Improvement of the QuEChERS extraction step by matrix-dispersion effect and application on beta-lactams analysis in wastewater sludge by LC-MS/MS

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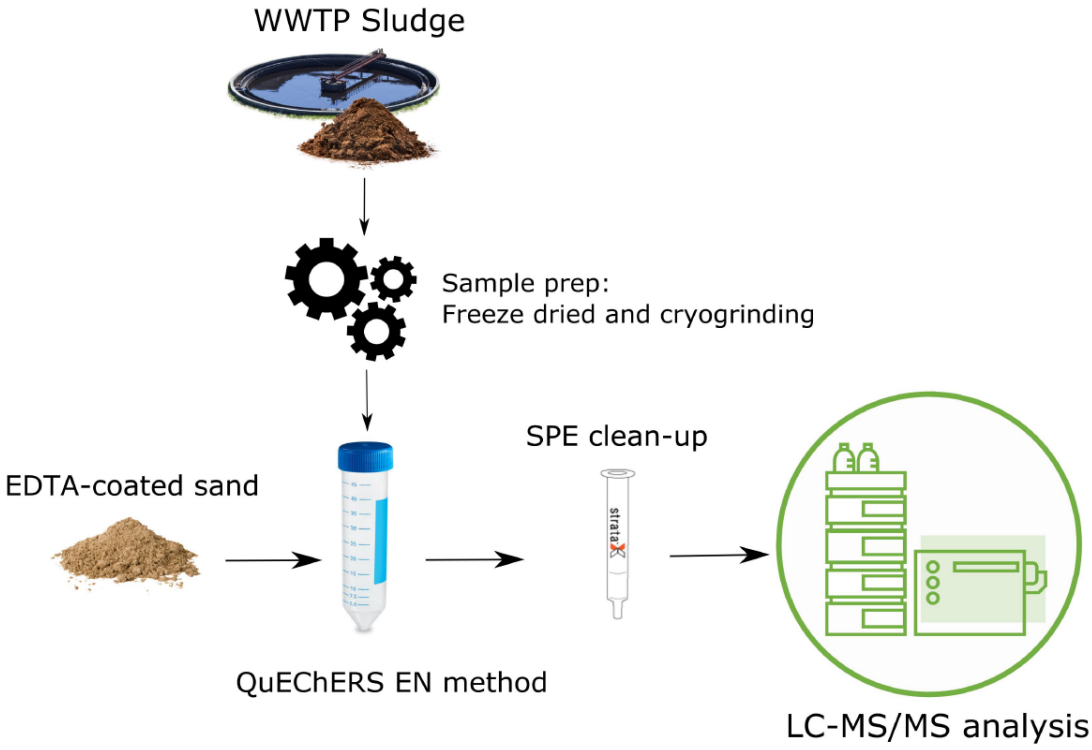
Graphical Abstract:



Abstract

In the last decade, beta-lactams use in veterinary and human medicine increased to represent today about 15% of the overall consumption. Beta-lactams tend to degrade and metabolize in the environment. Therefore, analytical methods must be sensitive enough to quantify low concentrations of the parent molecules and also allow detection of metabolites. This study presents the development of a modified QuEChERS method for the extraction of seven beta-lactams and one degradation product (Amoxicillin, Ampicillin, Cefapirin, Cefoperazone, Cefquinome, Ceftiofur, Cloxacillin, and Amoxicillin-Diketopiperazine) from sewage treatment plant sludge and their analysis by liquid chromatography coupled with tandem mass spectrometry. Before the QuEChERS extraction, a dispersion step of the sample with EDTA-treated sand was optimized and added, allowing to facilitate the exchanges between the matrix and the extraction solvent. Then, to decrease the interferences present in the extract, a fast and efficient pass-through SPE was implemented.

The optimized method was validated and showed satisfactory performances, in adequacy with the analysis of beta-lactams in solid environmental matrices. Limits of quantification lower than 20 $ng.g^{-1}$ for all analytes, high accuracy (96%-114% quantification on spiked samples nominal concentration) and interday precision (2%-12% RSD) were obtained. This method was then applied to eight sludge samples. Cefapirin and amoxicillin-diketopiperazine were detected in four samples each, at concentrations of 10.2-53.3 $ng.g^{-1}$ and 3.0-9.5 $ng.g^{-1}$ respectively. Thus, the developed method is very effective for the extraction of beta-lactams from environmental solid matrices.

Keywords: antibiotics; beta-lactam; metabolite; sludge; dispersive-QuEChERS; LC-MS/MS

1. Introduction

Beta-lactams, which include penicillins and cephalosporins, are among the most widely used antibiotics in veterinary medicine over the last 15 years, mainly in pig and cattle farms.[1] Indeed, according to the European Surveillance of Veterinary Antimicrobial Consumption reports produced annually by the European Medicines Agency, their consumption has increased from 9% in 2005 to almost 15% in 2018[2,3]. However, over the last decade, the consumption of 3rd and 4th generation cephalosporin has been drastically reduced in France and globally in Europe [2–4]. They are also widely used in human medicine, averaging at 13% of the daily antimicrobials consumption this last 20 years[5], being one of the first medicine prescribed.

Due to their wide use, beta-lactams are often analysed in food- matrices, such as milk [6–11], honey [12] or meat[11,13,14]. In those matrices, Maximum Residues Limits (MRLs) are set and periodically adjusted for each molecule and matrix. In the environment, beta-lactams analysis have been extensively described in liquid matrices such as surface waters[15,16], rivers[17,18], wastewaters[19–21], or in urine[22,23].

Regarding liquid matrices, Solid Phase Extraction (SPE) is the most used sample preparation technique. Considering solid environmental matrices, such as manure or wastewater treatment plant (WWTP) sludge, the two most used extraction techniques for veterinary drugs are ultrasound-assisted extractions (UAE) and QuEChERS [24–28]. The principle of the QuEChERS methodology involves two main steps: (i) a liquid/solid extraction of the matrix, assisted by the addition of salts or buffers to promote phase separation and preferential transfer of analytes to the solvent; and (ii) a dispersive purification of the extract on a solid adsorption phase.

Peysson et al.[25] and Salvia et al. [24,26] developed multi-residue extractions in WWTP sludge and soil, respectively, including some beta-lactams (Penicillin G, Cefoperazone, Cloxacillin), using the acetate-based salts described in the Association of Analytical

Collaboration (AOAC) QuEChERS official method 2007.01 [29]. Salvia et al. obtained maximum extraction yields of 50% for Penicillin G, and Peysson et al. reported an average recovery of 15% for cefoperazone but no extraction recovery of cloxacillin. As both these methods were intended for multi-family analyses, compromises were made during the optimisation to achieve suitable recoveries for most of the included analytes. Bessaire et al. [27] proposed a specific QuEChERS buffer for veterinary drugs followed by a C_{18} based dispersive SPE, for the extraction of 23 beta-lactams in different food matrices, leading to extraction efficiencies of 34% on average. Matrix-matched calibration can then be used to compensate for the analytes losses but only when developing a method with a single matrix type, as suggested by Geis-Astaggiante et al.[30].

As beta-lactams are very likely to degrade[31–34], it is necessary to monitor their degradation products (DPs). One of the main DPs of amoxicillin in the environmental matrices is amoxicillin diketopiperazine (AMX-DKP), which result from cyclisation of amoxicillin after hydration and water removal. This metabolite has been detected and quantified by Gozlan et al. [32] in groundwater and by Hirte et al. [33] in WWTP effluent and river water. But, to the best of the authors' knowledge, the determination of AMX-DKP has never been reported before in solid matrices such as WWTP sludge.

In this context, the objective of this work was to develop an efficient and robust extraction and quantification of 7 beta-lactams and 1 DP (AMX-DKP) in wastewater sludge. These molecules were selected from the study realised by Soulier et al. [1], who determined these beta-lactams as the most often prescribed. An original adaptation of the first step of salting-out extraction of the classical QuEChERS method was developed: a dispersive-QuEChERS extraction. It was followed by a rapid SPE step for clean-up, then liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was employed for the identification and quantification. The final method was validated and applied on a few WWTP sludge collected in France.

