Development and validation of QuEChERS-based extraction for quantification of nine micropollutants in wastewater treatment plant

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Abstract
A modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) method was established for simultaneous quantification of eight pharmaceutical molecules (2-hydroxyibuprofen, diclofenac, ibuprofen, propranolol, ofloxacin, oxazepam, sulfamethoxazole, carbamazepine) and caffeine in environmental matrices. Analysis was performed by ultra-high-performance liquid chromatography with tandem mass spectrometry (UHPLC-MS-MS). Quantification was performed by using the ^{13}C internal standard method for each molecule. Two methods were firstly optimized on freeze-dried waste activated sludge and then applied and validated on real complex matrices, which have contrasted physicochemical properties, i.e., clarified wastewater and primary sludge. The combination of acetate buffer with $MgSO_4$ (protocol A) and citrate buffer with Na_2SO_4 (protocol B) was found necessary to recover the nine targeted compounds. Adding a higher salts quantity of Na_2SO_4 (protocol B) compared to $MgSO_4$ (protocol A) is crucial to increase the ionic strength of the aqueous solution and to obtain comparable extraction recoveries of the targeted molecules. Adding two times solvent volume to the aqueous phase leads to increased absolute recovery for all molecules and both protocols. After demonstration of the final protocol's performance on the control matrix, its robustness was tested on the matrices of interest. As a result, the two proposed detection methods exhibit good reproducibility, high sensitivity, and high reliability.

Keywords Pharmaceuticals · Extraction · UHPLC-MS-MS · Multi-class analysis · Complex matrices

Introduction

Pharmaceuticals and personal care products are classes of emerging micropollutants that find their way to the environment because of the lack of specific elimination conditions in wastewater treatment plants (WWTPs). Whether they are in natural waters or in soils amended with sewage sludge, these compounds can induce multiple effects on humans, animals, and other living organisms, such as endocrine disruption and antibiotic resistance, even at very low concentrations [1–5]. However, those molecules are much more studied in the aqueous matrices (effluents of WWTP) than solid matrices (sludge). It can be explained by the lack of appropriate methodologies for extraction and quantification and the difficulties to develop them [5].

Three main steps compose the analytical procedure: extraction, purification, and quantification. Concerning the first step, i.e., the extraction of the target analytes, until 2012, the most commonly used techniques for organic pollutants in sludge-like matrices were ultrasound-assisted extraction (USE) [6, 7], pressurized liquid extraction (PLE) [8–10], and microwave-assisted extraction (MAE) [11]. Those methods are usually followed by a clean-up procedure using solid-phase extraction (SPE) [12]. The analytical techniques used were gas chromatography (GC) coupled with an electron capture detector (ECD) [13], a flame ionization detector (FID) [14], or mass

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spectrometry (MS) [15, 16], for volatile or semi-volatile organic compounds, or by liquid chromatography (LC) coupled with mass spectrometry (MS) for other organic compounds [17, 18].

Another extraction method used for organic micropollutant determination in solid matrices is the quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction. This method was introduced as a technique for the determination of pesticide residues in fruits and vegetables [19]. The first step of the procedure consists in extracting 10 g of sample with 10 mL of acetonitrile (ACN). Thanks to the addition of 4 g of anhydrous magnesium sulfate ($MgSO_4$) and 1 g of sodium chloride (NaCl), a liquid-liquid partitioning is formed. Next, a purification step is achieved by a dispersive solid-phase extraction (d-SPE), which involves mixing 1 mL of ACN extract with 150 mg of $MgSO_4$ and 25 mg of primary secondary amine (PSA). PSA has been reported to lower the recovery of acidic compounds due to strong interactions [20]. However, this sorbent makes it possible to eliminate polar interferences due to volatile fatty acids, sugars, lipids, etc., just as many compounds present in the environmental matrices encountered in wastewater treatment plant. When extracting multiresidue in complex environmental matrices, the combination of different adsorbents should be used for optimal cleaning purposes. Finally, gas chromatography coupled with mass spectrometry (GC/MS) is used for quantitative analysis of pesticides [19]. This method has several advantages compared to other sample preparation techniques. Indeed, using a minimal amount of solvents and no specific extraction equipment, the authors were able to obtain high-quality results with recovery yields ranging between 94 and 102%. In addition, the d-SPE step is very simple compared to SPE: it uses less sorbent, no vacuum, and no preconditioning column, and it requires no training or careful attention from the user [19].

Since this first publication, the interest for the QuEChERS extraction method has increased continually. Indeed, many studies have revealed its versatility by extending its use to other matrices and analytes. Table 1 summarizes some examples of modifications implemented in order to adapt the basic method to pharmaceuticals in sludge-like matrices. Cerqueira et al. (2014) and Rossini et al. (2016) applied protocols very similar to the classical method, although Cerqueira et al. (2014) used an acidified ACN like Peysson and Vuilliet (2013) or Bragança et al. (2012). ACN is the most common solvent used for the first step of the QuEChERS method because of its selectivity (only few co-extractives from the matrix were extracted), its high polarity compared to other solvents, and its compatibility with the chromatographic applications [21]. The acidification of ACN (with acetic acid (AA) or formic acid) is often used in order to avoid increasing pH after adding PSA for the d-SPE step [21]. Regarding the addition of buffers, it is essential to adjust the pH and to have a compromised value, where most analytes, labile under acidic or alkaline conditions, are sufficiently stabilized [21]. Bragança et al. (2012) have used a citrate buffer, while Peysson and Vulliet (2013) have used an acetate one. The addition of EDTA solution during extraction improves complexation with interferents [2, 5]. Finally, even if the salt Na_2SO_4 leads to higher recoveries of fluoroquinolones in human and animal tissues compared to $MgSO_4$, the mix of $MgSO_4$ and NaCl is the salting-out condition used in studies involving environmental matrices (Table 1). About the purification step, the primary secondary amine (PSA) sorbent is an exchange phase that has a high affinity with polar interferents such as organic acids, some polar pigments, and sugars, explaining its wide use for fruit or vegetable samples [19]. In general, the combination of C18 and PSA leads to obtaining cleaner samples. These two sorbents are most commonly applied in d-SPE, where C18 is particularly effective to remove fats without affecting recoveries [22, 23]. As for the addition of salts during the purification step, it aims to remove the water from the organic phase, capture some interferents, and consequently decrease the matrix effects. $MgSO_4$ is the salt commonly used. In some studies, where polar molecules are not targeted, $CaCl_2$ is also added [21]. As a result, adding salts has a positive effect on the extraction quality but could decrease extraction recoveries depending on the physicochemical properties of the molecules.

In a context of improve environmental quality, the project "Separating Micropollutants at the Source" (SMS) suggests a demonstration platform with specific treatments such as a membrane bioreactor and a sludge digester focusing on the removal of pharmaceutical micropollutants from wastewater after urine separation [24, 25]. The matrices studied in this project are therefore very diverse, in particular in terms of suspended matter content. Nine micropollutants were chosen for this study: diclofenac (DIC), ibuprofen (IBP), 2-hydroxyibuprofen (2OH-IBP), carbamazepine (CBZ), sulfamethoxazole (SMX), ofloxacin (OFL), oxazepam (OXA), propranolol (PRO), and caffeine (CAF), because they cover a wide range of pharmaceutical classes and physicochemical properties.

