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Optimization, validation and application of a novel multiresidue method for prioritization of contaminants of emerging concern in biosolids

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ABSTRACT

Biosolids generated during municipal wastewater treatment are a source of organic matter and essential macronutrients, making them valuable to increase the fertility of soils. However, they also contain variable concentrations of micropollutants, particularly pharmaceuticals, which can pose environmental risks. An analytical methodology based on QuEChERS extraction and LC-MS/MS analysis was optimized and applied to the determination of 59 micropollutants, with large variations in their polarities and different uses, in biosolids from sewage treatment plants (STPs). Extraction efficiencies above 80%, and moderate variations in the efficiency of electrospray ionization were achieved for most of the target analytes in biosolids from different STPs. The accuracy of the final method ranged from 80% to 120% for 57 out of 59 micropollutants, with limits of quantification between 1 and 5 ng g^{-1}, except for chlorhexidine (50 ng g^{-1}). Forty-nine pollutants showed detection frequencies above 70%, with thirty-six compounds quantified in 100% of biosolids from 27 STPs. Biocides and psychiatric drugs represented more than 75% of residues in biosolids, with average accumulated concentrations of 5149 and 2425 ng g^{-1} (dry weight, d.w.), respectively. Within the first group, the maximum concentrations of azithromycin and chlorhexidine stayed above 2300 ng g^{-1} and 11,000 ng g^{-1} d.w., respectively. Ecological risk assessment of biosolids-amended soils identified fifteen compounds whose potential leaching into aquatic environments warrants further investigation. Significative accumulation in biosolids of several pharmaceuticals listed in the Wastewater Directive must be considered together, with their residues in raw and treated wastewaters, to estimate their real removal at STPs.

1. Introduction

Managing sludge in a cost, safe-effective way is a pending challenge of most urban sewage treatment facilities [1,2]. Among the families of micropollutants emitted by urban sewers, pharmaceuticals are of great concern given their inherent bioactivity and, thus, potential impact in the health of the environment. During sewage treatment, micropollutants are distributed between the aqueous phase and the solid fraction, consisting of inorganic materials, insoluble organic matter, and dead microorganisms. Low biodegradability, lipophilic substances are known to be strongly adsorbed onto sludge particles [3]. Moreover, compounds with ionizable moieties might be associated to sludge through electrostatic interactions, leading to residues ranging from ng g^{-1} to μg g^{-1} of sludge (dry weight, d.w., values) [4]. Knowing the content of micropollutants in biosolids (the final stabilized sludge, generated at sewage treatment plants, STPs) is essential for evaluating the performance of treatments applied in the line of sludge, at STPs, such as anaerobic digestion combined, or not, with thermal hydrolysis; or aerobic digestion [5]. It is also crucial for assessing the safety of this waste as a source of nutrients in forestry or agriculture (either directly or after being composted), and to understand the efficiency of other sludge valorisation approaches, including thermal treatments, under nitrogen atmosphere, at different temperatures [6,7]. All these alternatives avoid biosolids incineration and landfilling recognized as environmentally unsustainable practices.

A common problem during the determination of pharmaceutical residues in biosolids is the high complexity of sample extracts. Quite often, serious signal suppression effects during LC-MS/MS analysis impair their sensitive determination. The above drawback has been overcome exploiting the properties of certain pollutants to separate them from co-extracted species. In this regard, a selective sample preparation strategy for the on-line extraction and clean-up of basic

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compounds from freeze-dried biosolids was previously developed within our group [8]. Nevertheless, compounds fractionation strategies, based on mixed-mode sorbents, are not compatible with multiresidue, multiclass procedures (dealing with compounds with different functional groups in their chemical structure) demanded by monitoring laboratories, to which the sample workflow determines the cost of their services. Currently, most multiresidue methods consider generic, low selectivity extraction conditions, based on pressurized-liquid extraction [9] or sonication [10,11] usually followed by reversed-phase concentration of the primary extract after water dilution [4,12,13], leading to time-consuming sample preparation workflows, using expensive solid-phase extraction (SPE) cartridges.

Assuming that target micropollutants are not fractionated and separated from organic species present in biosolids, the complexity of the extracts obtained from organic-matter rich samples is mainly determined by operational conditions during sample preparation. Among the different techniques proposed for the extraction of organic compounds from biosolids and sludge, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) represents one of the simplest methodological alternatives, with the advantages of operating under mild conditions. Peysson et al. [14] firstly assessed the performance of QuEChERs for the multiresidue extraction of pharmaceuticals from sludge. Their pioneer application was followed by other studies combining QuEChERS and sonication to enhance the extraction efficiency [15], and investigating the feasibility to analyse directly the primary extract provided by QuEChERS without any additional clean-up [16].

In this study, the suitability of QuEChERS methodology for the extraction of 59 micropollutants, encompassing diverse chemical families, from dewatered biosolids produced at municipal STPs is evaluated, and parameters affecting sample preparation performance investigated in depth. Simultaneous, effective extraction of contaminants of emerging concern followed by a clean-up step based on dispersive solid-phase extraction (d-SPE), enhances analytical workflow efficiency and reduces operational costs during the characterization of multiclass pharmaceuticals and pesticides accumulated in biosolids. In combination with LC-MS/MS, the analytical procedure was applied to investigate the distribution of target compounds in biosolids obtained from 27 STP facilities, serving medium and small populations in Spain. Average residues of micropollutants determined in these biosolids were employed to assess the ecological risk derived from application of this waste as soil fertilizer.

2. Material and methods

2.1. Solvents, sorbents and standards

Methanol (MeOH), acetonitrile (ACN) and formic acid (FA), for LC-MS analysis, were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced in the laboratory using a Geni U system (Rephile, Shanghai, China). QuEChERS extraction sorbents (magnesium sulphate either combined with sodium acetate, or with sodium chloride and citrate salts, corresponding to AOAC and EN variations of QuEChERS procedure, respectively) [15] and 50 mL polypropylene tubes were obtained from Waters (Milford, MA, USA). C_{18} and graphitized carbon sorbents were provided by Sigma-Aldrich (Milwaukee, WI, USA). Oasis HLB cartridges (200 mg, 6 mL) were from Waters.

Selection of targeted compounds was made based on different criteria, such as their previous detection in biosolids [6,8,14,17,18], inclusion in successive revisions of the EU watching lists for Union-wide monitoring in the field of water policy, the EU wastewater treatment directive [19], and a preliminary suspect screening of extracts by LC-QTOF-MS. In a first approach, a group of 59 organic micropollutants (all employed as pharmaceuticals except the pesticides imazalil, metconazole, tebuconazole and chlorpyrifos) and 29 analogues, labelled with deuterium, or ^{13}C, were considered. This suite of micropollutants includes substances with large differences in their polarities, with acid, basic and non-ionizable moieties in their structures, **Table S1**. Individual standards were acquired from Sigma-Aldrich and Cymit (Barcelona, Spain). Stock solutions of native compounds (ca. 1 mg mL^{-1}) were made in MeOH. Same solvent was used to dissolve isotopically labelled internal standards (ILIS) acquired as pure substances. Additional dilutions were also made in MeOH. A mixture of native compounds at a concentration of 1 μg mL^{-1} (5 μg mL^{-1} in case of chlorhexidine) was prepared in MeOH and employed for a maximum of 1 month. A second mixture with ILIS at 2 μg mL^{-1} (10 μg mL^{-1} for chlorhexidine-d_8) was prepared in the same solvent. Calibration standards at concentrations ranging from 0.1 to 100 ng mL^{-1} (0.5 to 500 ng mL^{-1}, chlorhexidine), with ILIS maintained at 20 ng mL^{-1} (except for deuterated chlorhexidine, 100 ng mL^{-1}) were prepared in ACN, 2.5% in FA. Concentrated stocks and calibration standards were maintained at −20 °C.

