Analysis of 60 pharmaceuticals and personal care products in sewage sludge by ultra-high performance liquid chromatography and tandem mass spectroscopy

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A B S T R A C T

This paper presents a comparison of three different analytical proposals for the determination of 60 pharmaceuticals and personal care products (PPCPs) in solid urban sewage sludge. Two fast sample pretreatments, i.e., online solid-phase-extraction (online SPE) and direct injection (DI), were tested against the conventional offline solid phase extraction (offline SPE). In all cases, subsequently, extracts underwent ultra-high-performance-liquid chromatography coupled to tandem-mass-spectrometry (UHPLC-MS/MS), simultaneously operating in both positive and negative electrospray ionization (ESI) mode. In addition, as solid matrices, clean-up steps were necessarily preceded by ultra-sound-assisted extraction (UAE) in all cases. Matrix-matched quantification was combined with internal standard providing high reliability to all three approaches. Best performance was observed for the fully automatized and non-pretreated DI method, showing limits of detection below 30 ng g^{-1} for many of the target compounds, and recoveries between 80 and 120%. Finally, the best working method was validated and applied to the analysis of PPCPs in different dewatered digested sludge samples from the wastewater treatment plant (WWTP) in Valladolid (Spain). Acetaminophen was found at concentrations above 1,000 ng g^{-1}.

1. Introduction

Pharmaceuticals, used to treat human and animal diseases, and personal care products include different classes of organic chemicals, which are used especially for a better quality of daily life. Once they reach the environment become pollutants that fall into the category of compounds of emerging concern (CECs) [1–3].

Wastewater treatment plants (WWTPs) serve to treat wastewater, reducing the adverse effects of various pollutants on the environment and maintaining a good quality of life for all living beings. However, they are designed to remove solid materials and reduce levels of metals, bacteria, and other pathogens [4], but are not efficient for the removal of PPCPs, so effluents discharged directly into aquatic systems may contain significant amounts of these substances [5]. The use of analgesics and anti-inflammatory drugs is high in southern European countries, such as Spain [6], and salicylic acid and ibuprofen are the compounds with the highest concentrations found in wastewater and sludge [7].

In recent years, there has been a boom in the development of analytical methods for the determination of PPCPs in environmental matrices such as sewage sludge. A literature review by Pérez-Lemus et al., [8] reported that LC-MS/MS is the main technique used for the analysis of PPCPs in environmental matrices because it is highly versatile, sensitive and selective [9]. Regarding sample pre-treatment, UAE is mostly used when PPCPs are determined in solid environmental matrices such as sewage sludge. This technique is easy and fast in preparation, and, in addition, reduces the usual requirements of high organic solvent volumes [8]. As this extraction step is not very selective, it is conventionally followed by a clean-up protocol. To this end, SPE, onto disposable cartridges in a manifold, is the technique most commonly used. It requires the subsequent cartridge elution, solvent evaporation and extract reconstitution before instrumental analysis [10–11]. All these steps are time consuming –they usually take two full

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working days-, and are a source of error introduction as they typically entail low automation as well as analyst manipulation and exposure to toxic chemicals. In addition, large quantities of disposable material and organic solvents are employed. In the last years, alternatives have been developed which instrumentalist SPE and even couple it to the chromatographic section [12–17]. However, their implementation in the routine analysis labs is still scarce. Even less has been explored regarding the possibility of relaying all the selectivity needed after the extraction step to the chromatographic section that is to dispense with the clean-up step by SPE [18–20].

The main objective of this study is to compare the performance of three analytical approaches in the determination of 60 PPCPs of several classes (analgesics/anti-inflammatory, antibiotics, lipid regulators, psychiatric and cardiovascular drugs, hormones, preservatives, surfactants, anti-parasites, plasticisers, triazine herbicides and antimicrobials) in sewage sludge. All of them included UAE and LC-MS/MS, in an attempt to represent most of the current methodologies. Two fast pretreatments, i.e., a fully automated SPE online coupled to LC-MS/MS (online SPE), and a direct injection of the UAE extract with no prior clean-up (DI) were tested against the conventional manual SPE-extract reconstitution clean-up protocol (offline SPE).

To the best of our knowledge, this is the first time the performance of sewage-sludge-extracts DI into the LC-MS/MS has been assessed. Novel is also the comparison of these two fast approaches, -online SPE and DI-with the conventional offline SPE clean-up which entails a real bottleneck in the throughput of this type of analytical methods. Pros and limitations of each proposal have been discussed. The best working methodology was validated and applied to the analysis of PPCPs in different dewatered digested sludge samples from the WWTP in Valladolid (Spain).