Quantifying the targeted molecules through mass balances in the different environmental matrices in order to evaluate the efficiency of the proposed solutions was the foreseen methodology. In fact, there is no protocol dealing with the nine target molecules at the same time and provided from so contrasted matrices. Thus, the objective of this study is the optimization of the QuEChERS extraction method followed by quantification using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), in order to obtain a simple procedure applied in complex environmental matrices for the analysis of nine pharmaceutical micropollutants.

Table 1 Summary of modifications implemented in order to adapt the basic QuEChERS method to pharmaceuticals in sludge-like matrices

Samples		QuEChERS extraction		d-SPE		Analysis			Reference
Matrices	Mol.	Solvents	Buffer/salts	Sample	Sorbent	Sample	Concentration	Preparation	
Soils and sediments	IBP 2OH-IBP	3 mL water at pH 2.5 (HCl) 7 mL ACN (1% HCOOH) Vortex 4 min US bath 4 min	Citrate buffer (1 g NaCit+0.5 g Na_2Cit) 4 g $MgSO_4$ +1 g NaCl Vortex 4 min	No d-SPE		5 mL in glass tube	Solvent evaporation under N stream 500 μL ACN (5%)	Filtration 0.2 μm PTFE	(Bragança et al., 2012)
Solid and liquid sludge	DIC IBP CBZ SMX OFL PRO CAF	10 mL 0.1 M EDTA Vortex 30 s 10 mL ACN 1%AA Vortex 30 s 1 mL heptane 10 metal balls	Acetate buffer (1.5 g NaOAc) 6 g $MgSO_4$ Hom. 15 s Vortex 45 s	9.5 mL in a 15-mL Falcon tube	150 mg PSA 900 mg $MgSO_4$ Hom. 15 s Vortex 45 s	8 mL in glass tube	200 μL DMSO (40%) Evaporation of ACN under N stream 25 μL ACN (5%) 300 μL water (55%)	Filtration 0.45 μm PTFE	(Peysson and Vulliet, 2013)
Solid sludge	CBZ PRO SMX CAF	10 mL ACN 0.1 mL AA	4 g $MgSO_4$ +1 g NaCl	2 mL extract	300 mg $MgSO_4$ 125 mg PSA	Dilution by 5 in ultrapure water	–	Filtration 0.45 μm	(Cerqueira et al., 2014)
Thickened and centrifuged sludge	DIC IBP 2OH-IBP	10 mL ACN Hom. 15 s Vortex 1 min	2 g $MgSO_4$+2 g NaCl Hom. 15 s Vortex 1 min	On-line SPE					(Rossini et al., 2016)
Porcine manure	SMX	20 mL MeOH/ACN/0.1MEDTA-McIlvaine buffer (12.5/37.5/50) Vortex 1 min US bath 15 min	4 g $MgSO_4$ +1 g NaCl Hom. 1 min	2 mL organic phase transferred in a 10-mL Falcon tube	40 mg PSA 20 mg C18 Vortex 1 min	All in glass tube	Evaporation under a N stream until 0.2 mL 0.8 mL ACN-0.1% formic acid/water (0.2/0.8, v/v)	Filtration 0.22 μm Nylon	(Guo et al., 2016)
Soil	SMX CBZ IBP	2 ml 0.1% formic acid+vortex+soaking+16 ml ACN/MeOH (1:1 v/v)+vortex	No	On-line SPE					(Montemurro et al., 2019)

US ultrasonic, *N* nitrogen, *Hom.* homogenization

Experimental method

In order to obtain an extraction protocol ensuring the quantification of targeted molecules in the matrices of the SMS project, a 3-parts approach was implemented. (1) From a water doped with micropollutants: a principal component analysis (PCA) allowed a qualitative analysis of the effect of extraction parameters on the extraction yields of the target molecules. It has shown the impacting parameters (buffers and salts) and leads to the application of 2 protocols. (2) From a complex reference matrix (freeze-dried activated sludge), the effect of a modification of the quantities of each chemical on the extraction yields and the matrix effects was studied in order to bring out 2 optimized protocols. (3) Finally, an application and validation of the final protocols was carried out on two complex real matrices from the SMS project (digestate and primary sludge).

Chemicals and reagents

A set of 9 chemical compounds encountered in wastewater (8 pharmaceuticals and 1 tracer of human activity) was selected, and the details on the target molecules (formula and physicochemical properties) and corresponding ^{13}C internal standards (IS) are presented in Supplementary information (ESM) Table S2. Analytical standards of ≥ 98% purity were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), with the exception of OXA and IS for OFL, PRO, SMX, OXA, and 2OH-IBP furnished by Alsachim (Illkirch Graffenstaden, France). All products were supplied in powder except IS for CAF and CBZ that were obtained as methanolic solution at a concentration of 1 mg/mL and 100 μg/mL, respectively.

Tri-sodium citrate dihydrate (Na_3Cit, $2H_2O$), sodium acetate (NaOAc), sodium chloride (NaCl), disodium sulfate (Na_2SO_4), and magnesium sulfate ($MgSO_4$) were obtained from Sigma-Aldrich. The LC-MS–grade solutions used, including methanol (MeOH), ACN, and AA were obtained from VWR Prolabo (Fontenay-sous-Bois, France) as well as ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA, $2H_2O$), citric acid monohydrate (Cit, $1H_2O$), primary secondary amine (PSA), and C18 adsorbents (SUPELCO).

Preparation of concentrated solutions

Individual stock solutions were prepared in MeOH at concentrations of 100 mg/L, except for OFL that was prepared at 20 mg/L. These solutions were immediately aliquoted in vials and stored at − 20 °C. Two fresh mix solutions (standards and IS) were prepared at 300 ng/mL by diluting appropriately the stock solutions in ACN/water 95/5 (v/v). They were used as spiking solutions during method development and in the validation study as well as to construct internal calibration curves in ACN/water 95/5 (v/v) from 5 to 60 ng/mL with a constant concentration of IS at 60 ng/mL.

For each extraction campaign, concentrated buffer and EDTA solutions were prepared to avoid adding the powder products to each QuEChERS extraction tube, which is commonly done in a conventional extraction. The acetate buffer contained 300 g/L NaOAc, the citrate buffer contained 232 g/L Na_3Cit, $2H_2O$ and 115.2 g/L Cit, $1H_2O$, and the EDTA solution was prepared at 75 g/L.

Matrix description

To optimize the protocol, a large quantity of freeze-dried secondary sludge called “reference matrix” was recovered in a wastewater treatment plant (Villefranche de Lauraguais, France) and stored at 4 °C. For the protocol validation step, two contrasted “real matrices” (in particular in terms of particulate matter), i.e., primary sludge (15 g_{TSS}/L) and clarified wastewater (0.5 g_{TSS}/L), were sampled in the wastewater treatment plant (Cugnaux, France) and immediately stored at − 20 °C until use. It has to be noted that a comparison between fresh and freezed samples led to differences lower than 15% except for SMX and CBZ (25% and 33% respectively) due to values close to QL (ESM Fig. S1).