2.2. Samples and sample preparation

Biosolids were provided in the spring of 2025 by companies managing 27 different STPs, operating in Spain. Selected facilities processed urban wastewater from low and medium size populations (from 10,000 to 30,000 habitant equivalents, h.e.) without hospitals connected to the net of wastewater sewers. These STPs include an activated sludge treatment unit, without quaternary treatments. The combined solid wastes, from primary and secondary units, are not submitted to any treatment apart from dewatering (received biosolids contained between 70% and 75% of moisture). One of the plants processed wastewater from an 80,000-population city, with several hospitals, and applies an anaerobic digestion step of combined sludge before dewatering. The exact origin of each sample is not disclosed due to confidentiality agreements.

After reception, biosolids were freeze-dried, homogenized using a mill grinder, and maintained at −18 °C until analysis. During preliminary optimization of extraction conditions, experiments were carried out using a non-spiked pool of biosolids prepared by combining samples from several STPs. Responses obtained for compounds noticed in this matrix, well above their procedural limits of quantification (LOQs), were used to identify the best sample preparation conditions. Spiked samples (500 ng g^{-1}, equilibrated overnight) and fortified sample extracts (50 ng mL^{-1}) were considered to calculate extraction efficiencies (EEs) and matrix effects (MEs) for the set of micropollutants involved in the research. Moreover, samples spiked at three different concentrations were used to assess the accuracy of the method. Thus, 0.5 g of biosolids were accurately weighed in polypropylene extraction tubes and fortified with ILIS (200 ng g^{-1}). The mixture was submitted to vortex agitation for 10 min, and re-hydrated by addition of 4 mL of ultrapure water. After homogenization of the slurry, 5 mL of organic solvent, either ACN or ACN:FA (97.5: 2.5, *v*/v), were added followed by vortex agitation, addition of salting-out sorbents (corresponding to AOAC or EN variations of QuEChERS method), centrifugation (3500 rpm, 10 min) and additional clean-up. Under final operating conditions, the sodium citrate buffer sorbents were combined with ACN:FA (97.5: 2.5, v:v) as salting out sorbents and extraction solvent, respectively. For the clean-up step, d-SPE was performed in 2 mL of the organic extract, vortexed with 40 mg of C_{18} and 20 mg of graphitized carbon. The liquid supernatant was passed through a 0.22 μm PTFE hydrophobic syringe filter before LC-MS/MS analysis.

2.3. Determination conditions

Quantification of micropollutants was conducted using an Agilent (Wilmington, DE, USA) 1290 ultra-performance liquid chromatography (UPLC) system, connected to a triple quadrupole MS instrument (QqQ-MS/MS, Agilent 5495D) through an electrospray ionization source (ESI). Compounds were separated using a C_{18}-type column (Zorbax Eclipse Plus, 50 mm × 2.1 mm, 1.8 μm) purchased also from Agilent. Ultrapure

water (A) and MeOH (B), both 0.1% in FA, were combined as mobile phases, at a constant flow of 0.3 mL min^{-1}, using the following gradient: 5% B (0–2 min), 100% B (15–16 min), 5% B (16.1–19 min). The temperature of the column was set at 30 °C and the injection volume was 1 μL. Whenever possible, two transitions were selected for each compound (either native substances, or ILIS). The transition presenting the highest intensity (higher S/N ratio) was selected as quantification transition (Q1), while the second in intensity was selected as qualification transition (Q2). The ESI source was operated in positive mode (ESI+) for all compounds, except for hydrochlorothiazide (ESI-). **Table S2** compiles retention times and MRM transitions, with their collision energies, for native compounds. Those corresponding to ILIS are summarized in **Table S3**. Identification of compounds in the biosolid extracts was made based on retention times and response ratios between Q2 and Q1 transitions matching those observed for calibration standards, within limits of ±0.1 min and ± 30%, respectively.

The analytical procedure was characterized in terms of extraction efficiencies (EEs), matrix effects (MEs) during ESI ionization, accuracy, and limits of quantification (LOQs). EEs, independent of MEs, were calculated as the ratio between the difference of responses for spiked and non-spiked fractions of the same biosolid matrix divided by the difference between spiked and non-spiked extracts multiplied by 100. MEs (%) were estimated as the quotient between differences in responses for spiked and non-spiked extracts referred to a standard of the same concentration (50 ng mL^{-1}) and multiplied by 100. Normalized values close to 100% point out to similar ionization efficiencies at the ESI source independently of the sample matrix [20]. Values below and above 100% correspond to signal suppression and enhancement, respectively. Accuracy was examined using solvent based standards containing the same concentration of ILIS as that expected in biosolid extracts (20 ng mL^{-1} except for chlorhexidine-d_8, 100 ng mL^{-1}). Spiked samples were equilibrated overnight before extraction. As for instrumental LOQs, they were calculated for solvent-based standards with the aim of evaluating the performance of the LC-MS/MS analytical platform without potential matrix effects from co-extracted compounds.

2.4. Risk assessment in biosolid-amended soils

The potential impact of micropollutants quantified in biosolids on the terrestrial environment was evaluated using two different approaches. The first one involves direct comparison of environmental concentrations predicted in soil (PEC_{soil}) with non-observed effect concentration (NOEC) for soil micro-invertebrates [21]. PEC_{soil} were calculated considering an application rate of 0.5 Kg of biosolids per m^2 of soil, assuming the perfect homogenization of biosolids and soil, within the top 20 cm layer, and an average soil density of 1600 Kg m^{-3}. Alternatively, when NOEC are not available, the environmental risk on biosolid-amended soils was assessed using the equilibrium partition method. To this end, the lowest predicted non-effect concentration (PNEC) for aquatic organisms ($PNEC_{water}$) of each drug, available at Norman database [22], was combined with its organic carbon-water partition coefficient (Koc) [23] to estimate their PNEC in soil ($PNEC_{soil}$). Considering standard values of wet soil density (1700 Kg m^{-3}), solid soil fraction (2500 Kg m^{-3}) and organic carbon content (2%), the relationship between both parameters is given by Eq. (1) [24].

$$PNEC_{soil} = (0.1176 + 0.01764*Koc)*PNEC_{water} \quad (1)$$

Risk quotient values (RQs) were calculated either as the ratio between PEC_{soil} and NOEC, or $PNEC_{soil}$, when NOEC values were not available. RQs in the range from 0.1 to 1 correspond to a moderate environmental risk, with values above 1 identifying compounds posing a high risk for the environment.