2. Material and methods

2.1. Standards and reagents

The standards for all PPCPs (Table S1 **and List S1 as Supplementary Material** Data) were of high purity grade. Individual stock solutions at 1,000 mg L^{-1} for most PPCPs standards were prepared in methanol (MeOH). PPCPs such as danofloxacin, enrofloxacin, marbofloxacin, ciprofloxacin, ofloxacin, levofloxacin, and norfloxacin were prepared in 0.2% hydrochloric acid (HCl) MeOH/MilliQ® water (1:1) and apramycin in MilliQ® water. From them, two mix stock solutions with 33 and 27 compounds, respectively, were prepared in MeOH at 20 mg L^{-1}. Subsequently, serial dilutions (2, 0.5, 0.05, 0.005) mg L^{-1} in MeOH contained all the analytes. All solutions were immediately stored at −80 °C in darkness until use in the analysis. Sixteen internal standards, such as the isotopically labelled sulfadiazine-d4, sulfadimidine-d4, ciprofloxacin-d8, sulfamethoxazole-d4, danofloxacin-d3, enrofloxacin-d5, rac ibuprofen-d3, rac naproxen-d3, diclofenac-d4, salicylic acid-d4, clofibric acid-d4, methyl-d4-paraben, ethyl-d5-paraben, propyl-d7-paraben, triclosan-d3, bisphenol A-d8 (LGC Standards (Barcelona, Spain) (Table S1), were used. Individual and a mixture of isotopically labelled internal standards were prepared in MeOH as well as its corresponding serial dilutions (2, 0.5, 0.05, and 0.005 mg L^{-1}). All of them were also stored at −80 °C in darkness.

High purity solvents, i.e., LC-MS-grade MeOH, LC-MS-grade acetonitrile (ACN) and 98%-purity formic acid (FA), were provided by Labbox (Madrid, Spain). Ethylenediaminetetraacetic acid (EDTA), with 99% purity, and aluminium oxide (Al$_2$O$_3$) were purchased to Sigma-Aldrich (Madrid, Spain). Concentrated (37%) HCl and sodium hydroxide (98% purity) were supplied by Panreac (Barcelona, Spain). Oasis HLB (60 mg, 3 mL) SPE cartridges from Waters (Barcelona, Spain). All aqueous solutions were prepared in deionised water with a resistance of not<18 MΩ cm. Nitrogen (N$_2$), with 99.999% purity, was provided by Abelló Linde S.A. (Madrid, Spain).

2.2. Sewage sludge analytical methodology

2.2.1. Sample collection and conservation

Sludge samples were collected from the municipal WWTP in Valladolid (Spain). It serves a population of approximately 350,000 inhabitants and treats around 101,000 m^{3}day^{-1} of wastewater. In addition, it generates approximately 9,600 m^3 day^{-1} of biogas from the digestion of 2,500 m^3 day^{-1} of sludge. Activated sludge process is the secondary treatment taking place and responsible of the removal of most part of the macro-pollutants present in the urban wastewater. The surplus biological sludge generated during this aerobic treatment is mixed with the primary sludge and fed to the anaerobic digester. The digestion process considerably reduces the total mass of solids, destroys pathogens, and facilitates dewatering or drying of the sludge. The digested sludge, often called as biosolid, has the appearance and characteristics of a rich potting soil and is dewatered before disposal. The dewatered sludge (DS), which was selected as the matrix for this study, still contains a significant amount of water (up to 80%) but, even at this moisture content, the sludge no longer behaves as a liquid and can be handled as a solid material, making it easier to handle and less expensive to transport

DS samples were collected in high density polyethylene drums with polypropylene screw caps and immediately transported to the laboratory at 4 °C in darkness, where they were stored at −20 °C in darkness. Once they were fully frozen, they were freeze-dried, then, back to store at −20 °C in darkness until analysis.

2.2.2. Offline SPE proposal methodology

Sample pre-treatment for the offline SPE methodological version was based on Argüeso-Mata et al., 2021 [21] and Perez-Lemus et al., 2020 [12], with further optimization to fully adapt the methodology to the matrices and analytes of the present study. Hence, **1)** An exact amount of freeze-dried DS (~0.3 g) was placed in a plastic centrifuge tube (50 mL), spiked with 200 μL of a methanolic solution containing all the isotopically labelled internal standards at 0.5 mg L^{-1}, and then, homogenized. The mixture was, then, let it settle in darkness overnight in the extraction hood for solvent evaporation and internal standard fixation. **2)** Subsequently, the sample underwent UAE in a Sonorex Digitec 160 W 35 kHz ultrasonic bath (Navarra, Spain) for 30 min at 25 °C to assist for the desorption and extraction of the analytes. With this purpose, twelve millilitres of a MeOH/MilliQ® water mixture, 5:95 (v/v) and pH-adjusted to 9 was added as extracting solvent. At this pH, most of the target analytes were expected to be present in their ionic form (Table S1) encouraging their desorption and extraction towards the extracting solvent. Additionally, one hundred milligrams of activated alumina (Al$_2$O$_3$, dried at 105 °C for 48 h) were added to the resulting suspension too, for matrix in-situ clean-up during the UAE. After the UAE, the extract was centrifuged for 10 min at 10,000 rpm and 10 mL of the supernatant were collected with a glass pipette. Then, another fresh 12-mL aliquot of the same extraction solvent was added, and a new extraction cycle was carried out. **3)** The pooled supernatant (20 mL) was filtered through a 0.45-μm polytetrafluoroethylene (PTFE) syringe filter and placed in a 100 mL glass bottle. **4)** A 2 mL aliquot of a 5% EDTA solution and 80 mL of desionized water were added too. The resulting solution was pH 3 adjusted to increase the analyte lipophilia by shifting their acid-base equilibrium towards neutral molecules in preparation for the offline SPE clean-up step. SPE was performed onto Oasis HLB SPE cartridges which were previously activated and conditioned with 3 mL MeOH and desionized water, respectively. After extraction (assisted by vacuum in a manifold) was carried out, cartridges were rinsed with 3 mL of a 5% MeOH solution, and then dried for 20–30 min. Cartridges were stored at −20 °C in darkness if not immediately eluted. For the elution, 2 × 3 mL of ACN were used, with no vacuum assistance. Extracts were then dried under a N$_2$ stream using an Organomation N-EVAP 11,250 evaporator and stored at −20 °C in darkness until the instrumental analysis by LC-MS/MS. Then, the extracts were reconstituted with 1.0