2. Experimental

2.1. Chemicals and reagents

Ampicillin (AMP) trihydrate, Amoxicillin (AMX) trihydrate, Cloxacillin (CLX) and Cefoperazone (CFZ) were bought from TCI Europe (Zwijndrecht, Belgium). Ceftiofur (CEF), Cefquinome (CFQ) and Cefapirin (CFP) were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). AMX-DKP, CEF-d3, AMP-d5 and CFP-d4 were bought from TRC (Toronto, Canada). All standards were at least 97% purity. The structure of each beta-lactam is reported in Fig. S1.

Stock solutions (1 $mg.ml^{-1}$) of each beta-lactam were prepared by dissolving about 10 mg powder, accurately weighted in 10 ml of acetonitrile (ACN) in glass Wheaton vials and were stored at -18°C for 3 months. Accurate concentration was then calculated taking each standard purity into account. Calibration solutions of each analyte (500 $ng.ml^{-1}$) were prepared by diluting individual stock solutions in water/ACN (1/1, v/v).

Water (LC-MS grade) was obtained from Fisher Scientific (Illkirch, France), ACN (LC-MS grade) from Honeywell (Seelze, Germany) and formic acid (UPLC-MS grade) from Biosolve (Dieuze, France). Ethylenediaminetetraacetic acid (EDTA) and Fontainebleau sand were purchased from VWR (Fontenay-sous-Bois, France), and Strata X™ SPE cartridges from Phenomenex (Le Pecq, France).

The compositions of the different QuEChERS extraction kits (from Agilent, Massy, France) were as follow: the European Committee for Standardization (EN 15662:2008) kit contains 4 g of $MgSO_4$, 1 g of NaCl, 1 g sodium citrate dihydrate and 0.5 g sodium hydrogencitrate sesquihydrate; the AOAC kit contains 6 g of $MgSO_4$ and 1.5 g of sodium acetate, the Original kit contains 4 g of MgSO4 and 1 g of NaCl; and the Veterinary Drugs kit contains 4 g of Na_2SO_4 and 1 g of NaCl. Clean-up d-SPE kits (from Macherey-Nagel, Düren, Germany) PSA contains

900 mg of $MgSO_4$ and 150 mg of PSA phase, and the PSA/C_{18} contains 900 mg of MgSO4, 150 mg of PSA phase and 150 mg of C_{18} phase.

2.2. EDTA-treated sand

Fontainebleau sand was used to promote dispersion of the matrix. Before use, sand was treated with EDTA to remove metal impurities. Briefly, 180 g of sand were put in contact with 360 ml of 0.2 M EDTA water solution and stirred for 2h. After stirring, sand was left to settle for 10 min and excess water was dumped. Finally, sand was dried overnight in a heat chamber.

2.3.Sample collection

Sludge samples were collected from a wastewater treatment plant (WWTP) located in Haute-Savoie, France. The layout of the WWTP was described in detail by Chonova et al [35]. Briefly, this WWTP treats effluents from both urban wastewaters and hospital wastewater for an actual capacity of 32,000 Population Equivalent and operates on activated sludge treatment. After collection, samples were transported to the laboratory where they were immediately freeze-dried (Alpha 2-4 LD Plus, Christ) then homogenized and pulverised using a cryo-grinder (6770 Freezer/Mill, SPEX Sample Prep). The resulting powders were stored at -20°C before extraction. All method developments were realised on Sludge 1, in which no beta-lactam was detected, except AMX-DKP.

2.4.Sample preparation

The optimised extraction was performed in the following successive steps: first, 0.5 g of sludge were weighed into a 50 ml centrifuge tube and mixed with 2 g of EDTA-treated sand. Then, 5 ml of 0.1M EDTA water solution were added, the mixture was swirled with a vortex mixture for 30 s then 10 ml of ACN were added and swirled again for 30 s. EN QuEChERS salts were then added, agitated for 15 s by hand and by vortex for 45 s. Extraction tubes were then centrifuged for 5 min at 10,000 rpm. The supernatant was then transferred to a 12 ml glass tube.

After QuEChERS extraction, a clean-up was performed using SPE (Rapid Trace SPE Workstation, Caliper). First, a Strata X™ cartridge (3 ml/200 mg) was conditioned with 5 ml of ACN at 5 $ml.min^{-1}$. Then, 10 ml of the QuEChERS ACN extract was flowed through the cartridge at 1 $ml.min^{-1}$ and collected in a new 12 ml glass tube.

Finally, the purified ACN extract was evaporated to dryness under a gentle N_2 flow, in a water bath heated to 40°C. The dry residue was later dissolved in 1 ml of 97/3 H_2O/ACN, mixed for 1 min and centrifuged at 1,500 rpm for 2 min to allow potential non dissolved particles to settle to the bottom of the tube. Particle-free supernatant was finally transferred to a 2 ml vial for LC-MS/MS analysis.

2.5.LC-MS/MS method

The system used was an Agilent (Massy, France) 1290 Infinity Series system equipped with a quaternary pump. The column was a Kinetex F5, 100×2.1mm, 1.7 µm from Phenomenex (Le Pecq, France). Optimized chromatographic conditions were as followed: a binary mobile phase was used with a flow rate set to 300 $µl.min^{-1}$ for a run time of 16 min, with the column maintained at 50°C. Mobile phase A was an aqueous solution of 0.1% formic acid, and B was ACN with 0.1% formic acid. The separation was performed with a programmed gradient: 0

min: 3% B; 10-12 min: 100% B; 12-13 min: 3% B. An equilibration time of 3 min was realised before each injection. The sample injection volume was 40 µl.

A 5500 QTrap from Sciex® (Les Ulis, France) was used in scheduled-Multiple Reaction Monitoring (s-MRM) mode with positive electrospray ionization. The mass spectrometer source was operated at 600°C, with a ionisation tension of 5,500 V. Curtain gas pressure was set to 30 psi, nebulisation and turbo gas were set to 40 and 60 psi respectively. For all compounds, the entrance potential was set to 10V. MS/MS detection was optimized by infusion of individual standard solutions at 100 $ng.ml^{-1}$ in 50/50 (H_2O/ACN) + 0.1% formic acid via syringe pump at a flow of 10 $\mu l.min^{-1}$. Optimized parameters are presented in Table S1.

2.6.Method validation

Limits of quantification (LOQs) were evaluated as the concentrations leading to a signal-to-noise ratio of 10. The method linearity for each molecule was determined by injection of six matrix extracts spiked before extraction, from 10 $ng.g^{-1}$ to 200 $ng.g^{-1}$. Calibration curves and samples were spiked with a mixture of internal standards (IS) at 100 $ng.g^{-1}$. Quantification was done based on calibration curves representing A_{STD}/A_{IS} as a function of C_{STD}/C_{IS}, with A_{STD} being the area of the standard, A_{IS} the area of the IS, C_{STD} and C_{IS} being the concentration of the standard and the IS respectively. Intraday repeatability was based on 4 replicates and intermediate precision was evaluated on five days. Intraday repeatability was determined for each level by calculating the relative standard deviation (RSD) of replicates and interday precision was determined by calculating the RSD on five days measurements.

Extraction efficiencies were calculated as the ratio between the analyte peak area of a matrix spiked before extraction and the analyte peak area of a matrix spiked after extraction, at the

same concentration. Matrix effects were evaluated by calculating the ratio between the analyte peak area of a matrix spiked after extraction and a solvent standard of the same concentration.

3. Results and discussion

3.1. Development of the extraction method

For the development of the extraction method, the first steps were the selection of the QuEChERS sorbent and the sample mass. Then, the addition of a matrix-dispersion component in the QuEChERS extraction was studied and optimised. Different extraction solvents were also tested. Finally, as sludges are very complex matrices, an addition of a sample clean-up step was considered.

3.1.1. Extraction salt selection

Four different commercially available QuEChERS extraction salts were tested: AOAC, EN, Original and Veterinary Drugs (VET). AOAC and EN differ in the nature and amount of salts, and result in different pH or buffering power in solution. The resulting pH in buffered sludge water extracts was measured: between 4.5-5 for EN method kit and a basic pH (8-8.5) considering the AOAC. The two other extraction salts do not contain any buffer, only desiccating agent ($MgSO_4$ or Na_2SO_4) and NaCl to promote H_2O/ACN by salting out effect and phases separation. The VET extraction kit was designed to allow softer extraction conditions, with the replacement of $MgSO_4$ by Na_2SO_4. Indeed, the absorption of water by Na_2SO_4 is a lesser exothermic reaction than the one with $MgSO_4$ and thus less likely to degrade the analytes.