3. Results and discussion

3.1. LC-MS/MS determination conditions

LC and MS/MS parameters were optimized to enhance the separation of compounds and to prevent interferences among targets, including native species and their labelled analogues. The chromatographic retention times ranged between 3.3 min, for hydrochlorothiazide, and 14.6 min for chlorpyrifos. The use of FA as modifier in aqueous and organic phases improved the peak shape of lipophilic, basic compounds, particularly for chlorhexidine, versus ammonium acetate. Acidification reduced non-reversible interactions of these compounds with negatively charged silanol groups in the stationary phase of the LC column. Occasionally, chlorhexidine presented some chromatographic problems even using FA as modifier. In solvent standards, and in the extracts from most biosolids, a symmetric peak was obtained for this biocide; however, in some samples, both the native compound and its deuterated analogue lead to twin peaks, Fig. 1. Likely, double peaks are the result of complexes and/or ion pairs between the positively charged pollutant and ligands present in certain samples. In these situations, the sum of responses for both peaks, for the native biocide and its deuterated analogue, were used. For the macrolide antibiotics azithromycin and clarithromycin, the use of FA permitted their chromatographic separation (conversely to ammonium acetate), preventing misidentification problems since the MS/MS spectra of both compounds share several product ions (ions at *m/z* values 158, 116 and 83, **Fig. S1)**.

As regards MS/MS detection conditions, climbazole-d_4 showed two common transitions with native imazalil (297 > 69 and 297 > 169) and very close retention times, **Tables S2 and S3**. This problem was overcome using the less intense, but more selective transition of imazalil (297 > 255) for quantification purposes. Azithromycin ionized as a mixture of single and double charged features (*m/z* 749.5 and 375.3, respectively), with the latter ion presenting the highest intensity. Despite this fact, the single charged species was preferred as precursor ion to minimize interferences in the response of azithromycin-d_3 ($[M + H]^+$ and $[M + 2H]^{2+}$ ions at 752.5 and 376.8 *m/z* units), given that native and deuterated species share same product ions. Some authors have reported problems with the stability of this antibiotic due to, sorption to glass material, including autosampler vessels [25]. In this research, using standards prepared in ACN, or ACN:FA, stable responses were observed for the macrolide antibiotics azithromycin and clarithromycin for at least one week.

A critical pair of compounds to determine due to isobaric interferences and co-elution problems were sertraline and its *N*-demethylated derivative, norsertraline. Sertraline and sertraline-d_3, initially considered as ILIS for the parent drug and its demethylated derivative, experienced in source-fragmentation leading to an ion at *m/z* 275, identically to the most intense feature in the ESI(+)-MS spectrum of norsertraline [8]. Moreover, sertraline-d_3 and norsertraline show identical retention times. Given this situation, $^{13}C_6$-norsertraline, instead of sertraline-d_3, was employed as ILIS for both compounds. This selection avoids cross-talking problems of native compounds with this ILIS; nevertheless, partial co-elution of norsertraline and sertraline (figure not shown) made challenging the quantification of the first compound, particularly in samples containing much higher signals of the parent drug than of its metabolite.

Under optimized LC-MS/MS conditions, considering an injection volume of 1 μL, instrumental LOQs, calculated using solvent-based standards, varied between 0.05 and 1 ng mL^{-1} (**Table S2**), with determination coefficients above 0.99 for calibrations standards ($n = 8$ concentration levels) in the range from 0.1 to 100 ng mL^{-1}. Chlorhexidine presented serious contamination problems, whose exact origin could not be identified. For this compound, the instrumental LOQ was set at 5 ng mL^{-1} and the linear response range extended up to 500 ng mL^{-1}.

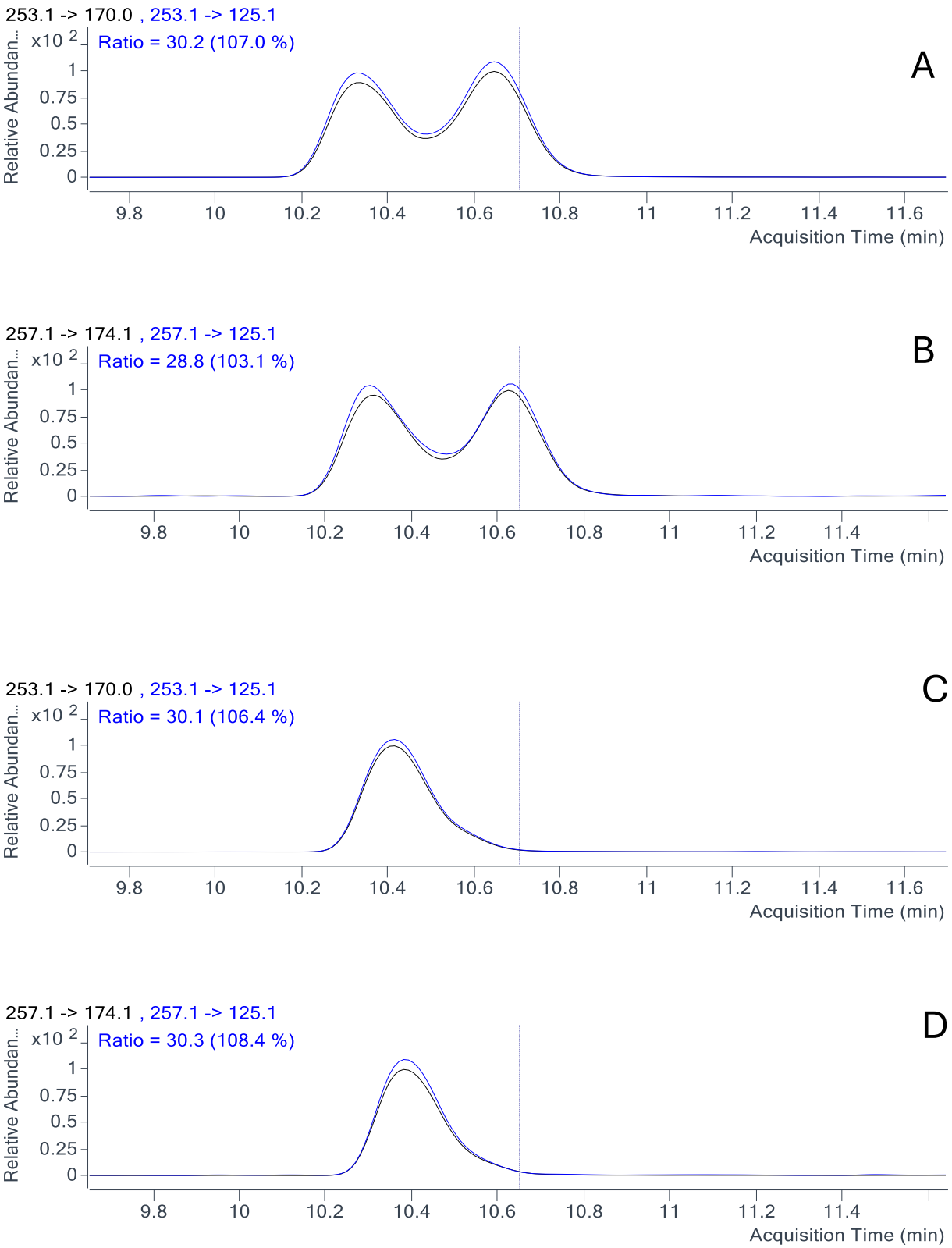

Fig. 1. MRM chromatograms corresponding to Q1 (blue) and Q2 (black) transitions of chlorhexidine (A and C) and chlorhexidine-d_8 (B and D) in the extracts from two different biosolids, showing double peaks (A and B) and the usual chromatographic behaviour of the biocide (C and D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Sample preparation conditions

QuEChERS is conceived as a two-step sample preparation technique. Its extraction efficiency is controlled by factors affecting the partition of compounds between moisturized biosolids and ACN. Thus, salts employed in this step could affect the yield of the extraction. Furthermore, addition of FA might help to disrupt the structure of the sample and to break electrostatic interactions between basic compounds

(existing in protonated forms) and opposite charged entities present in biosolids. In case of polar species with acidic groups, FA enhanced their affinity by the organic phase during extraction. Using the same pooled matrix of biosolids, acetate and citrate versions of QuEChERS method produced equivalent responses for 90% of compounds detected in this matrix. Only the antihypertensive agents olmesartan and candesartan, together with chlorhexidine, and the highly lipophilic compounds amiodarone and chlorpyrifos, showed higher normalized responses using citrate salts, **Fig. S2**. Whatever the employed salts (acetate or citrate) the combination of ACN with FA presented a positive effect in the responses of many compounds found in the extracts from a non-spiked pool of biosolids, Fig. 2.