Experimental approach for condition optimization

A first study screening 36 extraction conditions was carried out in duplicate from 2.5 mL of water spiked with 80 ng/mL for each compound (CAF, OFL, SMX, CBZ, PRO, OXA, DIC, IBP, and 2OH-IBP). The effect of salts (1 g $MgSO_4$, 1 g $MgSO_4$ + 0.25 g NaCl, and 1 g Na_2SO_4) and 1.25 mL of buffers (acetate, citrate, and no buffer) was evaluated. The presence or absence of EDTA, i.e., 1.25 mL of concentrated solution or water, has been tested. Finally, ACN was chosen as the solvent used for extraction and the effect of 2.5 mL ACN acidified with AA (2%) or not was evaluated. The corresponding matrix/aqueous/solvent ratio is 0.1/2/1 (g/mL/mL). The chain extraction on spiked water was performed without the purification step.

Principal component analysis (PCA) based on the recovery yields of the nine target molecules revealed that buffer and salts were key parameters of the QuEChERS extraction step (see ESM Fig. S2).

Considering a recovery higher than 60%, 2 extraction protocols were defined: the combination of acetate buffer with the salt $MgSO_4$ (protocol A) and citrate buffer with the salt Na_2SO_4 (protocol B). For both protocols, parameters have been optimized to become adapted to the reference matrix. Thus, the amount of salts was also optimized (0.05 g, 2 g, 4 g, 6 g) comparing absolute recoveries and the matrix effects. Three different matrix/water/solvent ratios were also tested by

modifying the solvent and aqueous volume: 0.05/2/1, 0.1/1.44/1, and 0.05/1/1, as presented in Table 2. Standard conditions of purification, evaporation, and concentration were applied without specific optimization (see "Final protocols").

Final protocols

From the development procedure, 2 protocols have been defined. Both protocols consisted in introducing either 0.25 g of reference matrix and 2.5 mL of distilled water or directly 2.5 mL of real matrices in a 50-mL polypropylene (PP) Falcon® tube. Reference matrix samples were spiked at 40 ng/mL by adding 0.35 mL of the standard solution. For method validation, real matrix samples were spiked at several levels with standard solution (see "UHPLC-MS-MS analysis"). Quantification of the analytes was performed by the internal standard approach. A volume of 0.2 mL of IS solution was spiked to samples. The PP tubes was left for 2 h on a shaking table (MultiReax Heidolph) at 1000 rpm and room temperature to ensure the homogeneity of the sample, and more over the adsorption of the internal standard in the sludge.

1.25 mL of EDTA, 1.25 mL of buffer (acetate or citrate for protocol A or B, respectively), 5 mL of acidified ACN, and 1 g $MgSO_4$ for protocol A or 4 g of Na_2SO_4 for protocol B are added in tubes that are immediately vortexed (Heidolph™ Multi Reax Vortex Mixer from Fisher Scientific) at maximum speed for 1 min and then centrifuged at 7100 g for 5 min. Purification of the organic phase and recovery were performed as suggested in Gonzalez-Salgado et al. [26]. It has to be noted that the effect of sorbents was studied by using PSA and C18 alone (ESM Fig. S3) and results show an improvement of absolute recoveries when PSA and C18 were used in combination. Moreover, as in the SMS project, we faced contrasted matrices (wastewater, primary and secondary sludge, digestate, permeate from the membrane bioreactor, urine) we kept the mixture PSA + C18 which allows efficiency on a wide range of molecules and matrices.

For the real matrices, a volume of 1 mL of a solution of ACN/water 95/5 (v/v) was added while for the reference matrix, to be able to assess the extraction recovery and matrix effects ("Analytical calculations and validation of the method"), 0.8 mL was added and supplemented with 0.2 mL of the IS solution (same final concentration than IS calibration samples, i.e., 60 ng/L). Pyrex tubes were vortexed at maximum speed for 1 min, and the liquid was filtered through a 0.2-μm cellulose acetate membrane (Minisart RC 4, Sartorius, France) and transferred in vials before analysis [26]. The operating parameters of the extraction conditions of the control and final protocols are given in the ESM Table S1. Resulting protocol B has been successfully employed to evaluate the performances of anaerobic digestion [26].

UHPLC-MS-MS analysis

LC separation was carried out using an Ultimate 3000 UHPLC System (Thermofisher, USA) following the protocol proposed by Gonzalez-Salgado et al. [26]. Sample aliquots (10 μL) were injected onto an ACQUITY UPLC HSS (High Strength Silica) T3 (100 mm × 2.1 mm, 1.8 μm) column from Waters. Detection was achieved with an Applied Biosystems Sciex QTRAP® 4500 hybrid linear ion-trap triple quadrupole mass spectrometer (Foster City, USA) equipped with a Turbolon-Spray source. The instrument was operated in ElectroSpray (ESI) positive (+) or negative (−) in multiple reaction monitoring (MRM) mode (dwell time, 80 ms). The operating parameters were as follows: capillary voltage,

Table 2 Quantities of sludge, water, and ACN for 0.25 g of sludge and corresponding ratios

		Control	Tested conditions to optimize protocol			
			Modified salts $MgSO_4$ and Na_2SO_4	Modified ratios		
				"ACN/mat"	"ACN/aq"	"ACN/mat/aq"
Calculated ratios	ACN/mat (v:w)	10	10	***20***	10	***20***
	ACN/aq (v:v)	0.5	0.5	0.5	***0.7***	***1***
Detail of quantities and volumes of parameters	Matrix (g)	0.25	0.25	0.25	0.25	0.25
	Salts (g)	1	**0.5–2–4–6**	1	1	1
	ACN (mL)	2.5	2.5	**5**	2.5	**5**
	Water (mL)	2.5	2.5	**7.5**	**1**	2.5
	EDTA (mL)	1.25	1.25	1.25	1.25	1.25
	Buffer (mL)	1.25	1.25	1.25	1.25	1.25

Bold values correspond to parameters modified compared to the control protocol

mat matrix, expressed in g; *aq* aqueous phases represent the sum of water, EDTA, and buffer solution, expressed in mL

5500 V and −4500 V for the positive and negative mode respectively; source temperature, 500 °C; gas, N_2; curtain gas, 20; ion source gas 1, 20; and ion source gas 2, 70. MS and MRM conditions are summarized in ESM Table S3. For MS spectra and chromatogram acquisition and exploitation, Analyst® 1.6.2 software from Applied Biosystems Sciex (Foster City, USA) was used.

For quantification, MRM transitions were used. Five-point (from 10 to 100 ng/mL) calibration curves were generated. The calibration standards were prepared in water/ACN 95/5 and filtered at 0.2 μm. Calibration curves were performed at the beginning and at the end of each batch process. Curves were built by calculating the ratios between the peak area of each analyte and the peak area of corresponding IS using weighted 1/x model for linear regression. Along the sequence, quality control (QC) samples (medium concentration level of the curves) were also analyzed to confirm their validity. No significant (< 6%) deviation has been observed. As sludge extracts may contain many interfering compounds, blank samples (mobile phase mixture without analytes) were included every 10 injections or between two different matrices. Non cross-contamination has never been observed.

Analytical calculations and validation of the method

To find the best conditions for sample preparation based on QuEChERS, the absolute recoveries (AR) and matrix effects (ME) must be considered. Blanks (unspiked samples) were previously analyzed using IS calibration to evaluate the eventual presence of analytes and quantify it if possible. To dissociate AR and ME, the matrices were spiked at 40 ng/mL with standard solution prior to QuEChERS extraction and the QuEChERS upper phases were spiked at 60 ng/mL with the IS solution prior to analysis. All tested conditions are performed in duplicates.