mL of 0.1% FA in MeOH/water mixture (5:95, v/v), as it constitute the mix of mobile phases at initial conditions in the LC gradient used during the chromatographic gradient described below. Extracts were filtered through a PTFE 0.22-μm syringe filter before instrumental injection. **5)** The instrumental LC-MS/MS analysis was performed using a UHPLC Sciex Exion system connected to a Sciex 6500 + triple-quadrupole mass spectrometer from Sciex equipped with an ESI source operated in both positive and negative mode in the same run. Chromatographic separation was achieved by a Phenomenex reversed-phase column Kinetex EVO C18 (50 × 2.1 mm, 1.7 μm), which was temperature-controlled at 40 °C along the entire chromatogram. The gradient run at 500 μL min-1 with 0.1% FA (v/v) in water and 0.1% FA in MeOH as mobile phases starting with 5% of the organic phase for 1 min and then increasing to 95% in 2 min, held at 95% for 3 min, and finally returning to the initial conditions, which were kept for 4 min. The total run time for each injection was 10 min. Injection volume was set at 10 μL. Mass spectrometry acquisition was performed in selected-reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte, thus achieving four identification points per compound (2002/657/EC) [22]. Table S2 shows the specific details of the UHPLC-MS/MS conditions. In addition, ESI operational settings were: capillary voltage, 4500 V; capillary temperature, 400 °C; both gas 1 and 2, 45 psi. Data acquisition and evaluation were performed by SciexOS software.

2.2.3. Online SPE proposed methodology

Protocol for the online SPE methodological approach was identical to the one described in section 2.2.2, except for the step 4) which was substituted by the addition of 400 μL of 5% EDTA to the extract. The resulting solution was pH 3 adjusted and then filtered through a 0.45-μm PTFE syringe filter. The filtrate underwent instrumental online SPE coupled to the LC-MS/MS method described above. Four injection volumes, i.e., 50, 100, 200 and 300 μL, were tested. A Strata-X 25um 20 × 2.0 mm online reusable cartridge by Phenomenex was used. Extract was conveyed to the cartridge by a 5% MeOH solvent at a flow rate of 1 mL min^{-1} for 1 min. In this conditions, extract was fully seeded in the cartridge and rinsed. Subsequently, UHPLC gradient described above would elute backwards the cartridge towards the analytical column.

2.2.4. DI proposed methodology

Protocol for the DI methodological option was identical to the one described in section 2.2.2, except for the steps 3) and 4) which were substituted by pH 3 adjusted and then a filtration through 0.22–μm with a PTFE syringe filter. No SPE clean-up was taking place, and thus, no EDTA was added. Hence, extracts were directly injected in the LC-MS/MS. Four injection volumes, i.e., 50, 100, 200 and 300 μL, were tested.

3. Results and discussion

3.1. Injection volume optimization in online SPE and DI methodologies

In this study, the injection volume (Vinj) was optimized to achieve high resolution and reproducibility in chromatographic analysis as it has a significant effect on peak shape and retention time. Four injection volumes, i.e., 50, 100, 200 and 300 μL, were tested for each proposed methodology. If the linear range is sufficient and the peak shape is good, the Vinj selected will be the one that provides the highest number of compounds with a Method Limit of Quantification (MLQ) < 70 ng g^{-1} (Fig. 1). The calibration curves acquired were linear with R^2 greater than 0.99 within the indicated concentration range for all the contaminants of interest. Therefore, volume of 100 μL was found to be the most appropriate for the online SPE and DI methodologies.

After data evaluation, some initial PPCPs proved to be unsuitable for analysis by the different analytical methods at any Vinj as they showed a very weak response or even no response at all. These PPCPs included antibiotics such as (oxytetracycline, doxycycline, tetracycline, among others), hormones (β-estradiol, 17-α-ethinylestradiol), and surfactants (4-*tert*-octylphenol and 4-nonylphenol) or others such as bisphenol A. In total 16 initial target compounds were ruled out.