The primary extract of QuEChERS showed an intense, brownish colour, preventing its systematic, direct analysis by LC-MS/MS. To reduce its complexity two different clean-up approaches were tested. First, dilution of the extract with ultrapure water (1:10 ratio) followed by SPE extraction using OASIS HLB, 200 mg cartridges (previously conditioned with MeOH followed by ultrapure water, 5 mL each), was tested [4,26,27]. Thereafter, the sorbent was washed using 5 mL of MeOH:H_2O (90:10) followed by elution with 5 mL of MeOH. Under these conditions, some polar pharmaceuticals, i.e. tramadol, venlafaxine, and their metabolites, were not effectively trapped by the OASIS HLB sorbent. Although retention of these compounds might be improved using a larger amount of sorbent, this will increase the cost of analysis. Another alternative is the use of d-SPE. This approach was tested as a faster and a cheaper clean-up approach. To this purpose, C_{18} and graphitized carbon were added to a fraction of the acidified extract in ACN. **Fig. S3A** shows the normalized responses after addition of 40 mg of C_{18} and 20 mg of graphitized carbon to 2 mL of QuEChERS extract versus those observed for a raw fraction. Signal reduction effects were not noticed, except in case of chlorhexidine. On the other hand, the visual complexity of the extract decreased after the d-SPE clean-up, **Fig. S3B.**

Table 1 summarizes the EEs of QuEChERS extraction followed by d-SPE clean-up. Most compounds (except atorvastatin, EE 68%) were recovered to an extent above 70%. Ciprofloxacin could not be effectively recovered (average values below 10%) under any of the tested variations of QuEChERS method; thus, it was excluded from the list of target micropollutants. This antibiotic presents a high polarity (log D -2.5 at pH 7) and a zwitterionic behaviour, **Table S1**, which probably hamper its transfer from the aqueous to the ACN phase during QuEChERS extraction. This finding matches previous drawbacks reported during application of QuEChERS to the extraction of fluoroquinolone antibiotics [14], and with the low extraction yields of other solid-liquid extraction alternatives, i.e. sonication, using different organic solvents [28]. MEs observed for pools of biosolids prepared by combining samples from different STPs are also summarized in Table 1**.** Signal suppression percentages between 40% and 60% were observed for chlorhexidine and several lipophilic compounds (chlorpyrifos, ketoconazole, norsertraline and sertraline). Moderate enhancement (ca. 30%) was noticed in both pools of biosolids for azithromycin and the antiviral drug oseltamivir. For the rest of compounds, normalized responses in spiked extracts ranged from 70% to 120% of those corresponding to a standard of the same concentration prepared in ACN:FA (97.5:2.5).

Changes in the composition of biosolids from different STPs might affect the efficiency of QuEChERS extraction and ESI ionization limiting the robustness of the analytical procedure. To evaluate this effect, without considering the contribution of native existing in the different biosolids, the responses of ILIS in the extracts of 8 different biosolids were compared with their mean responses for calibration standards. The normalized responses of ILIS in sample extracts ranged from 50% (amiodarone-d_4 and chlorhexidine-d_8) to 111% (azithromycin-d_3), with RSDs below 18%, except chlorhexidine (28%), (Fig. 3). As far as these data are coherent with EEs and MEs compiled in Table 1, the performance of extraction and clean-up steps seems to be similar for different biosolids.

The accuracy of the analytical procedure using solvent-based calibration standards was investigated with a pool of biosolids fortified at three different concentration levels: 500, 200 and 50 ng g^{-1} (2500, 1000 and 250 ng g^{-1} in case of chlorhexidine) and extracted in triplicate, **Table S4**. Average recoveries corresponding to above concentrations are also compiled in Table 1**.** From the selection of 58 compounds, the method provided quantitative recoveries (between 80 and 120%) except in case of the pesticide chlorpyrifos, which was affected by signal suppression problems and no isotopically labelled analogue was available. For some compounds, accuracy could not be assessed at the lowest concentration fortified due to the high signal obtained in the non-spiked samples, **Table S4.** Procedural LOQs were estimated as the instrumental LOQs of the LC-MS/MS (**Table S2**) multiplied by the ratio between sample extract (5 mL) and the amount of sample (0.5 g), considering average values of MEs summarized in Table 1**.** Obtained procedure LOQs ranged from 1 ng g^{-1} to 10 ng g^{-1}, except for chlorhexidine with a LOQ of 50 ng g^{-1}.

3.3. Occurrence in biosolids

Table S5 compiles the concentrations of the investigated micropollutants in a total of 28 samples of biosolids obtained from 27 different STPs. Out of the selection of 58 compounds included in the final protocol, only three species (fluconazole, metconazole and oseltamivir) remained below their LOQs (from 3 to 10 ng g^{-1}) in all samples. Fluconazole and oseltamivir are polar compounds (Log D values below the unit at pH 7), without ionizable groups permitting to establish electrostatic interactions with oppositely charged particles of biosolids. Metconazole is a medium polarity pesticide authorized for agricultural uses; however, no data could be obtained regarding its market-share in Spain. Four additional pharmaceuticals (atenolol, carbamazepine, clindamycin, and metoprolol) and the pesticides chlorpyrifos and imazalil were found above their LOQs in less than 70% of the analysed biosolids, with median values below 20 ng g^{-1}. The rest of compounds (49 substances) presented detection frequencies above 70%, with 36 pollutants quantified in 100% of biosolids, Table S5. The chromatograms corresponding to selected pharmaceuticals in the extracts from biosolids are shown as supplementary information, **Fig. S4**.

Fig. 4 presents median and maximum concentrations of different micropollutants attending to their pharmacological applications. As for psychiatric pharmaceuticals, four active drugs (sertraline, amitriptyline, trazodone, and citalopram) and the metabolite of sertraline (norsertraline) presented median concentrations above 100 ng g^{-1} d.w. in biosolids**,** Fig. 4. This group of substances includes from moderate to highly polar species, such as amisulpride (log D –0.0796), existing in the protonated form at neutral pH, and detected in 100% of samples, confirming the accumulation of non-polar and polar compounds in biosolids due to adsorption and electrostatic interactions, respectively. The presence of relevant concentrations of citalopram in biosolids is a significant concern, as this substance is used as a marker pollutant designed to verify the performance of STPs based on concentrations measured in inlet and outlet aqueous streams [19]. That is, a reduction of 80% in the concentration of this compound in the effluent of STPs does not grant an equivalent removal efficiency given its accumulation in biosolids. Other psychiatric drugs included in this directive were also ubiquitous in biosolids (case of amisulpride and venlafaxine) although at lower concentrations than those measured for citalopram. In comparison with previous studies involving the analysis of biosolids from a representative number of STPs, the mean residues of sertraline and norsertraline in the samples processed in this research (807 ng g^{-1} and 554 ng g^{-1}, $n = 28$ samples) were two-fold higher than those found in central Europe (521 and 205 ng g^{-1} for sertraline and norsertraline, respectively, $n = 40$ samples), whilst those of citalopram were lower (127 ng g^{-1} vs 382 ng g^{-1}, median residues) [16].

