4	415.1451	40	301.0294	0.9989	119 ± 12	95 ± 12	0.02	0.5
Irbesartan	IRB-d_4	429.2397	20	207.0917	0.9975	98 ± 4	104 ± 4	0.27	5.4
Telmisartan	IRB-d_4	515.2442	40	497.2336; 276.1396	0.9993 (0−2000)	81 ± 13	74 ± 3	0.13	4.7
Dronedarone	AMI-d_4	557.3044	40	435.2676; 142.1596	0.9834	87 ± 5	81 ± 5	0.56	11.2
N-Desethylamiodarone	AMI-d_4	617.9997	20	546.9261; 72.0807	0.9870	106 ± 17	85 ± 4	0.02	0.5
Amiodarone	AMI-d_4	646.0310	40	201.0910; 100.1121	0.9989	112 ± 77	67 ± 2	0.02	0.6
Azithromycin	IRB-d_4	749.5158	40	591.4215; 158.1175	0.9919	123 ± 12	135 ± 3	0.04	0.8
Lamotrigine	SER-d_3	256.0151	40	210.9824; 144.9606	0.9959	103 ± 8	57 ± 2	0.01	0.4
Tramadol	SER-d_3	264.1958	20	58.0651	0.9958	103 ± 5	98 ± 2	0.46	9.2
Amitriptyline	SER-d_3	278.1903	20	233.1325; 91.0542	0.9917	95 ± 8	82 ± 1	0.014	0.3
Benzydamine	SER-d_3	310.1914	20	86.0964; 58.0650	0.9901	95 ± 8	87 ± 2	0.20	4.0
Clomipramine	SER-d_3	315.1623	20	86.0964; 58.0650	0.9971	79 ± 5	80 ± 2	0.68	13.6
Berberine	SER-d_3	336.1235	40	320.0917; 292.0968	0.9840	95 ± 10	90 ± 2	0.02	0.4
Propafenone	SER-d_3	342.2064	20	116.1069; 72.0807	0.9957	101 ± 6	93 ± 1	0.17	3.5
Cinnarizine	SER-d_3	369.2325	20	167.0856	0.9984	84 ± 4	85 ± 1	0.97	19.3
Haloperidol	SER-d_3	376.1474	20	165.0708; 1123.0241	0.9932	100 ± 10	60 ± 2	1.35	45.0
Carvedilol	SER-d_3	407.1965	20	224.1281; 100.0757	0.9993	100 ± 14	103 ± 3	0.39	7.9

non-target acquisition modes considered in the current study were prone to report false negatives, particularly when compounds elute in chromatographic regions with a high number of molecular features and/or when they do not lead to formation of pseudomolecular ions during ESI ionization. Keeping in mind these limitations, when used in the iterative mode, DDA was found to perform slightly better than DIA. Most of the compounds identified in the study are pharmaceuticals or their human excretion metabolites. The obtained semi-quantitative concentration values highlighted a group of 10 compounds combining high detection frequencies and median concentrations above