Matrix effects (ME) were evaluated according to the following equation:

$$ME\ (\%) = \left(\frac{A_{IS\_matrix}}{A_{IS\_water}} - 1\right) \times 100 \quad (1)$$

where A_{IS_matrix} is the IS peak area in the spiked QuEChERS upper phase of the sample and A_{IS_water} is the IS peak area in the samples for calibration. A positive value indicates an enhancement of the signal and a negative value a suppression of the signal.

AR were determined by the following equation:

$$AR = \frac{[Matrix]_{IS\_end} - [Blank]}{[Water]} \quad (2)$$

where $[matrix]_{IS\_end}$ is the analyte concentration in the upper phase of the spiked sample (calculated by adding IS before analysis), [blank] is the analyte concentration in the non-spiked sample, and [water] is the analyte concentration added to the samples.

Relative recovery (RR), which considers both AR and ME, was determined by internal calibration according to the following equation:

$$RR = \frac{[Matrix]_{IS\_ini} - [Blank]}{[Water]} \quad (3)$$

where $[matrix]_{IS\_ini}$ is the analyte concentration in the spiked matrix determined when IS and standards were spiked in the sample before extraction.

To assess the robustness of the analysis procedure, an evaluation of the performance of methods was realized on 2 real matrices (primary sludge and clarified wastewater). The precision, the accuracy, the detection limit (DL), and quantitation limit (QL) were determined for both protocols A and B. Method accuracy (estimated by means of RR experiments) and precision (expressed as intra-day repeatability in terms of relative standard deviation (RSD)) were studied by spiking samples at different concentrations according to the concentration levels found in real samples. When the analyte concentration was less than 10 ng/mL, spiking levels were 1, 2, 4, and 15 ng/mL, and when it was superior, spiking levels were 5, 10, 20, and 75 ng/mL. All experiments were performed in duplicate. RR values between 70 and 120%, with RSD lower than 20%, were considered acceptable.

The limit of detection was calculated according to the EPA's method [27] by considering Student's t value, and the standard deviation determined by analyzing 8 blank samples or spiked samples at low levels when it was necessary. The DL was calculated using the following equation:

$$DL = t_{n-1,\ 1-\alpha=0.99} \times SD \quad (4)$$

where n is the number of replicates, $t_{n-1,1-\alpha}$ the Student's t value appropriate for a single-tailed 99th percentile statistic and a standard deviation estimate with n − 1 degrees of freedom, and SD the sample standard deviation of the replicate sample analyses [27]. QL is calculated by considering 3 times the DL. The analyte concentration of blank or spiked samples must be 1 to 5 times the estimated DL as recommended by the guidelines given in the European Reference Laboratory (EURL) experts' report [28]. Thereby, if the analyte concentration of the blank sample is higher than 5 times the estimated DL [28] or with a signal-to-noise (S/N) superior to 20 [27], no DL and QL can be defined (indeed real matrix without analyte is not available). Finally, if the ratio S/N of the quantitative ion was between 3 and 10, the concentration of the molecule was therefore just detected (concentration between DL and QL), and if the ratio was higher than 10, the concentration of the molecule could be determined. S/N was determined from Analyst 1.6.2 software using the "peak to peak" method where the background noise (N) is calculated at a distance of 10 times the width of the peak at half height before the onset of the peak.

Results and discussion

Preliminary study: screening of influencing parameters on the extraction step

Determination of conditions on spiked distilled water

A first study screening 36 extraction conditions was carried out on spiked distilled water, to investigate the effects of salts, EDTA, buffer, and acidification of ACN on the absolute recovery (AR) of the nine targeted molecules. The conducted principal component analysis (PCA) underlined that acidification of ACN and EDTA had negligible effect on recovery (see ESM Fig. S2). As preconized by Peysson and Vulliet (2013), EDTA 1% was maintained in this study and ACN was acidified as done in most of the studies summarized in Table 1.

Evaluation of acetate/citrate buffer and $MgSO_4$/$MgSO_4$ + NaCl/Na_2SO_4 as salts to improve the transfer towards the organic phase resulted in six combinations whose AR are presented in Fig. 1.

Figure 1 summarizes the absolute recovery obtained for 6 combination salts-buffer. The targeted molecules differ in their structures and their chemical properties, which results in behavior differences during the extraction. The compounds CAF, CBZ, and PRO were the only molecules satisfactorily extracted regardless of the couple buffer/salt used, with AR higher than 75%. None of the evaluated operating conditions allowed a satisfying recovery for all the molecules. The combination acetate/$MgSO_4$ allows a good extraction on the largest number of compounds. Unfortunately, OFL is not extracted with this method (AR < 4%). Divalent cations such as Mg^{2+} are known to make a complex with fluoroquinolones and quinolones [29]. Finally, to quantify the nine molecules, 2 extraction protocols will have to be implemented: (i) acetate buffer + $MgSO_4$ (protocol A) and (ii) citrate buffer + Na_2SO_4 (protocol B).

Application on control activated sludge

The combination of protocol A and B for each sample allows obtaining AR more than 60% for the nine compounds based on extractions of molecules spiked in water. However, activated sludge is a complex matrix and interfering substances may significantly change the trends. Absolute recoveries and matrix effects must be considered to assess the quality of sample preparation based on QuEChERS when tackling such matrices. This was done by spiking standard solutions in the raw sample and IS solution prior to the UHPLC-MS-MS analysis. Results obtained on sludge ("reference matrix") for protocols A and B are compared with the ones obtained with water on Fig. 2, all analyses being duplicated.

It is worth noting with Fig. 2 that even with water, matrix effects were quantified underlining the effect of the extraction protocol itself on the signal. Indeed, matrix effects, quantified when extracting analytes from water, comprised between + 6% (OFL) and − 37% (PRO) for protocol A, and between + 4% (OFL) and − 73% (PRO) for protocol B. In general, the observed matrix effects were more impacting with protocol B (citrate buffer + Na_2SO_4) than with protocol A. As expected, extractions performed on the reference matrix induced a significant suppression of the signal, but in this case, it did not appear to be a clear trend, since, except for OFL, the matrix effects obtained with the two protocols were in the same range. This tends to emphasize that in sludge, the matrix effects are more related to the compounds present in the original matrix than to those resulting from the extraction protocol, probably due to the use of the same purification procedure for both control protocols (same sorbents).