To test their extraction capacity, triplicate extractions on 1 g of WWTP sludge were performed with each sorbent, 5 ml of water and 10 ml of ACN. Extraction efficiencies were calculated for each beta-lactam and are presented in Fig. 1.

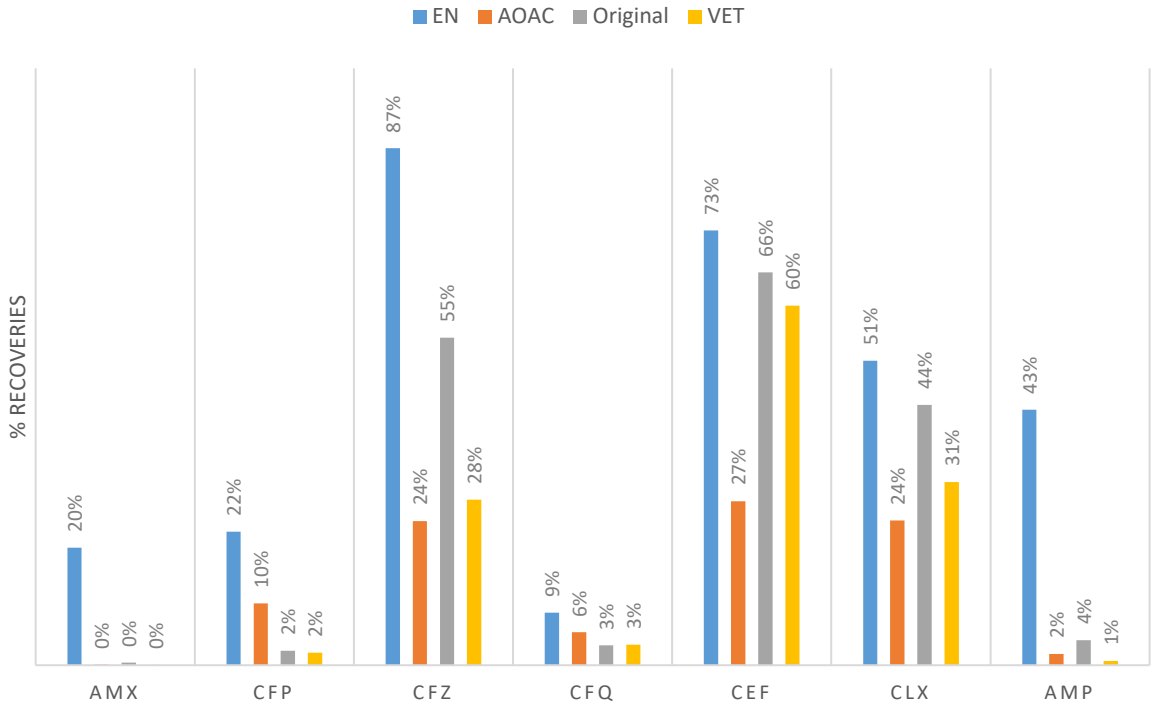


Figure 1 : Recoveries with 4 different QuEChERS Extraction kits (1 g sludge, extraction with 5 mL H2O and 10 mL ACN)

The EN method buffer was the only one allowing amoxicillin to be detected. It also yielded better extraction for all other beta-lactams, so this buffer was selected for further optimisation. One explanation for this difference could be the variation of pH induced by the extraction kits. Indeed, each compound logD is lower at pH 8 than at pH 5, as presented in Table S2. As logD describes the polarity of the molecules as a fonction of pH, beta-lactams are more polar at pH 8. Their ability to transfer to the organic phase is then reduced, explaining lower recoveries of the analytes.

Another explanation would rather be the presence of citrate salts in the EN buffer. Indeed, these have a chelating capacity that breaks the interactions between metal cations and organic compounds, which improves their extraction [36,37]. In conclusion, the EN buffer was selected for the following experiments.

3.1.2. Sample mass

The sample mass is a critical parameter. On the one hand, increasing the sample mass increases the analyte presence in the extract, lowering detection limits. However, on the other hand, it also raises the risk to extract matrix interferences, thus increasing overall matrix effects. Three different matrix masses were selected: 500 mg, 1 g and 2 g. All three were spiked at 50 $ng.g^{-1}$ with all analytes and extracted. A diminution of 50% of extraction recovery for all beta-lactams on average when increasing sample mass from 500 mg to 1 g was observed (Fig.S2). This reduction was stronger for the penicillins (AMX, AMP, CLX) than the cephalosporins. A further increase in sample mass to 2 g decreased the signal by a factor between 10 and 20%. The largest decrease was observed for CFQ, for which the extraction recovery decreased by 85% when going from a mass of 500 mg to 2 g. The matrix effect induced by increasing the sample mass thus outweighed the gain in sensitivity. To minimize this signal suppression, a mass of 500 mg was chosen for the following development steps.

3.1.3. Matrix dispersion with sand

The first step in the process of extracting a solid matrix is to bring the solvent into contact with the matrix particles. To do this, the contact surface must be maximised to increase interactions. In addition to fine grinding of the sludge and the use of a homogeniser, it is possible to increase the contact surface by adding an inert matrix. This is used for example in pressurized liquid extraction (PLE) in which the sample to be extracted is placed in a cell, supplemented with diatomaceous earth. Due to the toxicity of diatomaceous earth, we opted for Fontainebleau sand, a material that is also readily available and has been shown to facilitate the extraction of aminoglycoside antibiotics from milk [38]. Fontainebleau sand were mixed with the sludge to add a dispersive component to the extraction. The sand was previously treated with EDTA, as it has been proven that its chelating capacity can improve extraction efficiencies [39]. First, a 1

to 3 ratio between sludge and sand was applied [40]. The improvement of the extraction efficiency was significant but remained relatively moderate (20% on average for all beta-lactams) compared to a simple QuEChERS extraction (Fig. 2A). On the other hand, we noted an impact on the matrix effect for most molecules. Indeed, except for CEF and CLX, a strong reduction of matrix effects was observed (Fig. 2B), which were finally between -20% and 20%.