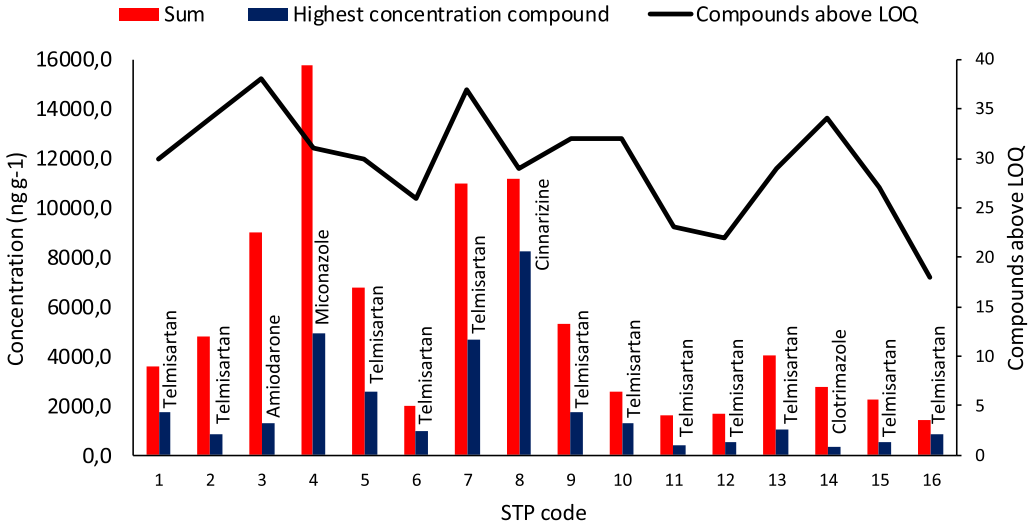

Fig. 3. Total concentration, concentration of the most abundant pollutant and number of compounds above LOQ in sludge extracts from 16 STPs.

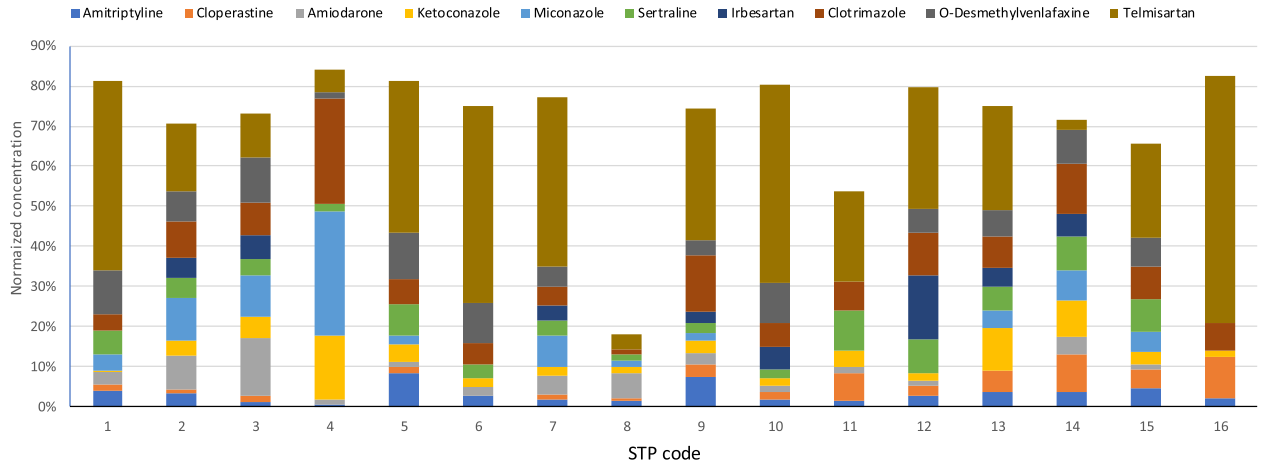

Fig. 4. Normalized concentrations of compounds showing median values above 100 ng g^{-1} and detection frequencies higher than 50% in the set of sludge samples from 16 STPs.

100 ng g^{-1}. Some of these compounds (*O*-desmethylvenlafaxine and the antimycotic drugs: miconazole, clotrimazole and ketoconazole) are concerning species, which have been included in the recent revision of the watch list of pollutants to be monitored in the aquatic environment based on their potential chronic toxicities. Thus, the analysis of sludge from STPs is mandatory to obtain an integrated overview of their mass balances during municipal sewage treatments.

CRediT authorship contribution statement

G. Castro: Investigation, Methodology, Writing – original draft. **M. Ramil:** Data curation, Formal analysis, Writing – review & editing. **R. Cela:** Project administration, Funding acquisition, Writing – review & editing. **I. Rodríguez:** Conceptualization, Supervision, Funding acquisition, Writing – original draft.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146256.

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