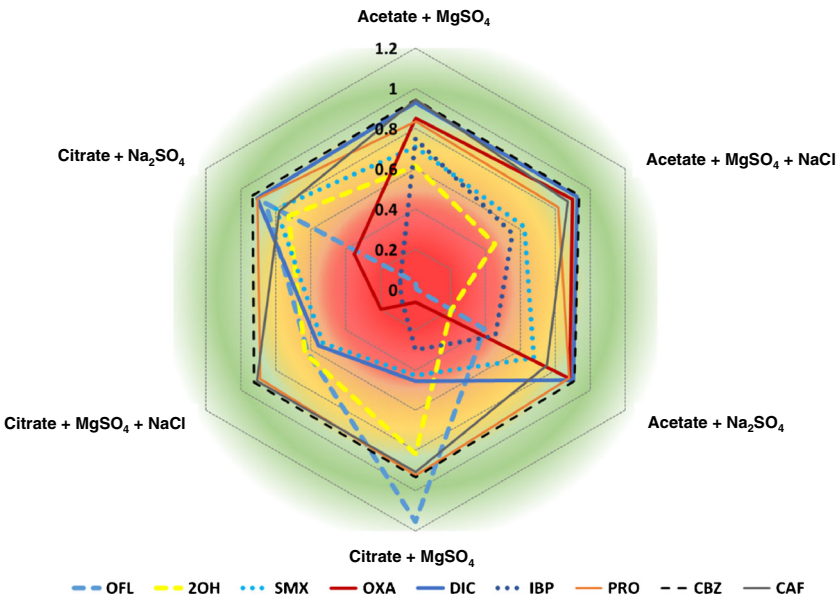

Fig. 1 Effect of salt (Na_2SO_4/$MgSO_4$/$MgSO_4$ + NaCl) and buffer (acetate/citrate) type on the absolute recovery of the nine compounds from spiked water. Six combinations were evaluated

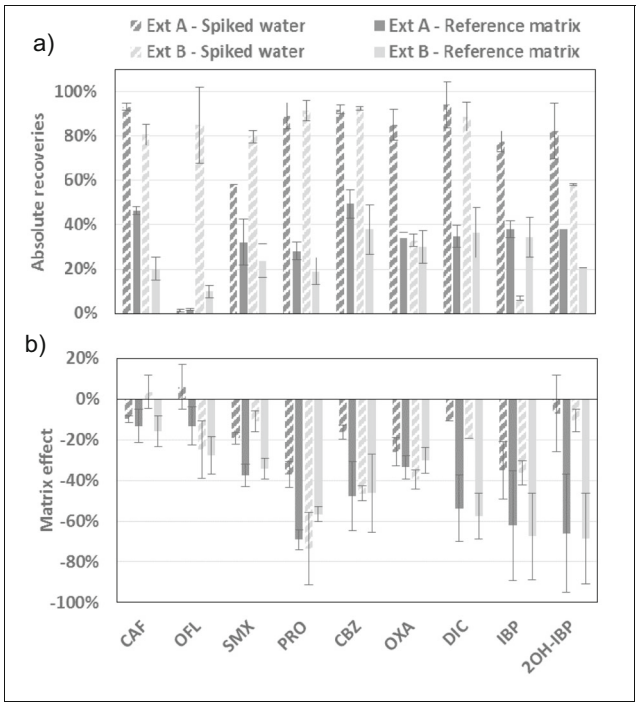

Fig. 2 Comparison of the effect of the extraction protocol on absolute recovery (**a**) and matrix effects (**b**) on water and the reference matrix

On the same way, except for IBP with protocol B and for OFL with protocol A which were poorly extracted from spiked water, the recoveries were significantly lower when the molecules were extracted from the reference matrix compared to water. The decrease was observed from a factor of 1.8 (SMX) to 3.2 (PRO) for protocol A and reached 8.6 for OFL with protocol B. In all cases, the recovery of the molecules did not exceed 46% and, more importantly, OFL was only 10% recovered with protocol B (2% with protocol A) likely due to the presence of divalent cations in the reference matrix, as it will be the case with environmental samples. An optimization of these protocols is therefore necessary to both increase the absolute recovery and reduce the matrix effects, which will result in lowering the quantification limits.

Optimization of extraction conditions on control activated sludge

Based on literature, two parameters were investigated, namely the quantity of salts and the ratio between solvent, aqueous phase, and matrix.

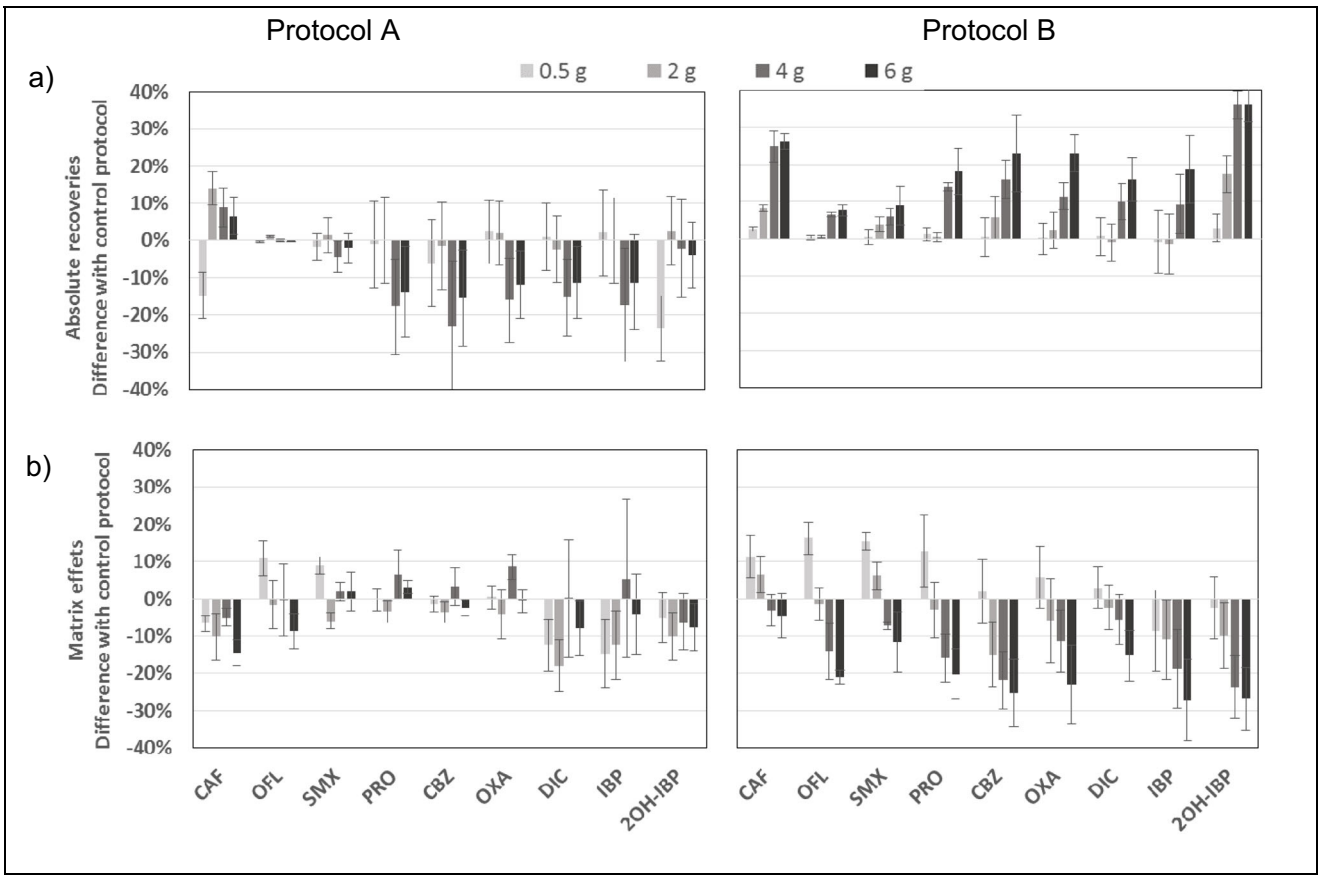

Fig. 3 Effect of salt quantity on absolute recoveries (**a**) and matrix effects (**b**) (expressed in terms of difference with the control protocol, i.e., 1 g) for protocol A ($MgSO_4$) and B (Na_2SO_4)

Quantity of salts

The addition of salts is expected to decrease simultaneously the aqueous solubility and the amount of water in the organic phase, influencing the transfer of analytes to the organic phase. Therefore, the QuEChERS extraction with 0.5 g, 2 g, 4 g, and 6 g of $MgSO_4$ (protocol A) or Na_2SO4 (protocol B) was evaluated and compared to the control protocol (1 g of salt). Figure 3 presents the effect of salt quantities on absolute recovery and matrix effects expressed in terms of difference with the control condition. An improvement in the extraction will therefore result in positive values for AR and for ME.