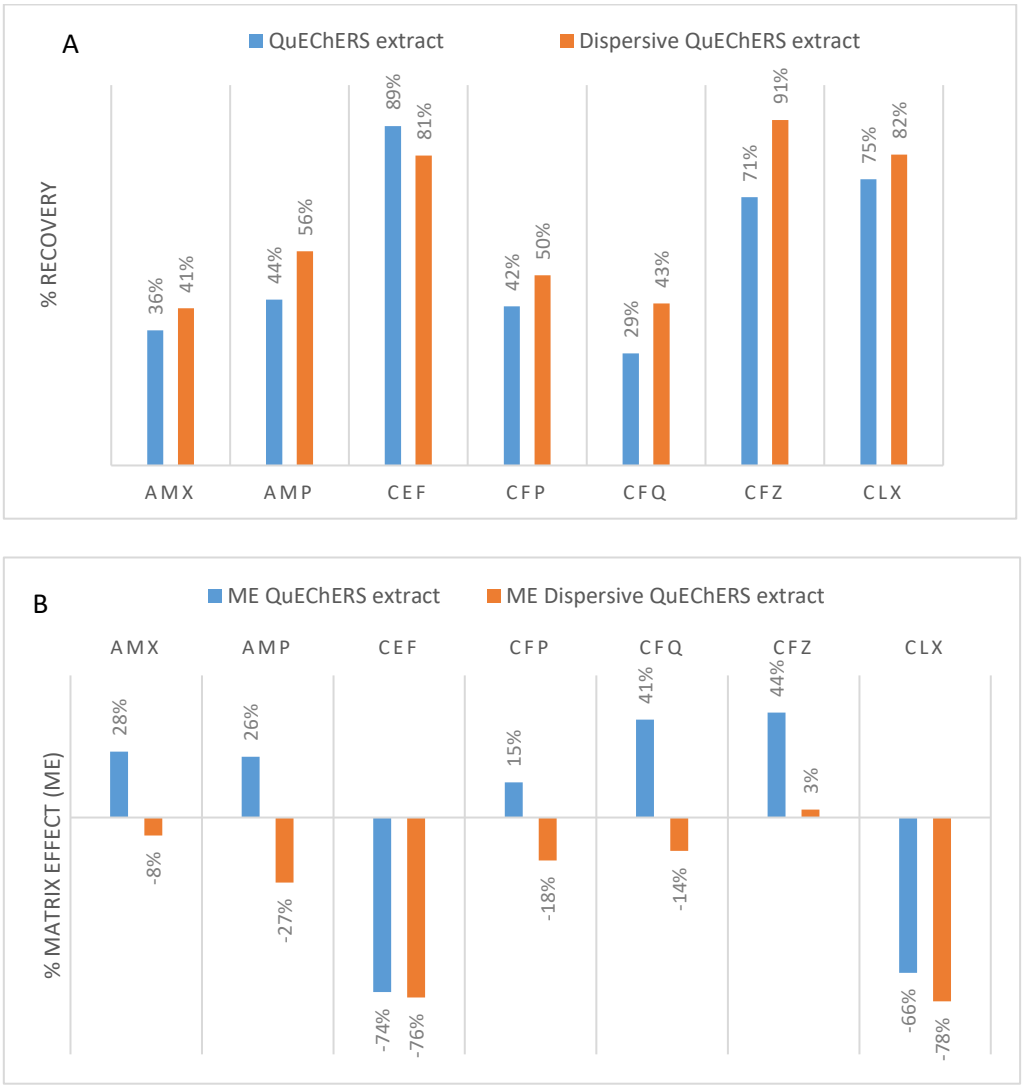


Figure 2 : Recoveries (A) and matrix effects (B) obtained with or without EDTA-treated sand as dispersive agent (500 mg sludge; extraction solvent: 5 mL H2O and 10 mL ACN; mass ratio sludge/sand)

Secondly, the sludge/sand ratio was optimised. For all extracts, 500 mg of sludge were spiked at 100 $ng.g^{-1}$ and extracted with different masses of EDTA-treated sand (TS) to obtain the

following sludge/sand ratios: 2/1;1/1;1/2;1/4. Fig. 3 shows that adding the sand as a dispersing component resulted in a 20% signal increase when going from a 1/1 to a 1/4 sludge/sand ratio. This increase was greatly observed for CLX and AMX, with a 30% signal increase.

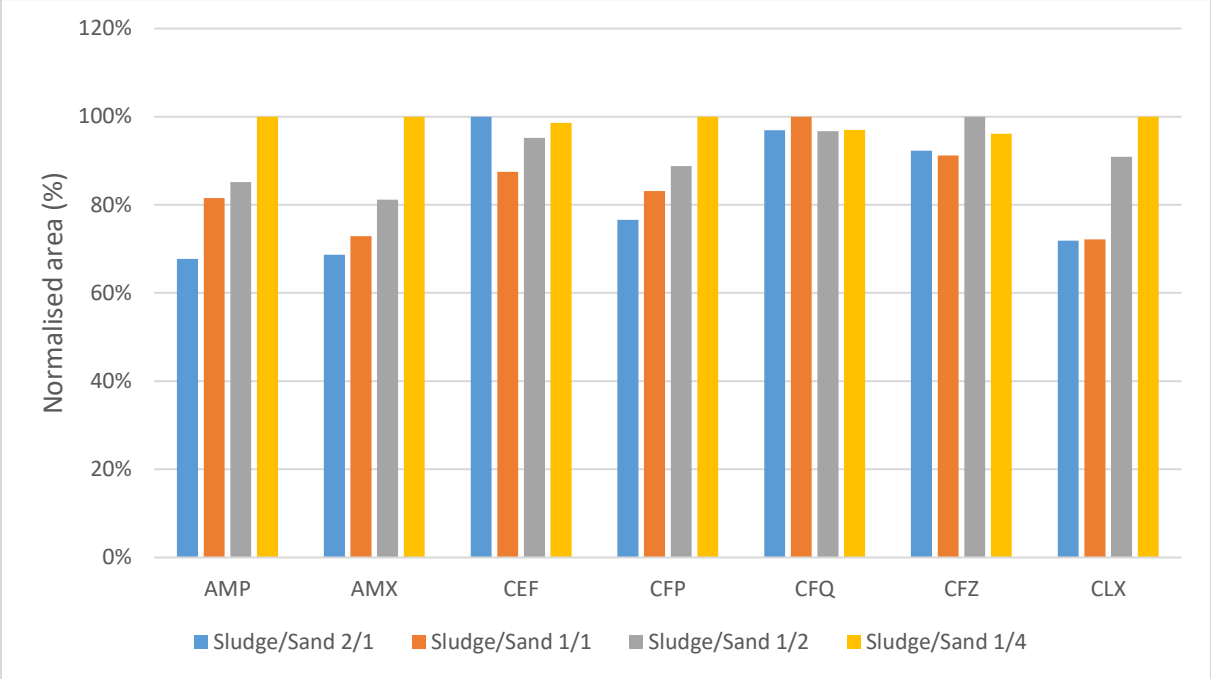


Figure 3 : Normalized area obtained for the extraction of the analytes with various sludge/sand ratio (500 mg sludge; extraction solvent: 5 mL H2O and 10 mL ACN).

Supplementary experiments showed that both the addition of sand, and the treatment with EDTA had a positive impact on extraction efficiencies. Extraction recovery comparison between extraction with not treated sand and without sand indicated an area increase between 31% for CLX and 72% for AMX. The comparison between non treated sand and TS extraction revealed gains between 21% for CFP and 34% for AMX. These results show that using sand helped to disperse the sludge samples, increasing the surface area available for the extraction solvent.

3.1.4. Improvement of the acetonitrile-based extraction conditions

First, we evaluated the addition of formic acid (FA) and EDTA as aqueous phase additives. Signal areas obtained with water, water at 0.1% FA, water at 100 mM EDTA, and water at 0.1% FA + 100 mM EDTA were compared (Fig. 4).

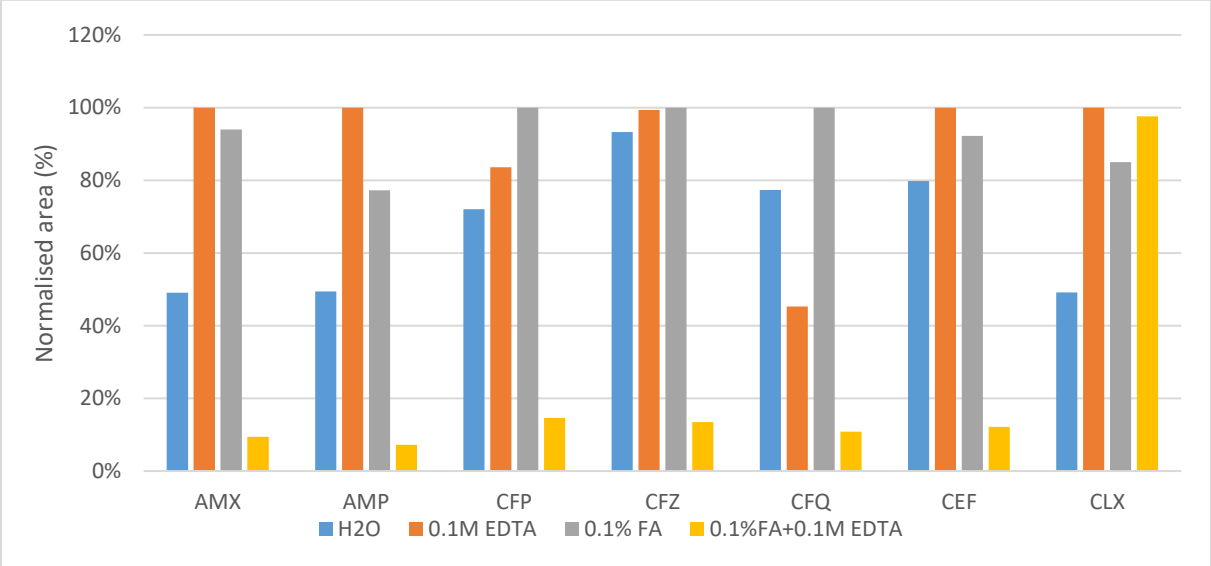


Figure 4 : Normalized area obtained for the extraction of the analytes, as a function of the addition of EDTA and formic acid (FA) in the aqueous phase: (500 mg sludge, 5 mL aqueous phase, 10 mL ACN)

When adding 100 mM EDTA to the aqueous phase, the signal is double for the penicillin subfamily. It also increases for cephalosporins, except for CFQ whose area is reduced by 40%. It is now recognised that complexation between metallic and organic cations, such as antibiotics can occur [41]. Metallic cations are often present in wastewater in large quantities, as shown for example by Östman et al.[42]. EDTA is known to be a powerful chelating agent for metals: when the sludge sample is exposed to an EDTA-containing solution, metal-antibiotics complexes are broken to form metal-EDTA complexes. Adding EDTA to the solvent then enables better extraction of beta-lactams.