3.2. Performance comparison for the three methodological proposals

The analysis time for each methodological approach is different. The protocol for the offline SPE methodology took much more time than for the other two approaches. For the three methodological proposals, the first three steps of the sample pre-treatment mentioned in section 2.2.2 were performed in a day. However, offline SPE was performed for two days before LC-MS/MS analysis and the total run time for each injection was 10 min. On the other hand, online SPE was performed for 2 min,
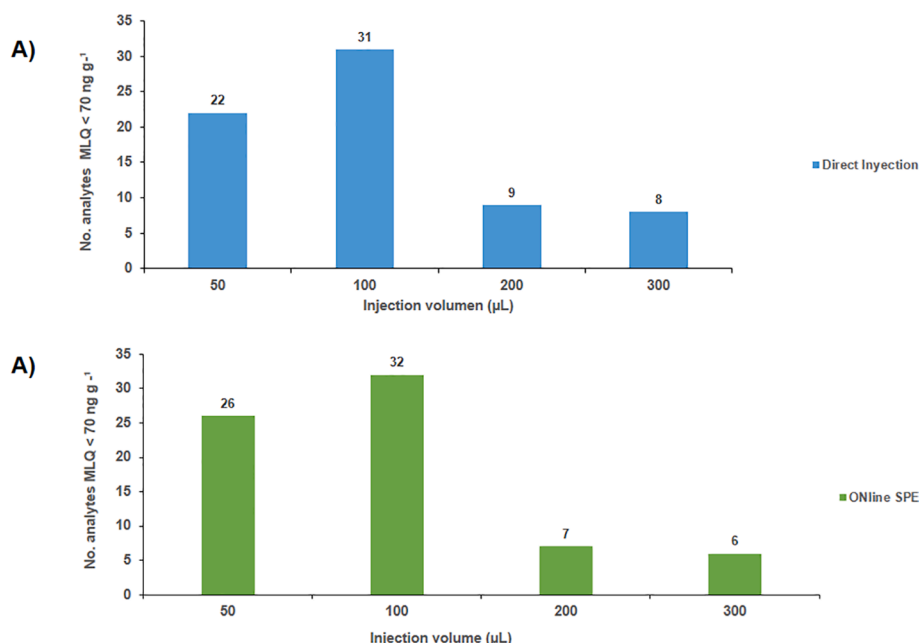

Fig. 1. No of analytes with MLQs below 70 ng g^{-1} in different injection volumes tested for each methodological proposal **A)** ONline SPE and **B)** DI.

based on the protocol mentioned in section 2.2.3, before LC-MS/MS analysis and for both online SPE and DI proposals, the total run time for each injection was also 10 min. Finally, the alternative methodologies to the conventional offline SPE protocol are faster methods to prepare and analyse a larger amount of samples per unit time.

Theoretical nanograms introduced in each injection for each sample on each point of the calibration curve were calculated, taking into account the optimized Vinj of each analytical proposal. The values calculated are shown in Table S3 and they indicated that the offline SPE samples introduced, at least theoretically, a higher amount of analytes into the chromatographic system. In contrast, the online SPE and DI samples introduced the smallest amount of analytes. Fig. 2 shows a representation of the theoretical nanograms introduced into each injection of each point of calibration curve for clofibric acid (isotopically labelled clofibric acid-d4 internal standard) and apramycin. The results reported a better slope of the calibration curve for DI samples than for the other two proposals. In addition, recoveries for the DI approach should be somewhat higher than for the online SPE approach, not performing a clean-up stage prior to samples analysis. However, the results showed similar values and this may be due to the fact that the matrix effect in the online SPE samples is reduced thanks to the clean-up stage mentioned above, generating better recoveries and more signal from the analytes. Calculated recoveries revealed satisfactory values between 80% and 110%, with a Relative Standard Deviation (RSD) lower than 18% for almost all compounds of interest in DI samples. In contrast, recovery values between 70% and 120%, with a RSD < 20% and recoveries between 60 and 105% with a RSD < 23% were observed for most of the analytes of interest in the online and offline SPE samples, respectively.

MLQs were experimentally determined for target compounds in each methodological proposal. DI samples provided MLQs lower than 70 ng g^{-1} for 70% of the analytes in dewatered digested sludge. In contrast, MLQs below 70 ng g^{-1} were obtained for 73% and 43% of studied compounds in online and offline SPE samples, respectively (Fig. 3). Based on sensitivity (and linear range and peak shape), the best approach for the determination of PPCPs in dewatered digested sludge is the DI proposal methodology.

The disadvantage of online SPE is chromatography. Offline SPE and DI methodologies provided chromatograms with similar skylines. Retention times and peak shape were similar. In these versions, the

compounds over 5 min, in particular between 0.72 min (metronidazole) and the 5.12 min (progesterone). In addition, the peak width range around 0.18 min with a few exceptions. However, in the chromatograms obtained with online SPE, all the compounds agglomerated and eluted within 2.5 min. The clumping in the elution of the compounds may hint at the clumping of the matrix elution, which could be related to an increase in the matrix effect. However, the clean-up stage carried out by online SPE seems to compensate. In addition, the average peak width is approximately triple in online SPE compared to the other two methods, which could have a negative effect on sensitivity, but it is not observed, so the matrix elimination effect by online SPE seems to compensate or better recovery with respect to offline SPE. Fig. 4 shows an Exchange Ion Chromatogram with the first transition of some compounds, including the first and last analyte to elute, for each proposed methodology.

Finally, a review of the data obtained from the method quality parameters mentioned above concluded that the most suitable method for the analysis of the PPCPs of interest (Table S1) corresponded to a DI in LC-MS/MS. This proposed method showed the best results for linearity range, accuracy, precision, Method Limits of Detection (MLDs) and MLQs. This is despite the fact that the lowest amounts of theoretical nanograms were introduced in each injection of each sample as are presented in Table S3. This resulting methodology stands out for its environmental and analyst safety and short time consumption. In addition, it allows to investigate the fate of a large number of CECs in sludge matrices.

4. Best working method validation and applications

4.1. DI methodology validation

Validation parameters (accuracy, precision, linearity range, MLD and MLQ) were determined for all target compounds contained in the analytical method.