For protocol A, except for CAF, an increase of salt quantity led to a decrease of the recovery of molecules concomitantly with a limited impact of matrix effects. Thus, the amount of salt was maintained at 1 g/L of $MgSO_4$ as in the control protocol. For protocol B, an increase of salt quantity was associated to a simultaneous increase of recovery and matrix effects. Especially the increase from 2 to 4 g generates a significant step on recoveries. Considering that ME is negatively impacted between 4 and 6 g, without significant gain on the recovery (in particular for OFL for which protocol B was implemented), a quantity of 4 g of Na_2SO_4 was preferred for the rest of the process. The differences of behavior observed with the two salts may be explained by the ionic strength which is higher with a bivalent salt facilitating the salting out of the targeted molecules.

Ratios

The choice of respective volumes for the aqueous and organic phases in regard to the matrix quantity is important to obtain a satisfying transfer and consequently recovery of molecules. Thus, the volumes of aqueous phase and solvent were changed, for a same quantity of sludge (i.e., 0.25 g), to vary ACN/aqueous, ACN/matrix, or both ratios as summarized in Table 2.

Figure 4 presents the effect in changing the ratios on absolute recovery and matrix effects expressed in terms of difference with the control condition for both protocols.

We can note in protocol B that the increase of amount of solvent alone did not allow obtaining a clear phase separation, thus making impossible the sampling of the organic phase. Whatever the protocol employed, the simultaneous increase of both ratios leads to an increase of recovery for most of the analytes with an average improvement of 15% (between 4 and 27%) and 28% (from 14 to 45%) compared to the control protocol for protocols A and B respectively.

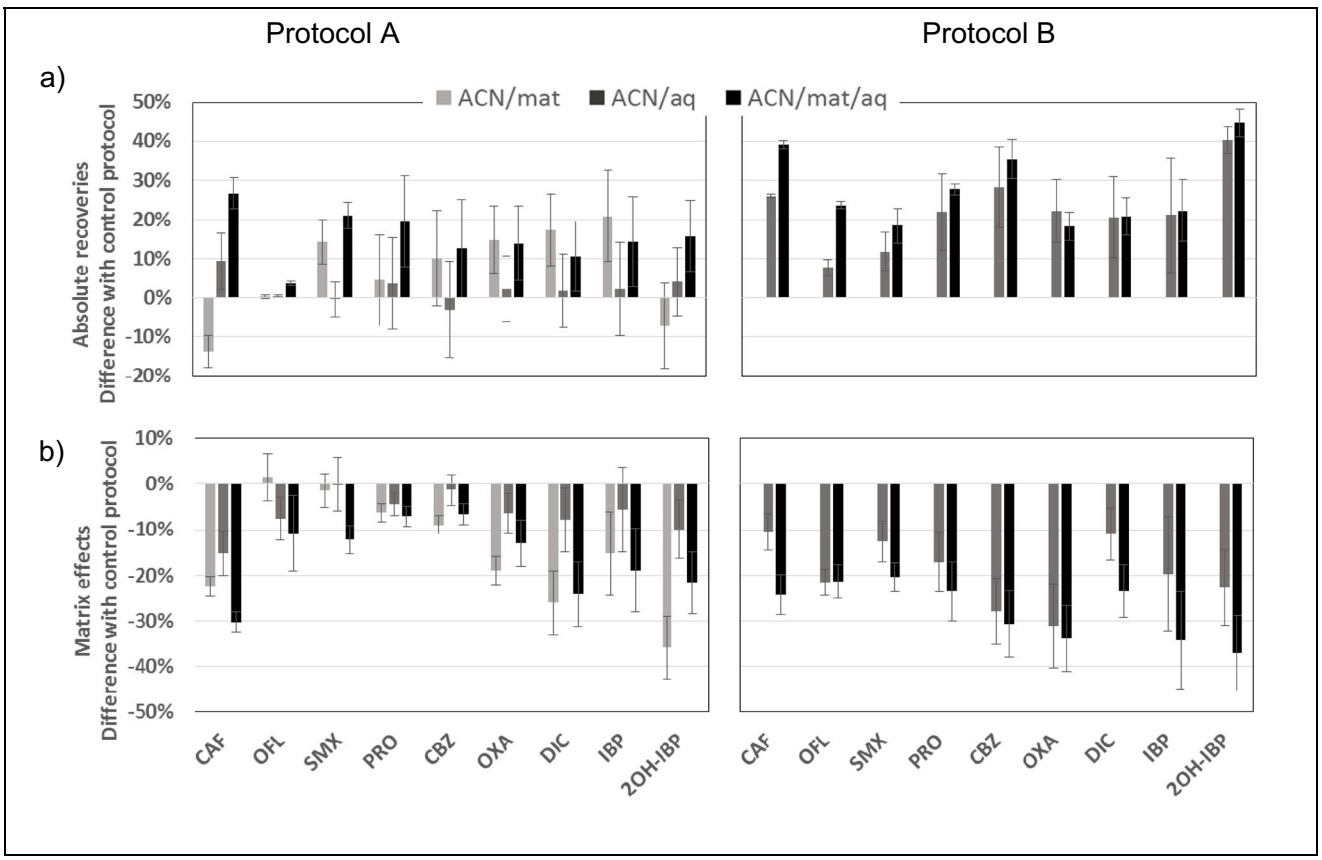

Fig. 4 Effect of doubling the ratios solvent to matrix ("ACN/mat"), solvent to aqueous phases ("ACN/aq"), and both ratios ("ACN/mat/aq") on absolute recoveries (**a**) and matrix effects (**b**) (expressed in terms of difference with the control protocol) for protocols A and B

Final protocol

Finally, all the successive improvements were implemented simultaneously to achieve the final protocols and a comparison of AR and ME with the initial ones is presented in Fig. 5.

Results underline that whatever the protocol, the optimization led to an improvement of analyte recovery that reached finally from 6% (OFL) to 77% (CAF) for protocol A and from 41% (SMX) to 70% (DIC) for protocol B. The use of buffer has led to pH during extraction of 5.69 for acetate (protocol A) and of 4.87 for citrate (protocol B). Thus, a non-negligible fraction of acidic forms for compounds with low pka is expected but this can be compensated by the increase of ACN quantity to favor solubility. Lots of antagonist phenomena such as the ionization of the molecules that impacts the solubility but also the interaction with sorbents, the chelation of divalent cations by citrate that can modify cation bridging and affect the extraction [30], are likely to occur during QuEChERS extraction. Finally, it is important to notice that performing both protocols would allow recovering all the compounds to 43% at minimum. As expected, when extracting more, more interfering compounds are also extracted leading to similar or increased matrix effects. However, it is important to underline that for OFL, which was problematic even when extracting spiked water, the optimization of protocol B leads to a 4-fold increase in the recovery while slightly reducing the matrix effects. Extraction recoveries of CBZ, DIC, IBP, 2-OH-IBP, and SMX were similar to those of Malvar et al. [31].