The addition of 0.1% FA also increased the surface area of all the compounds. In sludge, most of the organic matter particle surfaces are negatively charged, and analytes are heavily bound

to the solid particles [43,44]. Acidifying the sample reduces the amount of negatively charged particles, thus returning beta-lactams in solution rather than bound to the matrix.

While EDTA or FA added separately had a positive impact on the signal, when combined, a massive signal decrease was observed for all beta-lactams except for CLX. This behaviour may be explained by the EDTA different ionisation state with pH modification. With only water, the solution is at a neutral state (pH 7) and EDTA is mainly in its trivalent anionic state (HY^{3-}), enabling organic and metallic cations complexation. With 0.1% FA in solution, the pH is around 2.7, where EDTA is more in its monovalent anionic state (H_3Y^-) and its capacity to complex cations is thus reduced. Unlike the other penicillins (AMP and AMX), the extraction of CLX was not massively influenced by the mixture EDTA/FA. This difference can probably be explained by the absence of primary amine in its structure. Both AMX and AMP have two ionisable sites resulting in two pKa values whereas CLX only has one cationic form. EDTA can thus complex CLX regardless of its mono-, di- or trivalent anionic state. Finally, for the following optimisation steps, EDTA was selected rather than FA due to the potential instability of beta-lactams in acidic media.

Secondly, the influence of the volumes of the aqueous and organic phases (5 ml or 10 ml) was evaluated. Increasing the volume of the aqueous phase enhanced the signal for CFZ, CFQ, CEF and CLX by about 40% on average with a higher impact for CFQ (+60%) (Fig. S3A). Beta-lactams being polar molecules (logD at pH5 between -3.25 and 1), adding more water favours their solvation and their migration from the sample to the extract solution. However, doubling the aqueous phase volume also led to a 40% reduction of the signal for AMX, AMP and CFP. This could be explained by the mass of desiccant added in the pre-mixed QuEChERS buffer (4g of $MgSO_4$) which can only absorbed about 4-5 ml of water, so the transfer between the aqueous and the organic phase may not be complete. Therefore, for the following extraction optimisation, a volume of 5 ml water was maintained. Finally, for the organic layer (Fig. S3B),

a volume of 10 ml yielded a signal increase of 35% on average for all the targeted compounds. Once again here, the larger increase was noted for CFQ with about 50%. A larger quantity of organic phase allows a larger fraction of analytes to be solubilised and the transfer between the two phases is then more efficient.

As a conclusion, the final dispersive-QuEChERS extraction step was realised on 500 mg of sludge mixed with 2 g of EDTA-treated sand as dispersive agent. The extraction was performed with 10 ml of ACN, 5 ml of 0.1M EDTA and the addition of the EN QuEChERS salt kit, which is composed of citrate salts.

3.1.5. Sample clean-up

The use of the dispersive-QuEChERS extraction step with sand limited matrix effects, except for the compounds CEF and CLX (see section 3.1.3), which experienced signal inhibitions in the range of 75-80%. In the original QuEChERS extraction, dispersive SPE (dSPE) is used to further clean up the extracts. We tested two d-SPE phases: PSA (Primary and Secondary Amines) and PSA/C18. For both, three sludge 10 ml extracts were supplemented at 100 $ng.g^{-1}$ and added to a d-SPE tube containing the phases. The tubes were swirled with vortex for 1 min then centrifuged for 5 min at 10,000 rpm. A volume of 10 ml of supernatant was sampled to another tube and evaporated to dryness under a nitrogen stream at 40°C. Then, 1 ml of 93/7 H_2O/ACN was added and each extract was injected into the LC-MS/MS system. We observed a strong adsorption of beta-lactams when using d-SPE phases, resulting in almost 100% attenuation for all compounds. This phenomenon could be explained by the strong hydrogen bounding interaction of the beta-lactams accessible carboxylic acid with the primary and secondary amines used for the clean up step. A similar fact was already noted by Zhi et al.[45] who reported no recovery of penicillin G and oxacillin when purifying swine manure extracts with PSA conditioned in SPE cartridges.

To circumvent this problem, a SPE clean-up based on a styrene-divinylbenzene polymer (Strata X™) phase was evaluated. To limit the sample preparation time and to avoid a preliminar dilution of the extract in water, a "pass-through" SPE was considered. This consists of a single step after the cartridge conditioning: a single loading of the extract to collect the eluate. Results are presented in Fig. 5. SPE had a positive impact on the diminution of interfering substances (expressed as matrix effect -ME), as shown in Figure 5A, except for CFP and CFZ, for which ME did not significantly decrease. The largest negative MEs observed on CEF and CLX without clean-up were halved and MES on AMX and CFQ were changed to small positive effects after SPE. SPE clean-up yields were also calculated (Figure 5B)

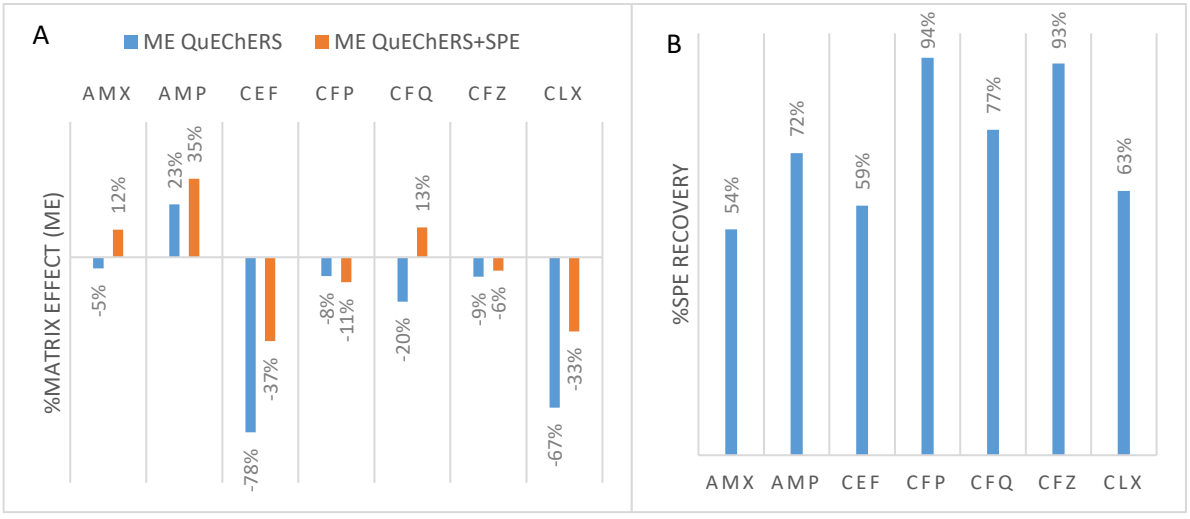


Figure 5 : Matrix effects (A) and SPE pass-through recoveries (B) obtained with or without the SPE pass-through clean up

They are quite different depending on the molecule, ranging from 54% to over 90%. The molecules with the lowest yieldsmay still be bound to dissolved matrix components and are removed during SPE loading of the cartridge or are not released from the polymeric phase of SPE, resulting in a loss of recovery. This clean-up step, therefore, allows better responses to mass spectrometry with removal of interfering compounds, lower limits of quantification for all selected molecules, and overall a more robust method.