In case of biocides, seven compounds showed median residues above 100 ng g^{-1} (d.w.), with chlorhexidine presenting median and maximum values above 1000 and 10,000 ng g^{-1}, respectively. The highest

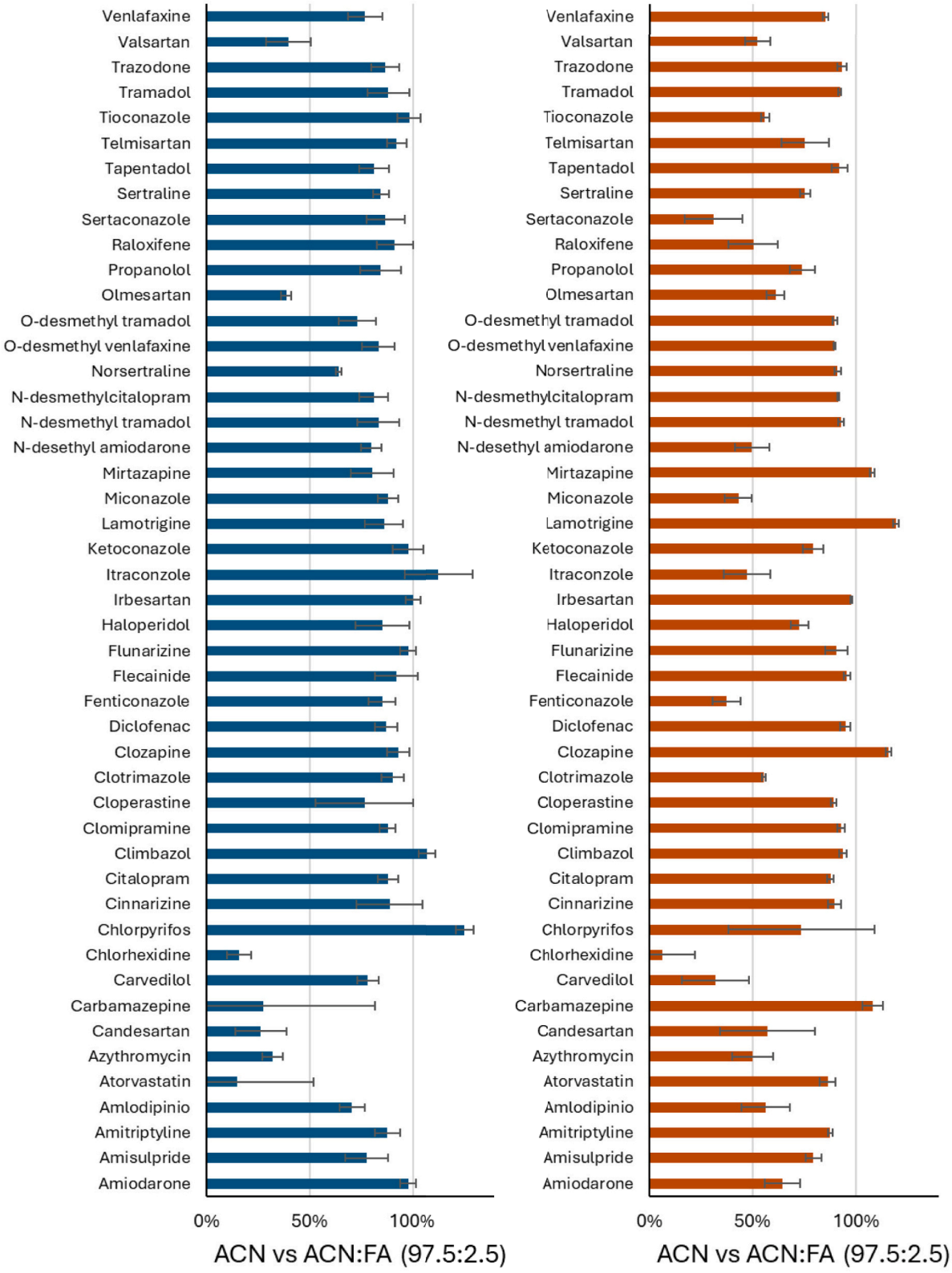

Fig. 2. Effect of extraction solvent (ACN vs ACN with 2.5% of FA) in the relative responses of micropollutants measured in extracts from a non-spiked pool of biosolids. Data for acetate (left) and citrate (right) QuEChERS modalities.

Table 1
Extraction efficiency (EE, %), matrix effects (MEs, %) during ESI ionization, average accuracy ($n = 9$ samples spiked at three different concentrations) and procedural LOQs. Data for a pool of biosolids from different STPs.