Quantification method was based on peak areas and performed by both internal standard and matrix-matched approaches in dewatered digested sludge. Each test was performed in duplicated and two significant concentration levels of 333 ng g^{-1} and 1333 ng g^{-1} were added. Seven-point calibration curves were generated using linear regression analysis covering the range from 2 to 13333 ng g^{-1} for all target analytes. Linearity was qualified by the linear correlation coefficient (R^2),
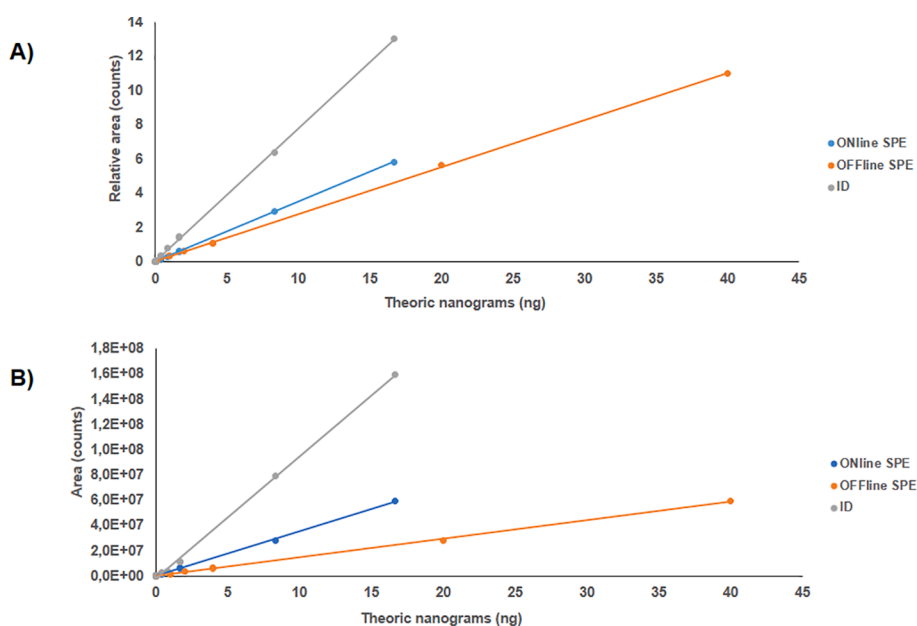

Fig.2. Theoretical nanograms introduced at each injection of each point on the calibration curve for two target compounds **A)** clofibric acid and its corresponding isotopically labelled clofibric acid-d4 internal standard and **B)** apramycin.
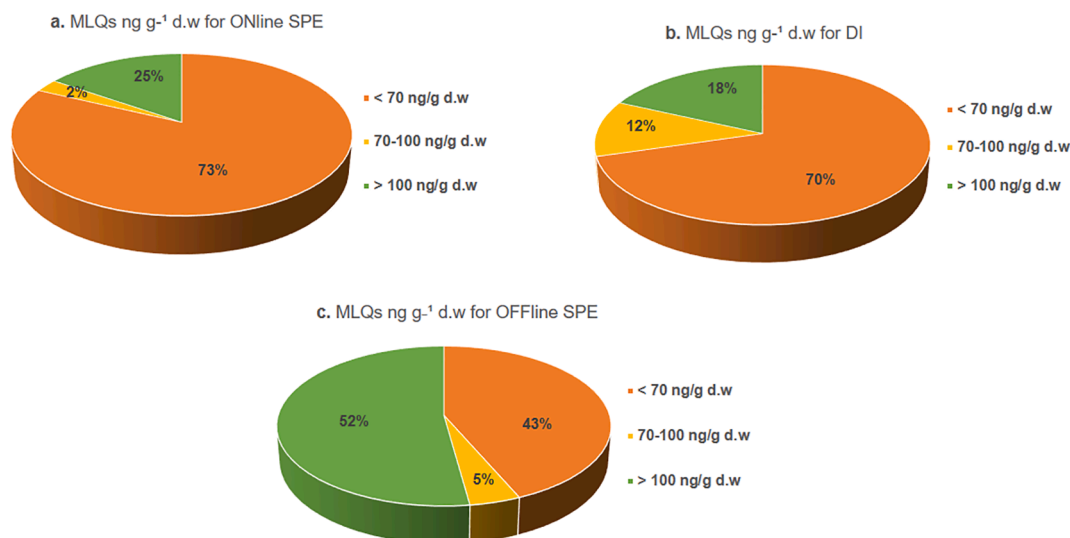
Fig. 3. Distribution of MLQs ng g^{-1} d.w for each proposed methodology **a.** ONline SPE **b.** DI and **c.** OFFline SPE.
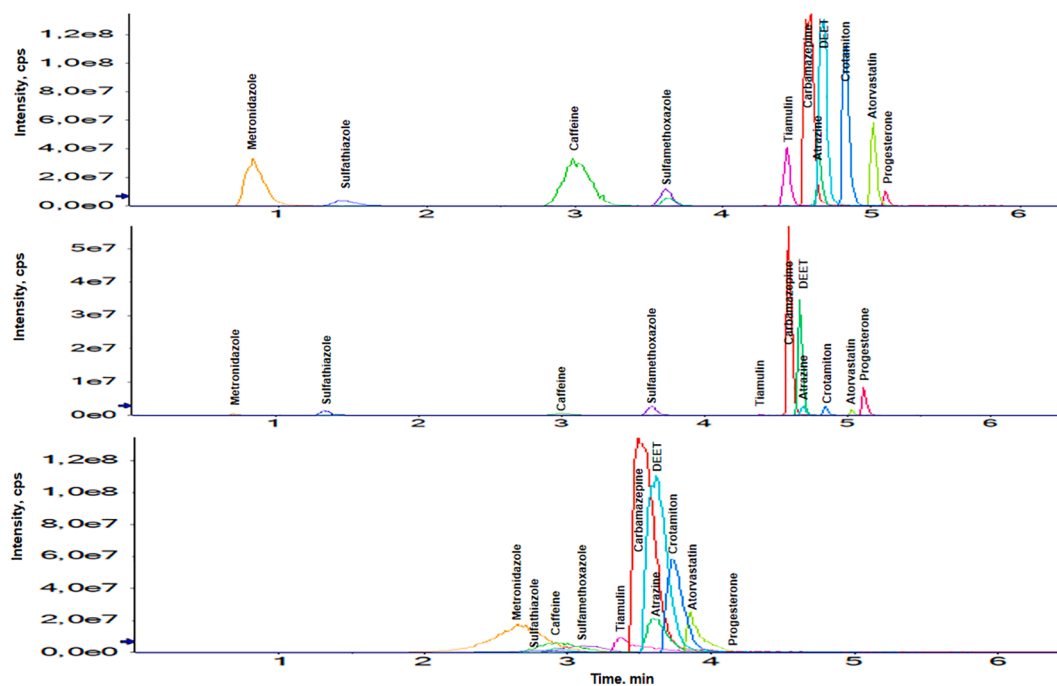