3.2.Method validation

For validation purposes and application, one degradation product of AMX, AMX-DKP was added in the final method. In complex matrices such as sludge, identification of analytes may often be difficult. Undeniable identification is realised with retention time stability, the monitoring of two specific MS/MS transitions and the consistency of the ratio between the two transitions. Both variations on the retention time and on the MRM transitions ratio were recorded, and are compiled in Table 2. Retention time variations were lower than 0.05 min, and ratio variations were mainly below 10% RSD, except for CFQ and AMX. These higher variations may be explained by the lower MS signal response of these two analytes.

Beta-lactams extraction efficiencies were evaluated with the whole final sample preparation method at low, middle, and high levels, in triplicate. Some beta-lactams exhibited absolute global recoveries below 30%, as showed in Table 1. However, for all molecules and all levels, extraction was repeatable with %RSD kept below 22% at 20 $ng.g^{-1}$ and even below 10% at 200 $ng.g^{-1}$. For these reasons, it was decided to validate the method with matrix-matched calibration to compensate for low extraction yield.

Table 1 : Overall beta-lactams extraction efficiency at three different concentrations

	20 $ng.g^{-1}$	**100 $ng.g^{-1}$**	**200 $ng.g^{-1}$**
	Mean recovery (%RSD)	Mean recovery (%RSD)	Mean recovery (%RSD)
AMP	43% (11)	32% (8)	30% (4)
CEF	64%(14)	62% (10)	60% (9)
AMX	12%(22)	12% (5)	11% (8)
CFP	27%(12)	26% (7)	25% (4)
CFQ	16%(15)	14% (13)	13% (8)
CFZ	65%(13)	76% (16)	67% (8)
CLX	47%(18)	46% (11)	38% (7)
AMX-DKP	68%(9)	67% (10)	67% (4)

For the validation of the linearity, approximate quantification limits of beta-lactams were determined by injecting replicates (n=3) of extracted spiked blanks solutions, based on signal-

to-noise ratios of 10. Beta-lactams response linearity was then determined from the injection of a matrix-matched calibration curve, starting from the previously determined LOQ to 200 $ng.g^{-1}$. Each compound displayed good linearity over the selected range, with determination coefficients (R^2) greater than 0.99 (Table 2).

Table 2 : Method validation results (linearity, LOQs, and precision at 3 different concentrations (a: intraday n=4; b: interday n=5 days))

	Retention time (min) (%RSD[b])	Transition ratio (%RSD[b])	LOQ ($ng.g^{-1}$)	Linearity		20 $ng.g^{-1}$		100 $ng.g^{-1}$		200 $ng.g^{-1}$	
				Range ($ng.g^{-1}$)	R^2	Mean (%RSD)[a]	Accuracy (%RSD)[b]	Mean (%RSD)[a]	Accuracy (%RSD)[b]	Mean (%RSD)[a]	Accuracy (%RSD)[b]
AMP	3.4 (1.1)	0.2 (5)	2.4	10-200	0.991	18.4 (12)	97 (7)	97.3 (9)	100 (10)	191.5 (4)	97 (4)
CEF	5.0 (1.0)	0.5 (3)	1.9	10-200	0.997	20.2 (5)	103 (5)	98.9 (5)	99 (5)	191.8 (2)	101 (3)
AMX	2.0 (3.1)	1.1 (8)	17.4	10-200	0.995	23.2 (23)	104 (7)	99.3 (8)	101 (8)	188.4 (7)	97 (6)
CFP	3.0 (1.3)	0.6 (10)	8.7	10-200	0.996	22.2 (11)	106 (7)	99.7 (3)	97 (7)	199.4 (2)	100 (2)
CFQ	3.5 (0.7)	0.4 (16)	11.8	10-200	0.994	23.7 (13)	105 (12)	101.9 (6)	98 (4)	196.0 (4)	99 (4)
CFZ	4.6 (0.9)	1.1 (2)	9.5	10-200	0.995	21.0 (7)	103 (4)	100.3 (6)	100 (7)	200.1 (2)	100 (3)
CLX	6.5 (0.8)	1.2 (9)	7.7	10-200	0.994	17.5 (15)	114 (11)	94.8 (11)	96 (7)	177.7 (11)	100 (4)
AMX-DKP	3.5 (1.2)	0.2 (7)	2.7	10-200	0.994	21.4 (19)	97 (8)	100.9 (8)	102 (9)	204.0 (8)	101 (3)

Quantification limits were calculated more precisely from the obtained calibration curves and the coefficients of variation. They were comprised between 1.9 and 17.4 $ng.g^{-1}$ (Table 2) therefore consistent with the literature for solid environmental matrices such as sludge (Table 3). It is difficult to compare methods that do not use the same mass spectrometer, or that have multi-residue analysis objectives. However, the method developed allows the lowest LOQs to be achieved.

Table 3 : Literature comparison for the determination of beta-lactams in solid environmental matrices

Analytes	Matrix	Sample preparation	Analysis	LOQs ($ng.g^{-1}$)	Ref.
CEF	Manure Sludge Sediment	UAE + SPE concentration	LC-MS/MS	17.4 15.4 5.8	[32]
CFP	Feces	UAE + SPE concentration	LC-MS/MS	4.0	[22]
AMX, CEF	Soils	UAE + SPE concentration	LC-MS/MS	10	[15]
AMX	Sediment	UAE + SPE concentration	LC-MS/MS	25	[20]
AMX	Manure	UAE + SPE concentration	LC-MS/MS	136	[34]
AMX	Manure	UAE	LC-MS/MS	50	[35]
CLOX,CFZ	Sludge	QuEChERS AOAC + d-SPE PSA	LC-TOF	50-5000	[23]
AMX,AMP,CEF,CFP, CFQ,CFZ, CLX,AMX-DKP	Sludge	Dispersive QuEChERS + SPE	LC-MS/MS	1.9-17.4	This study

Results of both repeatability and intermediate precision experiments are compiled in Table 2. Concerning the intraday repeatability, RSD were measured inferior to 20% at 20 $ng.g^{-1}$ for all compounds, showing good repeatability from one analysis to another. At the highest concentration, RSD are further reduced, below 5% for 5 of the followed molecules. For intermediate precision, at the lowest level, all RSD were measured below 11%. These variations

were deemed following the method validation guidelines given by the AOAC for environmental analysis[46,47]. At middle and high levels, all RSD were below 10% and 5% respectively.

For all levels, quantification was accurately realised with calculated values within ±6% of the nominal concentration, except for CLX at LOW level, evaluated at ±14% of the nominal value.

3.3.Applications

Eight sludge samples were collected at different times in the same site, across 3 years. Extractions were realised in triplicate. To measure the accuracy of the method developed and check for bias, extractions were also performed on spiked matrices at 100 $ng.g^{-1}$, midpoint of the calibration curves.

Regarding spiked samples, as presented in Table 4, quantification of all the target beta-lactams was accurate. For samples that contained beta-lactams in the non-spiked extract, the determined concentration was subtracted from the calculated spiked concentration before accuracy was computed. On average, the lowest accuracy was at 94% and the highest was 106% for CLX and AMX-DKP respectively. For all the samples and all the analytes, %RSD were kept below 15%.

Results of quantification of the non-spiked sludge samples are presented in Table 4. Amongst all beta-lactams followed in our study, only CFP and AMX-DKP were detected in half of the samples, quantified on average at 13.5 $ng.g^{-1}$ and 3.8 $ng.g^{-1}$ respectively. Sludge 8 exhibited about 4 times more CFP and Sludge 7 exhibited about twice AMX-DKP. Absence of other beta-lactams could be partly explained by a high metabolization or degradation capacity in sewage systems. Li and Zhang [31] determined that CLX has a short half-life of only 2h.