Compound	EE (%)		MEs (%)				Accuracy (%)		LOQs (ng g^{-1}, d.w.)
	Mean	SD	[a]Mean	[a]SD	[b]Mean	[b]SD	Mean	SD	
Amiodarone	95.7	1.0	75.4	0.9	76.3	0.3	98.3	3.9	1
[c]Amisulpride	105.1	1.0	111.9	1.2	107.6	1.4	98.7	2.8	0.5
Amitriptyline	105.2	1.1	99.1	1.0	95.1	0.1	93.0	3.0	1
Amlodipine	103.8	1.4	99.4	1.5	100.5	0.2	86.6	4.1	1
Atenolol	89.0	1.0	101.7	1.3	95.9	0.4	100.5	2.8	2
Atorvastatin	67.7	3.7	65.8	1.5	68.8	1.1	82.9	6.3	2
Azithromycin	74.0	8.0	132.0	1.3	127.0	0.8	97.5	5.7	5
[c]Candesartan	99.7	1.6	119.0	0.3	113.7	3.8	94.6	3.9	5
[c]Carbamazepine	104.4	0.7	74.4	0.7	65.8	0.2	99.2	3.0	2
Carvedilol	100.4	0.4	85.2	1.0	60.2	1.7	88.2	3.1	2
Chlorhexidine	90.8	1.4	32.6	7.6	46.4	1.2	116.2	2.7	50
Chlorpyrifos	101.7	2.3	59.7	0.1	57.5	2.4	54.7	5.5	2
Cinnarizine	103.9	0.6	104.6	0.4	91.3	0.9	102.3	5.2	1
[c]Citalopram	107.4	1.0	99.3	1.0	91.3	0.9	100.4	15.4	1
[c]Clarithromycin	103.8	1.5	97.9	0.1	86.4	2.2	86.7	3.2	1
Climbazole	94.1	1.2	88.7	1.2	80.1	0.9	90.3	2.8	1
Clindamycin	108.5	0.6	99.2	0.2	92.3	0.6	86.8	6.8	1
Clomipramine	103.9	0.5	100.9	0.7	89.7	0.6	93.9	2.2	0.5
Cloperastine	105.9	5.9	103.3	0.9	98.8	0.6	94.0	2.2	1
Clotrimazole	99.8	0.8	96.6	0.2	87.4	1.3	100.5	5.1	1
Clozapine	99.4	1.3	97.7	0.6	87.8	1.8	103.2	2.4	1
[c]Diclofenac	101.3	1.1	91.2	0.5	102.6	1.7	107.9	3.1	1
Fenofibrate	101.8	3.6	90.4	0.3	80.4	1.6	78.5	5.7	1
Fenticonazole	100.6	2.1	95.1	0.1	80.3	0.9	95.9	10.1	2
Flecainide	105.0	1.0	108.0	1.0	99.3	2.9	95.5	3.5	0.5
Fluconazole	101.8	2.5	120.7	1.1	108.7	1.6	91.4	4.0	5
Flunarizine	104.5	1.3	98.3	0.8	81.3	0.4	91.4	2.4	1
Haloperidol	103.8	0.8	101.8	0.1	89.5	1.7	83.6	3.3	1
[c]Hydrochlorothiazide	104.2	1.0	101.8	0.5	109.8	2.9	105.7	7.3	5
Imazalil	106.3	2.4	99.0	1.0	89.0	2.0	105.6	4.0	2
[c]Irbesartan	104.5	0.7	97.2	1.6	83.5	1.9	103.9	4.7	1
Itraconazole	110.3	9.0	95.9	1.2	121.6	1.0	99.1	8.3	10
Ketoconazole	103.4	1.5	67.5	2.0	58.2	1.8	87.5	3.1	10
Lamotrigine	97.0	1.3	76.9	0.4	67.1	0.2	91.7	7.8	10
Metconazole	107.0	1.0	107.0	2.0	90.0	2.0	96.5	3.2	2
[c]Metoprolol	105.4	0.9	95.9	1.3	90.4	0.8	106.5	1.9	5
Miconazole	99.5	1.0	89.1	1.3	81.2	0.4	99.1	3.6	2
Mirtazapine	96.5	0.9	106.0	0.6	95.8	1.4	101.9	4.1	1
N-desethylamiodarone	100.3	0.9	68.6	1.1	67.1	0.2	92.5	4.2	1
N-desmethyltramadol	106.0	1.1	107.3	1.1	103.2	1.8	97.0	3.3	1
N-desmethyl citalopram	103.4	0.9	92.4	0.6	83.4	0.5	80.2	7.7	2
Norsertraline	98.9	1.6	46.2	2.1	44.7	1.9	109.3	3.7	10
O-desmethylvenlafaxine	103.1	1.0	106.2	0.2	103.5	0.2	98.5	3.0	1
O-desmethyltramadol	103.2	0.6	110.7	0.9	98.4	0.5	90.7	3.1	1
Olmesartan	100.9	1.1	103.6	1.1	103.2	1.1	95.9	4.7	2
Oseltamivir	100.7	1.8	132.7	1.3	138.9	0.7	94.9	4.7	2
Propranolol	105.4	1.0	93.2	0.4	80.4	1.0	100.3	2.4	1
Raloxifene	100.3	1.4	89.2	1.9	78.5	2.2	79.2	2.5	1
Sertaconazole	96.6	0.9	91.7	2.7	80.9	2.0	87.5	2.5	2
Sertraline	100.4	1.4	46.4	1.0	53.8	0.1	70.1	3.5	2
Tapentadol	107.8	0.6	105.9	0.6	102.0	0.1	99.1	2.7	5
Tebuconazole	103.3	0.7	116.3	1.1	107.6	0.4	94.7	2.5	2
Telmisartan	113.9	3.0	75.7	0.2	72.3	3.4	95.0	5.2	2
Tioconazole	101.7	0.6	92.8	1.7	78.1	0.4	105.8	2.7	2
Tramadol	100.1	8.7	109.3	0.4	106.3	0.8	96.3	3.6	1
Trazodone	104.2	1.3	91.8	0.9	79.6	1.3	89.1	3.8	1
Valsartan	104.5	3.0	99.5	0.2	105.0	0.5	109.6	4.3	5
[c]Venlafaxine	106.5	0.7	102.1	0.1	86.3	1.7	98.9	5.9	1

[a] and [b] denote two different pools of biosolids.
[c] Compounds included in the Wastewater Directive (Directive EU 2024/3019) to monitor the removal efficiency of micropollutants during treatment of municipal wastewaters.

concentrations of chlorhexidine were found in the two biosolid samples obtained from STP7, the only facility receiving a combination of urban and hospital wastewaters and including an anaerobic digestion unit. This trend agrees with previous reports on the stability of this biocide during anaerobic digestion processes [26], and the very high concentrations of chlorhexidine in raw hospital wastewater (up to 89 μg L^{-1}) [29]. Average and maximum residues of this biocide in biosolids from several STPs in Sweden were 8900 and 19,000 ng g^{-1}, respectively [12].

The 2nd position within this group corresponded to the macrolide antibiotic azithromycin, with a median residue of 550 ng g^{-1}, followed by five azolic antimycotics, mainly used in topic applications, with mean residues above 100 ng g^{-1}. High concentrations of azithromycin, and in general of biocides (particularly antibiotics), in biosolids are concerning since they contribute to the development of resistant microorganisms [28]. Globally, biocides and psychiatric drugs represented more than 75% of residues found in biosolids, with average values of 5149 and

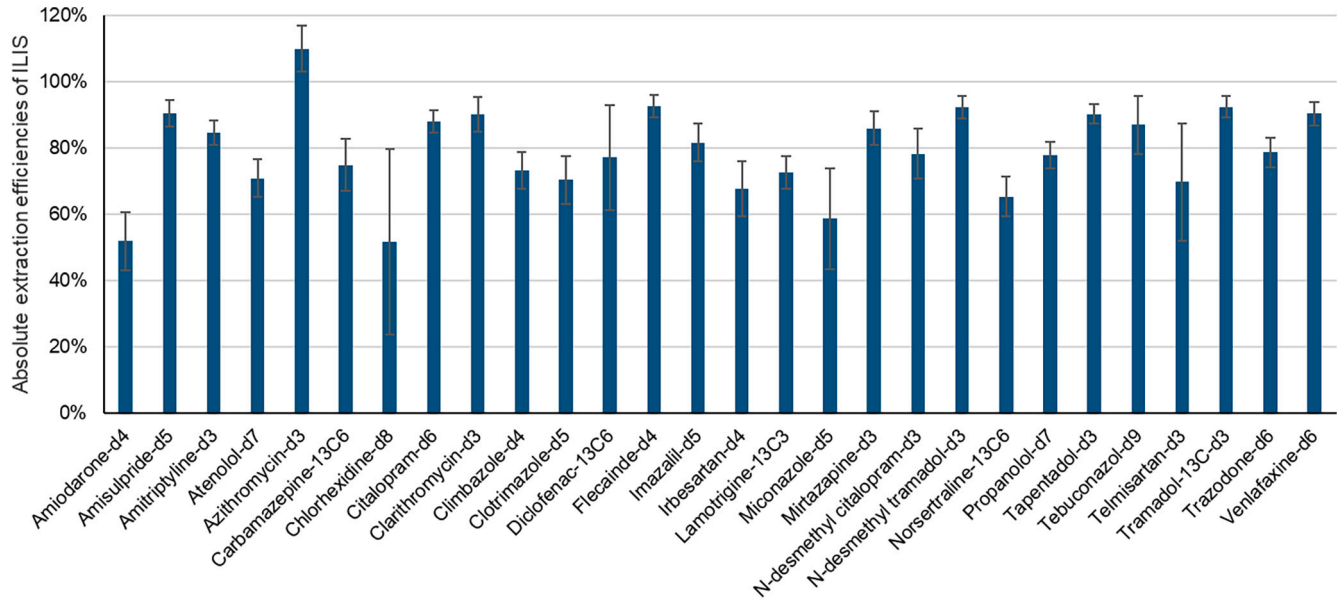

Fig. 3. Normalized responses of ILIS in the extracts from 8 different biosolids (n = 2 replicate extractions per sample) compared to those obtained in calibration standards.