Fig. 4. An Extracted Ion Chromatogram of some PPCPs from a dewatered digested sludge spiked to 13333 ng g^{-1} and analysed by DI in LC-MS/MS.

with R^2 greater than 0.99, for all the compounds within the indicated concentration range. The main quality parameters for developed method are summarized in Table 1.

In our specific case, method accuracy was expressed as % recovery and evaluated in samples spiked at two concentration levels (i.e., 333 and 1333 ng g^{-1}) for sludge samples. The observed recoveries were in the range 80–110% for most of cases. The values obtained were successful and very similar to those reported in other studies for the analysis of PPCPs in sewage sludge [23,24].

To ensure correct method precision, %RSD of the area observed for analogous samples prepared in duplicated with the developed method was calculated. A %RSD below 18% was observed for almost all analytes of interest (Table 1) when the analysis were performed in the same day (intra-day precision). The results indicate a good precision of the method and reported values similar or even better than previous publications for sludge samples [25,26].

MLDs and MLQs were experimentally determined as the providing a total signal-to-noise ratio (S/N) of 3 and 10, respectively, for each target compound. MLDs and MLQs values obtained were lower than 20 ng g^{-1} and 65 ng g^{-1}, respectively, for the 72% and 70% of the target compounds in the sludge samples (Table 1). The reported values were considered acceptable for trace analysis of target analytes in a solid matrix such as sludge. Moreover, these values were similar to, or even lower than, values obtained in analogue multicomponent methods based on gas chromatography coupled to mass spectrometry (GC–MS) [12,24] and even LC-MS/MS [23,27].

The multi-residue analytical method based on UAE followed by DI in LC-MS/MS has been satisfactory validated for 44 PPCPs, with different physical–chemical properties, achieving good results of linearity, precision, accuracy, and MLDs and MLQs.

Table 1
Lineal Range, Accuracy and Precision for Dewatered Digested Sludge.

				Accuracy		Precision			
			Lineal Range	Recovery (%) (n = 2)		Intra-day (%RSD) (n = 2)			
Chemical Name	[1]IS	R^2	Range (ng g^{-1})	333 (ng g^{-1})	1333 (ng g^{-1})	333 (ng g^{-1})	1333 (ng g^{-1})	[2]MLD (ng g^{-1})	[3]MLQ (ng g^{-1})
Penicillin G	Methylparaben-d5	0.997	LOQ-11760	122	115	5	8	8.5	28.3
Marbofloxacin	Sulfadiazine-d4	0.993	LOQ-13067	130	87	29	11	101.8	339.3
Enrofloxacin	Enrofloxacin-d5	0.999	LOQ-13333	60	70	17	2	52.8	176.0
Sulfadiazine	Sulfadiazine-d4	0.997	LOQ-13200	70	120	19	10	67.5	225.1
Sulfathiazole	Sulfadiazine-d4	0.992	LOQ-6667	112	83	17	19	15.7	57.5
Sulfadimidine	Sulfadimidine-d4	0.999	LOQ-6667	120	70	18	15	9.3	31.0
Sulfamethizole	Sulfadimidine-d4	0.991	LOQ-13333	102	93	12	17	2.3	7.7
Sulfamethoxazole	Sulfamethoxazole-d4	0.993	LOQ-6533	100	89	0.8	21	0.1	0.3
Tylosin	Methylparaben-d5	0.996	LOQ-11342	117	78	14	19	18.5	61.8
Tiamulin	Methylparaben-d5	0.999	LOQ-10550	95	98	0.8	0.9	2.5	8.5
Apramycin	Sulfadimidine-d4	0.995	LOQ-6903	112	73	14	8	14.5	48.2