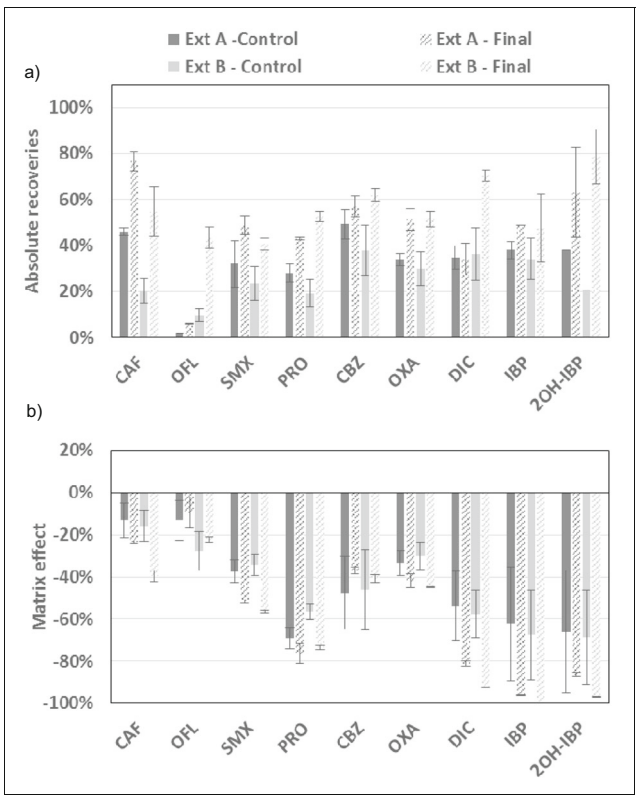

Fig. 5 Absolute recoveries (**a**) and matrix effects (**b**) for the reference matrix with control and final protocols

Validation and application on real matrices

The quality of the results obtained in terms of compound concentration in a sample of interest is crucial to have a relevant analysis of the process performances in a WWTP. Therefore, a validation study was carried out on contrasted (especially in terms of solid contents) real samples, namely clarified wastewater (TSS = 0.5 g/L) and primary sludge (TSS = 15 g/L). The relative recovery and repeatability of the method were established to evaluate the method performance. To do that, using spiked samples, standard curves were plotted for each targeted molecule, and for each real sample, in ranges depending on the concentration in the raw sample (up to 80 ng/mL for compounds found at concentrations higher than 10 ng/mL and up to 15 ng/mL for compounds encountered at concentrations lower than 5 ng/mL). The samples were extracted and tested according to "Final protocols."

The relative recovery for all molecules and the RSD (%) for each spiking level on clarified wastewater and primary sludge are summarized in Fig. 6.

For primary sludge, recoveries of the analytes ranged within 80–112% and 90–116% with extraction protocols A and B, respectively (with only 2 times above 110% for protocol A and 3 times for protocol B, out of 36 samples for each protocol). For the clarified wastewater, recoveries of the analytes ranged within 74–112% and 73–113% with protocol A and B respectively (with only 3 times below 80% for protocol A and 2 times for protocol B, out of 36 samples for each protocol). The intra-day RSD values were found to be lower than 20% with 131 values (144 in total) below 10% indicating a satisfactory repeatability. In conclusion, relative recoveries for target compounds were higher than 74% and lower than 116% (with a majority ranging from 80 to 110%) and RSDs were lower than 20%. These are highly acceptable values for environmental samples.

Table 3 showed micropollutant concentrations and DL determined in two samples of primary sludge and clarified wastewater by extraction A and B. The nine compounds were detected in both matrices. Differences between the two protocols were lower than 8% (excepted for OFL, 20.6%) when characterizing the primary sludge and lower than 3% (excepted for OFL, 28.6%) for clarified wastewater. These results underline that a single extraction protocol could be applied whatever the targeted molecule or the environmental matrix.

DL evaluated in sludge are lower for IBP and in the order of magnitude for SMX, PRO, CBZ, and DIC than Peysson and Vuillet (2013). More recently, Malvar et al. (2020)

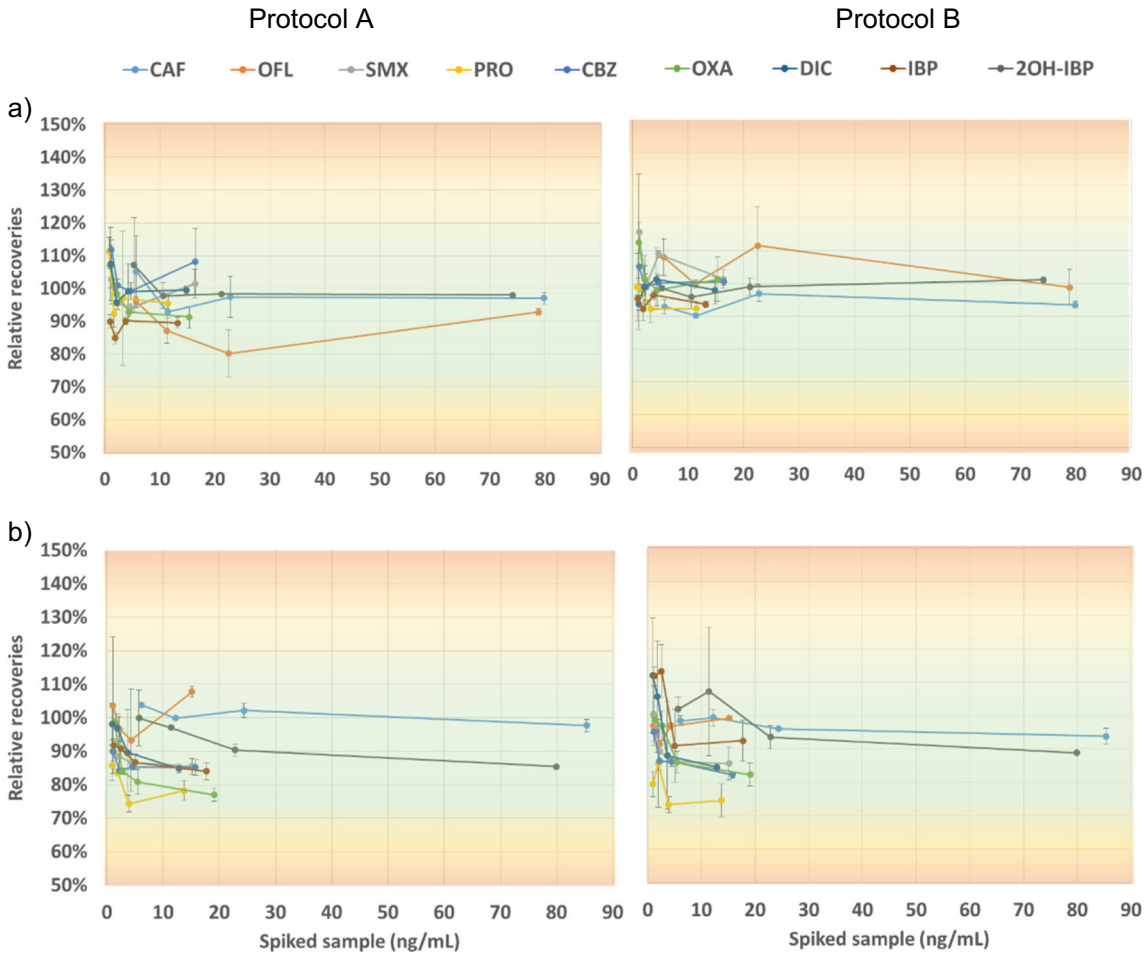

Fig. 6 Relative recoveries of standard additions of nine compounds for **a** primary sludge and **b** clarified wastewater