Table 4 : Application on 8 WWTP sludges, not spiked (concentrations in ng.g-1 (%RSD)) and spiked at 100 ng.g-1 (accuracy percentage (%RSD))

	Concentrations (ng.g-^{1})								**Accuracy at 100 ng.g^{-1}**							
Compounds	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
AMP	/	/	/	/	/	/	/	/	102 (1)	106 (9)	107 (3)	106 (2)	108 (10)	96 (2)	99 (8)	96 (3)
CEF	/	/	/	/	/	/	/	/	101 (1)	96 (2)	106 (0.2)	103 (4)	110 (2)	101 (6)	99 (4)	98 (4)
AMX	/	/	/	/	/	/	/	/	99 (7)	98 (4)	104 (8)	91 (10)	104 (12)	105 (2)	88 (9)	112 (7)
CFP	/	/	10.2 (6)	11.9 (6)	18.4 (10)	/	/	53.3 (3)	114 (6)	98 (2)	111 (2)	107 (3)	98 (2)	103 (3)	93 (3)	100 (1)
CFQ	/	/	/	/	/	/	/	/	86 (13)	91 (4)	114 (2)	113 (6)	104 (7)	112 (7)	80 (9)	95 (12)
CFZ	/	/	/	/	/	/	/	/	106 (11)	91 (4)	91 (2)	102 (6)	93 (5)	89 (7)	114 (4)	104 (7)
CLX	/	/	/	/	/	/	/	/	104 (12)	91 (10)	87 (13)	114 (3)	104 (6)	64 (7)	97 (15)	95 (12)
AMX-DKP	3.7 (8)	4.8 (1)	/	/	/	/	9.5 (11)	3.0 (1)	111 (4)	105 (2)	107 (7)	99 (4)	103 (4)	99 (6)	113 (5)	114 (7)

Furthermore, beta-lactams present poor adsorption on sludge [18], limiting their detection. As the transformation of AMX to AMX-DKP involves the addition then loss of an H_2O molecule by cyclisation [32], the metabolite may be present at higher concentrations in wastewater with a small portion adsorbed onto sludge particles. For all extracts where at least one beta-lactam was quantified, %RSD were determined to be below 11%, showing both a good matrix homogeneity and confirming method repeatability. It should be noted that the levels found in the sludge are below the limits of quantification of the previously described methods (Table 3), which shows the interest of the QuEChERS dispersive extraction to acquire data and knowledge on beta-lactams present in low concentrations.

4. Conclusion

The analytical method presented in this study allows determination and quantification of 7 beta-lactams and one metabolite in sludge, using a sample preparation method based on a new dispersive-QuEChERS extraction. One of the major improvements is the addition of EDTA-treated sand to the sample before extraction to disperse the sample and increase the surface available for exchange with extraction solvent. This method showed sensitivity and robustness coherent with environmental detection in solid matrices and allows accurate quantification (±6%).

The developed method presented here was validated and applied to 8 sludge samples, where CFP and AMX-DKP were detected in 4 samples, averaging 14 $ng.g^{-1}$ and 4 $ng.g^{-1}$ respectively. Due to their high metabolism capacity, the beta-lactam family has a wide range of degradation products: for example, Hirte et al. [31] identified 45 potential degradation products of AMX in wastewater, 23 of which have never been reported before. However, as standard molecules of the different metabolites are not widely available commercially, few analytical methods exist

at present. The quantification of the amoxicillin metabolite in our study demonstrates the need to look at metabolization or degradation products present in the environment.

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Supplementary materials : Improvement of the QuEChERS extraction step by matrix-dispersion effect and application on beta-lactams analysis in wastewater sludge by LC-MS/MS

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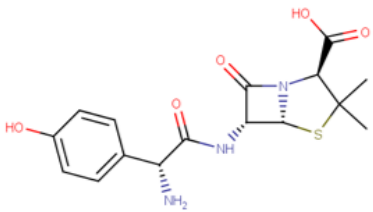
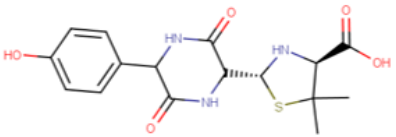
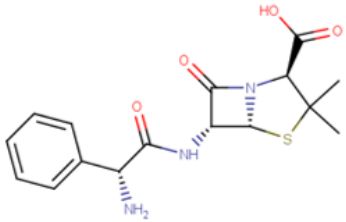
Fig. S1: Structures of beta-lactams in this study



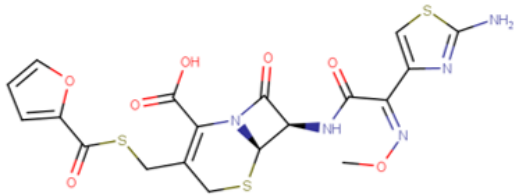
Amoxicillin (AMX)



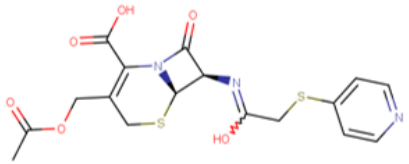
Amoxicillin diketopiperazine (AMX-DKP)



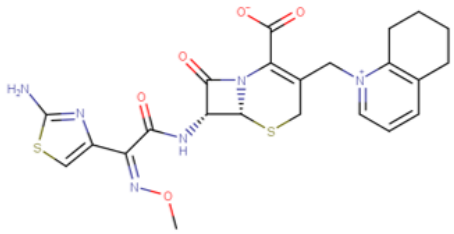
Ampicillin (AMP)



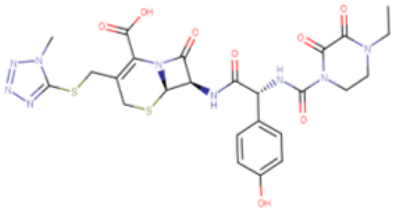
Ceftiofur (CEF)



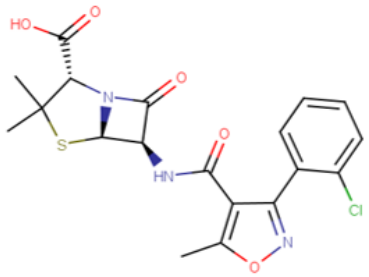
Cefapirine (CFP)



Cefquinome (CFQ)



Cefoperazone (CFZ)



Cloxacillin (CLX)

Fig. S2: Influence of increasing sample mass: normalized areas of each beta-lactam