				Accuracy		Precision			
			Lineal Range	Recovery (%) (n=2)		Intra-day (%RSD) (n=2)			
Chemical Name	[1]IS	R^2	Range (ng g^{-1})	333 (ng g^{-1})	1333 (ng g^{-1})	333 (ng g^{-1})	1333 (ng g^{-1})	[2]MLD (ng g^{-1})	[3]MLQ (ng g^{-1})
Trimethoprim	Sulfadiazine-d4	0.999	LOQ-13200	116	120	2	3	0.5	1.6
Florfenicol	Methylparaben-d5	0.999	LOQ-13309	107	80	9	6	15.0	50.9
Fenbendazol	Naproxen-d3	0.998	LOQ-13067	100	102	0.02	2	8.3	27.6
Dexamethasone	Propylparaben-d7	0.996	LOQ-13067	119	120	1	5	19.2	63.9
Progesterone	Ibuprofen-d3	0.999	LOQ-13200	99	120	3	4	22.8	76.0
Methylparaben	Methylparaben-d4	0.999	LOQ-13333	120	94	2	0.8	28.5	95.1
Acetaminophen	Sulfadiazine-d4	0.994	LOQ-13200	83	107	1	4	8.8	29.5
Carbamazepine	Ethylparaben-d7	0.996	LOQ-6667	83	106	0.3	0.7	1.4	4.8
Propranolol	Salicylic acid-d4	0.992	LOQ-5845	110	77	5	7	17.8	59.4
Metronidazole	Sulfadiazine-d4	0.996	LOQ-13333	112	108	6	1	4.8	16.1
Ofloxacin	Sulfadiazine-d4	0.998	LOQ-13333	125	91	2	11	35.2	117.2

				Accuracy		Precision			
			Lineal Range	Recovery (%) (n=2)		Intra-day (%RSD) (n=2)			
Chemical Name	[1]IS	R^2	Range (ng g^{-1})	333 (ng g^{-1})	1333 (ng g^{-1})	333 (ng g^{-1})	1333 (ng g^{-1})	[2]MLD (ng g^{-1})	[3]MLQ (ng g^{-1})
Naproxen	Naproxen-d3	0.994	LOQ-6667	79	99	4	2	2.6	8.7
Clarithromycin	Ethylparaben-d7	0.993	LOQ-13187	80	120	5	6	4.0	13.3
Erythromycin	Methylparaben-d5	0.997	LOQ-13067	101	103	11	0.7	18.5	61.7

Table 1 (*continued*)

			Lineal Range	Accuracy Recovery (%) (n=2)		Precision Intra-day (%RSD) (n=2)			
Clofibrate	Diclofenac-d4	0.996	LOQ-13333	105	120	14	7	233.6	778.7
Levofloxacin	Sulfadiazine-d4	0.998	LOQ-13067	72	93	2	0.8	95.2	197.1
Norfloxacin	Triclosan-d3	0.998	LOQ-6533	110	120	21	14	248.9	829.5
Atorvastatin	Diclofenac-d4	0.996	LOQ-6159	97	120	16	9	3.8	12.8
Atenolol	Triclosan-d3	0.990	LOQ-13333	111	116	20	2	14.1	46.9
Caffeine	Sulfadimidine-d4	0.996	LOQ-13133	118	97	5	2	3.2	10.7
Atrazine	Propylparaben-d7	0.996	LOQ-6640	106	71	8	21	1.8	6.1
Iohexol	Sulfadiazine-d4	0.991	LOQ-6500	73	74	21	15	17.9	59.6

Chemical Name	[1]IS	R^2	Range (ng g^{-1})	Accuracy Recovery (%) (n=2) 333 (ng g^{-1})	1333 (ng g^{-1})	Precision Intra-day (%RSD) (n=2) 333 (ng g^{-1})	1333 (ng g^{-1})	[2]MLD (ng g^{-1})	[3]MLQ (ng g^{-1})
DEET	Propylparaben-d7	0.996	LOQ-1307	120	102	3	4	1.3	4.5
Crotamiton	Naproxen-d3	0.995	LOQ-6467	88	79	0.5	0.4	1.0	3.3
Estrone	Naproxen-d3	0.999	LOQ-13333	110	109	4	10	10.3	34.5
Ethylparaben	Ethylparaben-d5	0.999	LOQ-13333	118	95	6	7	25.8	85.9
Propylparaben	Propylparaben-d7	0.995	LOQ-13333	120	98	9	4	20.0	66.8
Diclofenac	Diclofenac-d4	0.994	LOQ-6206	79	118	4	3	23.9	79.6
Ibuprofen	Ibuprofen-d3	0.995	LOQ-13067	116	99	1	21	24.2	80.6
Salicylic acid	Salicylic acid-d4	0.999	LOQ-13200	108	99	0.6	8	13.4	44.8
Clofibric acid	Clofibric acid-d4	0.999	LOQ-12933	102	102	7	1	2.7	9.1
4-Hydroxybenzoic acid	Sulfadimidine-d4	0.994	LOQ-1320	82	117	1	11	0.1	0.5

Chemical Name	[1]IS	R^2	Range (ng g^{-1})	Accuracy Recovery (%) (n=2) 333 (ng g^{-1})	1333 (ng g^{-1})	Precision Intra-day (%RSD) (n=2) 333 (ng g^{-1})	1333 (ng g^{-1})	[2]MLD (ng g^{-1})	[3]MLQ (ng g^{-1})
Gemfibrozil	Ibuprofen-d3	0.998	LOQ-13067	113	105	12	3	19.1	63.6

[1]IS: Internal Standard.
[2]MLD: Method Limit of Detection.
[3]MLQ: Method Limit of Quantification.