obtained lower DL from composted sludge (CAF 5.8 ng/g, CBZ 0.8 ng/g, DIC 0.5 ng/g, IBP 2.5 ng/g, 2OH-IBP 8.9 ng/g, SMX 1.3 ng/g). They obtained these results with triple quadrupole 6410 which is more sensible than the one used in our study. Furthermore, Malvar et al. (2020) did extraction from 2 g of dried sludge that is 50 times more than the quantity included in 2.5-mL samples. Other extraction procedures can be applied such as reported in a recent review [32]. In particular, ultrasound-assisted extraction was successfully applied on sewage sludge to quantify 148 pharmaceuticals allowing to reach low DL ranging from 0.9 (OFL) to 11.7 (IBP) ng/g [33]. Although DL were higher in the present study, this protocol has the advantage of making it possible to dispense with the time-consuming freeze-drying step. According to expected concentrations, it will be possible to increase the initial volume of sludge for extraction to decrease the DL.

Conclusions

Extraction by QuEChERS shows several advantages over traditional extraction techniques, requiring low sample and solvent volumes, as well as less time for sample preparation. The aim of this study was to develop a simple and reliable method that can be easily transferrable on diverse complex environmental matrices. Therefore, two optimized methodologies based on QuEChERS extraction that enable the determination of 9 molecules were developed. Extraction A was carried out with acetate buffer and $MgSO_4$ as extraction salt, and extraction B was performed with citrate buffer and Na_2SO_4 as extraction salt. The numerous parameters tested for the optimization of the sample preparation suggest the following changes: (1) the increase of ACN volume modifying the organic to aqueous and matrix ratios and (2) the addition of 4 g of Na_2SO_4 during extraction B instead of 1 g, as commonly applied. These procedures optimized from freeze-dried waste activated sludge were applied and validated on real complex matrices from the SMS project, i.e., clarified wastewater and primary sludge, which have opposite physicochemical properties. Matrix effects are between − 10 and − 100% and absolute recoveries are between 40 and 80%, except for OFL with extraction A which is less than 10%. The precision of both protocols was excellent for the 9 micropollutants. The lowest QL was obtained for CBZ at

Table 3 Micropollutant concentrations (μg/L) determined by extraction A and B, intra-day precision (RSD%) for each extraction method, mean and RSD (%) of concentrations determined with extraction A and B, and DL evaluated in samples of primary sludge and clarified wastewater (DL in μg/g was obtained dividing DL in μg/L by TSS expressed in g/L)

Date	Concentration (μg/L)			DL				QL			
				μg/L		μg/g		μg/L		μg/g	
Extraction	A (RSD%)	B (RSD%)	Mean (RSD%)	A	B	A	B	A	B	A	B
Primary sludge											
CAF	42.7 (6%)	42 (3%)	42.3 (1%)	<8.54	<8.4	<0.57	<0.56	<25.62	<25.2	<1.71	<1.68
OFL	27.9 (12%)	34.3 (6%)	31.1 (15%)	8.60	<1.43	0.57	<0.1	25.8	<4.29	1.71	<0.30
SMX	1.2 (1%)	1.3 (3%)	1.2 (3%)	0.46	<0.25	0.03	<0.02	1.38	<0.75	0.09	<0.06
PRO	3.2 (8%)	3.4 (5%)	3.3 (4%)	1.27	5.65	0.08	0.38	3.81	16.95	0.24	1.14
CBZ	1.3 (1%)	1.4 (4%)	1.4 (5%)	0.48	<0.13	0.03	<0.01	1.44	<0.39	0.09	<0.03
OXA	5.4 (5%)	5.4 (3%)	5.4 (0%)	<0.7	<0.73	<0.05	<0.05	<2.10	<2.19	<0.15	<0.15
DIC	1.8 (4%)	1.9 (5%)	1.9 (4%)	0.66	0.76	0.04	0.05	1.98	2.28	0.12	0.15
IBP	5.7 (1%)	5.8 (2%)	5.7 (1%)	<1.14	1.06	<0.08	0.07	<3.42	3.18	<0.24	0.21
2OH-IBP	9.2 (22%)	9.8 (1%)	9.5 (4%)	<1.83	<1.95	<0.12	<0.13	<5.49	<5.85	<0.36	<0.39
Clarified wastewater											
CAF	19.9 (10%)	20 (10%)	19.9 (0%)	<5.15	<9.34	<10.31	<18.68	<15.45	<28.02	<30.93	<56.04
OFL	0.8 (17%)	0.6 (5%)	0.7 (14%)	0.58	0.39	1.15	0.78	1.74	1.17	3.45	2.34
SMX	0.3 (7%)	0.3 (6%)	0.3 (5%)	0.08	0.13	0.16	0.26	0.24	0.39	0.48	0.78
PRO	0.3 (4%)	0.3 (6%)	0.3 (4%)	0.12	0.12	0.23	0.24	0.36	0.36	0.69	0.72
CBZ	0.2 (3%)	0.2 (3%)	0.2 (1%)	0.05	0.07	0.11	0.14	0.15	0.21	0.33	0.42
OXA	1.3 (3%)	1.3 (5%)	1.3 (0%)	0.30	0.32	0.60	0.64	0.90	0.96	1.80	1.92
DIC	0.6 (8%)	0.6 (6%)	0.6 (2%)	0.30	0.29	0.59	0.58	0.90	0.87	1.77	1.74
IBP	3.7 (3%)	3.8 (6%)	3.8 (2%)	<0.7	0.98	<1.49	1.96	<2.10	2.94	<4.47	5.88
2OH-IBP	8.5 (7%)	8.3 (2%)	8.4 (2%)	<1.7	<1.7	<3.39	<3.30	<5.10	<5.10	<10.17	<9.90

10 ng/g on primary sludge with extraction B and 50 ng/L on clarified wastewater with extraction A. The future choice of protocol A or B to be applied will depend on the matrices and QL of molecules that will be necessary to reach. The standard d-SPE (a mixture of PSA and C18) chosen in this study could be optimized according to the matrix in order to decrease the matrix effect and the QL.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-021-03489-z.

Declarations The authors attest that there is no conflict of interest. This work is part of the SMS project "Micropollutant source separation for health risks control and preservation of the environment" funded by the Adour-Garonne Water Agency and the French Agency for Biodiversity.

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