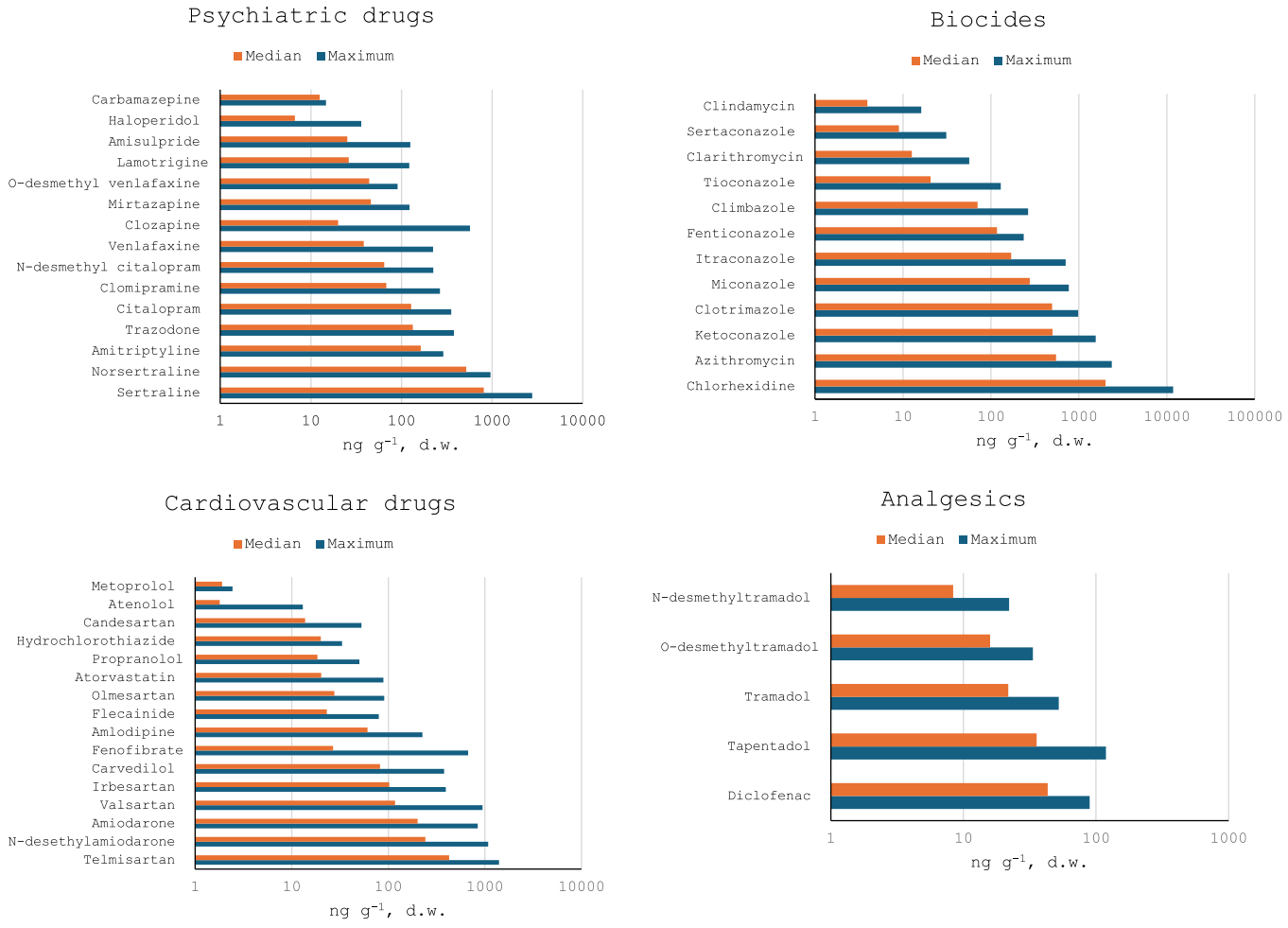

Fig. 4. Prioritization of pharmaceutical residues in biosolids as function of their therapeutic uses.

2425 ng g^{-1} d.w., respectively.

Cardiovascular drugs found in biosolids comprise antiarrhythmics (amiodarone, flecainide), antihypertensive agents (beta-blockers, angiotensin receptor antagonists, ARA-II) and cholesterol control drugs.

Median concentrations of the iodinated antiarrhythmic amiodarone, its desethyl transformation product and three ARA-II drugs (telmisartan, valsartan and irbesartan) stayed above 100 ng g^{-1}. Maximum concentrations of both compounds, telmisartan and valsartan stayed around 1000 ng g^{-1}. Telmisartan is recognized as one of the highest concentration drugs in biosolids with values up to 5000 ng g^{-1} [6]. Conversely, the average residues of valsartan in biosolids found in our study (140 ng g^{-1}, Table S5) are 6–7 times above those reported for STPs in Greece [15]. Similar to citalopram, irbesartan has also been selected as an indicator pollutant for assessing the efficiency of STPs [19]. Thus, it might be relevant to determine their relative fraction remaining in biosolids.

The non-steroidal anti-inflammatory diclofenac, and the opioid analgesics tapentadol, tramadol and the two major metabolites of the latter showed a limited contribution to total residues measured in biosolids, with median values between 8 ng g^{-1} (*N*-desmethyl tramadol) and 45 ng g^{-1} (diclofenac), Table S5. On the other hand, they were ubiquitous in all the processed samples.

As regards compounds not included in the above groups and detected in all samples, their mean residues varied between 28 ng g^{-1} for cinnarizine to 126 ng g^{-1} for the antitussive cloperastine.

3.4. Potential environmental implications

Application of biosolids as a source of nutrients, and more particularly of phosphorous (regarded as a critical raw material), in soil might pose ecological and human risks [30]. *Re*-introduction of micropollutants in the human food web can be minimized by disposing biosolids in gardening or forestry, instead of crop fields. On the other hand, the impact of this practice in the health of soil is more difficult to predict [31]. Considering the application rate of biosolids defined in section 2.4, PEC_{soil} are 680 times lower than the concentrations of micropollutants measured in biosolids [32]. Unfortunately, a direct comparison between these values and NOEC was possible only for pesticides. The maximum residues of the three pesticides determined in biosolids in this study (49, 21 and 93 ng g^{-1} for chlorpyrifos, imazalil and tebuconazole, respectively) were below their chronic exposure, non-observed effect concentration (NOEC) in earthworms and mites [21]. Based on these values and the application rate above discussed, their RQs remain below 0.1. Thus, they do not represent an ecological hazard. In case of pharmaceuticals, NOEC thresholds are not available, neither for soil microinvertebrates nor for bacteria, fungi, or archaea. Thus, a direct estimation of they risk quotients (RQs) is not possible, despite the potential bioaccumulation of same drugs in earthworms [33], and the link between biocide residues and changes in the microbiome of soil [34].