4.2. DI methodology application

Once validated, the best performing methodological option, the DI version, was successfully applied to dewatered digested sludge from the WWTP of Valladolid (Spain). Table S4 provides the average concentrations of PPCPs presented in dewatered digested sludge.

Carbamazepine, an anticonvulsant, was detected at a concentration of 36 ng g^{-1}. This pharmaceutical was found in Brazil at concentrations between 10.2 and 23.8 ng g^{-1} [28]. In France, at a concentration of 39 ng g^{-1} [13]. Trimethoprim, a bacteriostatic antibiotic, was found at concentrations below 10 ng g^{-1}. This antibiotic was detected in sludge samples at concentrations of 5 and 13 ng g^{-1} in France [29].

Analgesic and non-steroidal anti-inflammatory drugs such as salicylic acid, naproxen and diclofenac were present at levels < MLQ (45, 9 and 80) ng g^{-1}, respectively. In Italy, the analgesic salicylic acid was detected in sludge samples at concentrations between 12 and 57 ng g^{-1}

[30] and in Brazil at concentrations below 100 ng g^{-1} [31]. Naproxen, a non– steroidal anti-inflammatory drug, was also present in sludge samples at a concentration of 0.94 ng g^{-1} [30] in Italy and at a concentration of 39.8 ng g^{-1} in United Kingdom [10]. In addition, diclofenac, another non-steroidal anti-inflammatory drug very used, was found in Brazil at concentrations of 25 and 60.6 ng g^{-1} [28] and at an average concentration of 140 ng g^{-1} [13] in France.

Apramycin, a veterinary antibiotic, was found at a concentration of 172 ng g^{-1}. In Greece, this compound was also detected in sludge samples at concentrations of 16.3 ng g^{-1} [25]. Propranolol, a beta-blocker indicated for hypertension, was detected at concentrations of 135 ng g^{-1}. This compound was present in Brazil at concentrations ranging from 61.2 to 94.3 ng g^{-1} [28]. Concentrations between 82 and 849 ng g^{-1} [29] and concentrations of 46 and 54 ng g^{-1} [32] were also found in France and India, respectively.

Preservatives such as methylparaben, ethylparaben, and propylparaben, used to prevent or retard spoilage caused by chemical changes, were detected in sludge samples at concentrations between 48 and 131 ng g^{-1}. In United Kingdom, these personal care products were found at concentrations of 219 ng g^{-1}, below 0.57 ng g^{-1} and below 0.72 ng g^{-1} for methylparaben, ethylparaben and propylparaben, respectively [10].

Finally, the observed concentrations (expressed at the ng g^{-1} level) were found in the range < MLQ-2,163 ng g^{-1} for dewatered digested sludge samples. The observed results indicate contamination of sludge by PPCPs, as it is commonly used in the treatment of animal and human diseases, and the concentrations reported are similar to those in other countries.

5. Conclusions

In this work, three analytical methods based on the use of two different clean-up procedures (online or off-line SPE) or no clean-up stage prior to UHPLC-MS/MS determination of PPCPs in dewatered digested sludge were compared. The main conclusions are as follows:

1. A comparison of three different methodological proposals to determine 60 PPCPs in solid urban sewage sludge was carried out. In all cases, an in-situ clean-up stage, using 100.0 mg of activated Al$_2$O$_3$, was preceded by UAE. Two fast sample pre-treatments (i.e., online SPE and DI), were tested against the conventional offline SPE. In all cases, subsequently, extracts underwent UHPLC-MS/MS, simultaneously operating in both positive and negative ESI mode.
2. The Vinj in online SPE and DI methodologies was optimized to achieve high resolution and reproducibility in chromatographic analysis. After different volumes were tested for each analytical proposal, 100 μL was selected as the best performing volume for online SPE and DI, respectively.
3. Matrix-matched quantification was combined with internal standard, providing high reliability to all three proposals. Best performance was observed for the methodology consisting of UAE and DI in the LC-MS/MS with general relative recoveries between 80 and 110%, repeatability expressed as % RSD below 18%, MLDs under 30 ng g^{-1} and a linearity range of up to 5 orders of magnitude for almost all the target compounds.
4. The best environmentally friendly analytical method decreased the use of fungible material (i.e., small amounts of reagents and small solvent volume) and it was satisfactorily validated for 44 PPCPs, with MLQs below 70 ng g^{-1} for many compounds of interest. In addition, this methodology applied was fast and analyst appropriate for the analysis of PPCPs in dewatered digested sludge samples.
5. This method was successfully applied to different dewatered digested sludge. An analgesic and antipyretic as acetaminophen was reported at concentrations above 1,000 ng g^{-1}. PPCPs were observed in the range < MLQ to 2,163 ng g^{-1} for sludge samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2021.107148.

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