Table S6 summarizes RQ values calculated using the equilibrium partition method applying Eq. (1), based on mean concentrations of compounds quantified in the processed samples of biosolids. Seven compounds showed moderate ecological risks (RQs between 0.1 and 1)

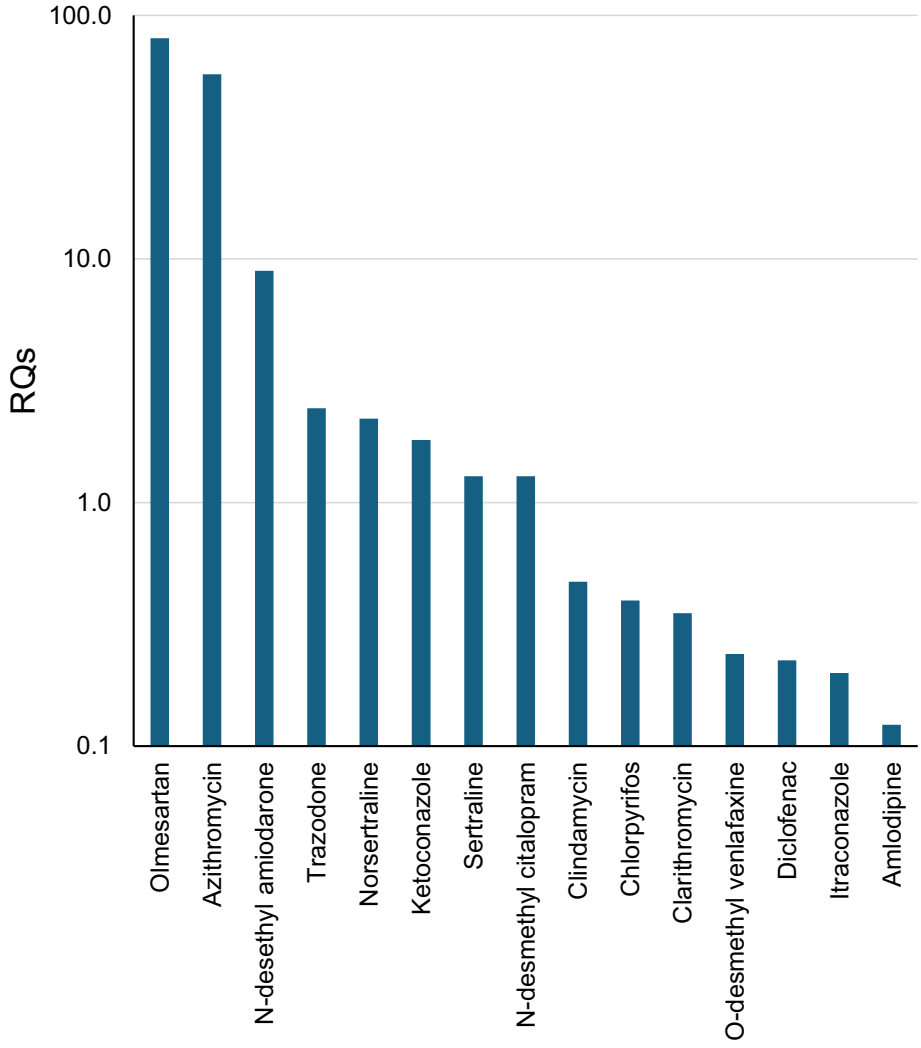

Fig. 5. Risk quotients (RQs) of selected compounds calculated from Koc coefficients predicted by EPA software tool (EPI SUITE 4.11) using the equilibrium partition method.

and eight drugs were classified within the high-risk category (RQs above 1), Fig. 5. Based on these predictions, urges to calculate the experimental soil water distribution constants (Kd) of those substances with RQs above 0.1 to fully understand the effect of additional parameters to soil organic carbon (i.e. pH, texture, cationic exchange capacity) in their leaching potential [35]. Likely, electrostatic interactions between charged moieties existing in the structure of ionizable pollutants with soil components will lead to lower leaching risks than those derived from predicted Koc values. As example, the experimental Kd values of azithromycin, in soils with different characteristics, varied between 0.47 L $Kg^{-1}$and 202 L Kg^{-1} [36,37]. Combining these experimental values with the $PNEC_{water}$ and the average residue of this antibiotic in biosolids, estimated RQs varied from 0.27 to 116, versus the 57.3 RQ value derived from the predicted Koc of this antibiotic, **Table S6.** The estimation of environmental risks based on RQs presented in **Table S6** does not consider the time dependent evolution of micropollutants in soil after application of biosolids [38]. Currently, reliable half-life data for pharmaceuticals in biosolids amended soils are still limited; however, some of the compounds highlighted in Fig. 5 have been recognized as persistent in biosolids amended soils [39,40], as is the case of sertraline (estimated half-life, DT50, from 48 to 84 days), or very persistent, as the antibiotic azithromycin (DT50 above 1 year) [39,40]. This scenario increases the risks of potential negative effects in the health of soils derived from punctual applications and accumulation in this compartment. Finally, cumulative, synergistic, or antagonistic interactions among co-occurring contaminants may result in either enhanced, or diminished, risks associated with the use of biosolids as soil amendments.

3.5. *Future challenges*

Alternatives to implement the treatment of biosolids generated at STPs, aiming among other issues a reduction of micropollutants residues, include anaerobic digestion, composting and pyrolysis. Using information compiled in a recent review [3], anaerobic digestion alone seems to have a limited efficiency to remove most pharmaceuticals identified in sludge. In case of chlorhexidine (the substance found at highest concentrations in this study) data published for biosolids from STPs applying anaerobic digestion in the North of Europe reflected similar residues to the highest values reported in this research [12]. At pilot scale, low temperature pyrolysis reactors reduced the concentrations of pharmaceuticals around 90% in biochar versus those existing in biosolids [6]. However, further research is required to calculate the presence of target compounds, and those of potential degradation products, in condensates and/or emissions generated during pyrolysis experiments before upscaling this technological approach to STPs.

4. Conclusions

QuEChERS extraction combined with d-SPE clean-up and LC-MS/MS, based on a QqQ instrument, provides a direct and sensitive approach for monitoring a broad spectrum of pharmaceuticals and pesticides in biosolids from urban STPs employing a reduced amount of clean-up sorbents compared to methodologies based on dilution and further concentration of the entire extract using SPE cartridges. The main limitation of this analytical methodology lies in its low extraction efficiency for small, zwitterionic compounds, such as fluoroquinolone antibiotics, which exhibit poor solubility in organic solvents. Based on the residues detected in biosolids from medium-sized municipalities, direct application of this waste to agricultural soils or other land uses (e.g., gardening, forestry) is not advisable. Biosolid analysis offers complementary insights to those obtained from liquid samples, enabling a more accurate assessment of STP performance. This information is essential for revising policies on the sustainable use of biosolids and for guiding technological improvements aimed at reducing the presence of chemicals of emerging concern in biosolid-derived products, such as biochar and compost.

CRediT authorship contribution statement

A. Barros: Writing – review & editing, Validation, Methodology, Investigation, Data curation. **G. Castro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **M. Ramil:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition. **I. Rodríguez:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation, Conceptualization.

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.microc.2026.118188.

Data availability

Data will be made available on request.

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