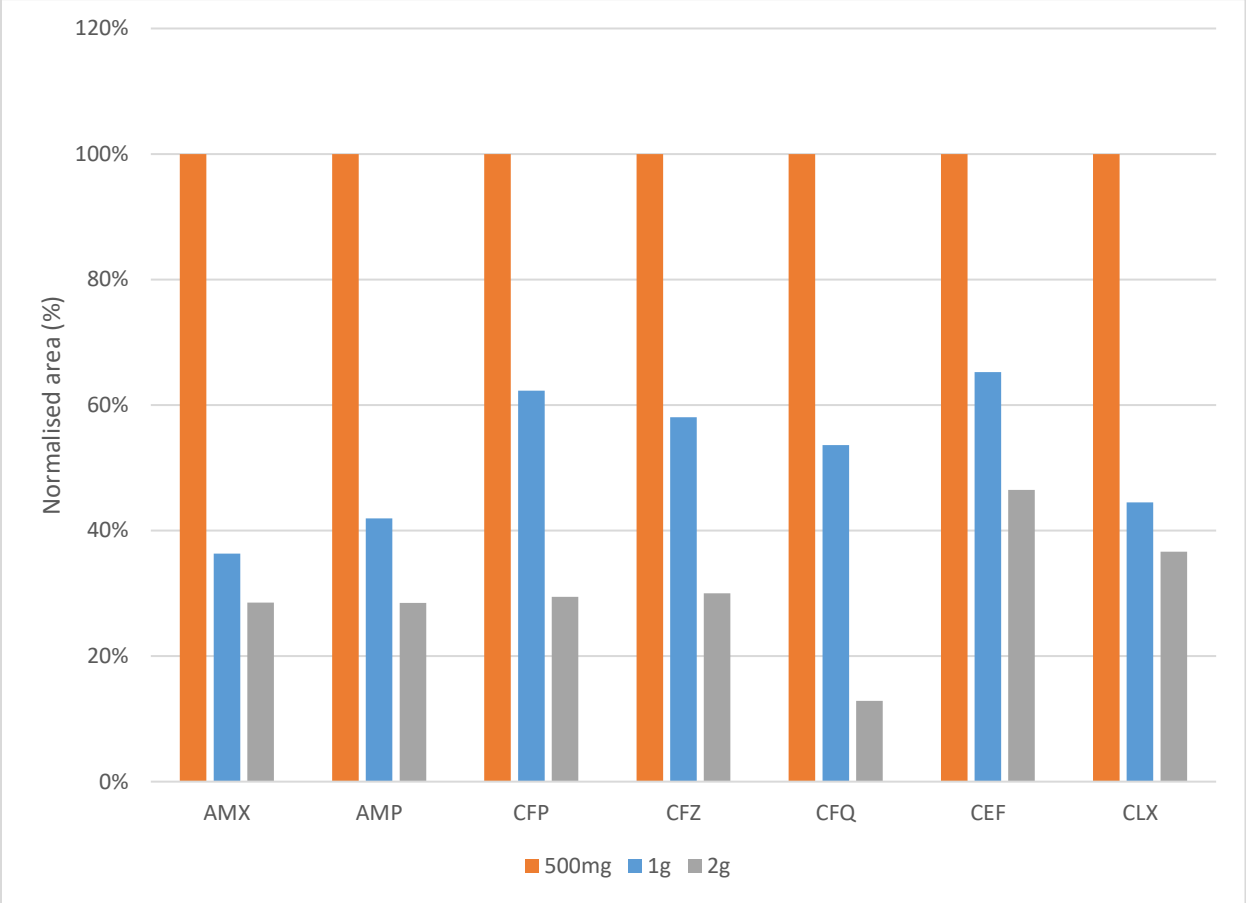
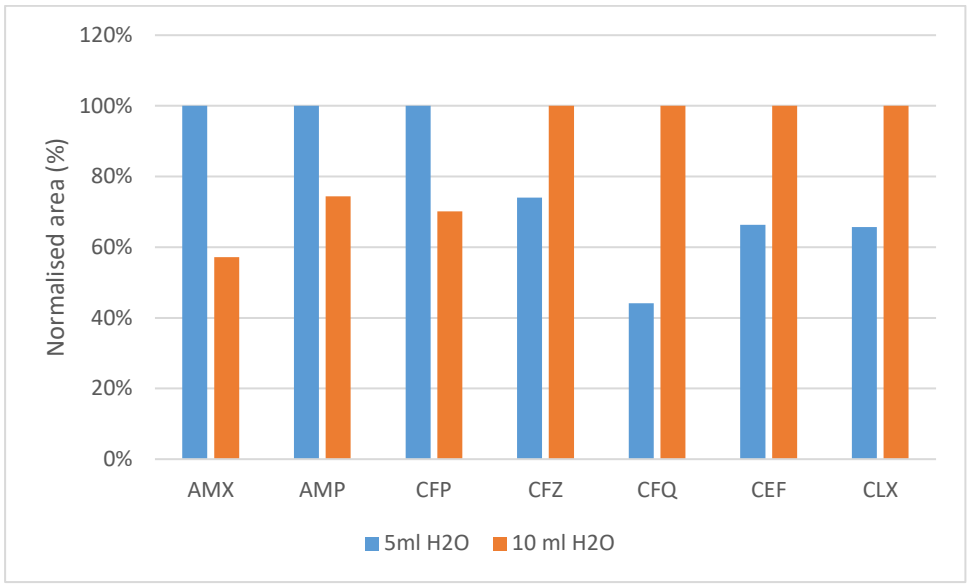


Fig. S3: A) aqueous volume selection and B) acetonitrile volume selection (500 mg sludge, H_2O+0.1M EDTA, normalised areas)

A



B

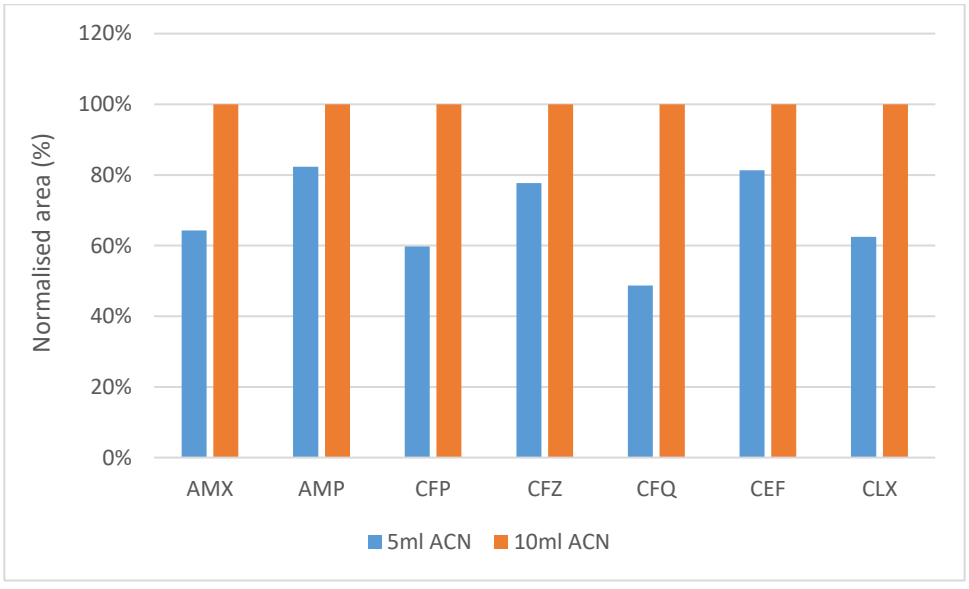


Table S1: MS/MS optimized parameters for beta-lactams analysis

Name	Corresponding Adducts	MRM Transitions (m/z)	Declustering Potential (Volts)	Collision Energy (eVolts)	CXP (Volts)
AMP [a]	$[M+H]^+$	350 → 106	81	31	8
		350 → 160		19	28
AMX [a]	$[M+H]^+$	366 → 349	51	11	54
		366 → 114		23	14
AMX-DKP [c]	$[M+H]^+$	366 → 160	121	21	16
		366 → 207		13	6
CEF [b]	$[M+H]^+$	524 → 241	81	23	8
		524 → 125		75	16
CFP [c]	$[M+H]^+$	424 → 292	71	19	24
		424 → 152		33	10
CFQ [c]	$[M+H]^+$	529 → 134	26	25	10
		529 → 396		15	28
CFZ [b]	$[M+H]^+$	646 → 530	31	17	10
		646 → 143		45	18
CLX [a]	$[M+H]^+$	436 → 277	151	21	12
		436 → 160		19	14
AMP-d5	$[M+H]^+$	355 → 197	147	23	12
CEF-d3	$[M+H]^+$	527 → 244	216	25	12
CFP-d4	$[M+H]^+$	428 → 296	161	21	18

[a] Quantification using AMP-d5 ; b Quantification using CEF-d3 ; c Quantification using CFP-d4

Table S2: Physicochemical properties of beta-lactams in this study

Molecule	Formula	MM (g/mol)	pKa 1/2/3	logD (pH5)	logD (pH8)
AMX	$C_{16}H_{19}N_3O_5S$	365.4	3.2/7.2/9.5	-2.4	-3
AMX-DKP	$C_{16}H_{19}N_3O_5S$	365.4	2.7/9.4/11	-1.25	-2
AMP	$C_{16}H_{19}N_3O_4S$	349.4	3.2/7.2/11.9	-2	-2.8
CLX	$C_{19}H_{18}ClN_3O_5S$	435.9	3.75/13.8	1	-1
CFP	$C_{17}H_{17}N_3O_6S_2$	423.5	3.4/5/11.5	-2.3	-4.5
CEF	$C_{19}H_{17}N_5O_7S_3$	523.6	2.5/3.5/10.7	-1.2	-2.5
CFQ	$C_{23}H_{24}N_6O_5S_2$	528.6	2.7/3.5/10.8	-3.25	-3.25
CFZ	$C_{25}H_{27}N_9O_8S_2$	645.7	3.2/9.5/11.0	-3	-4.5

logP at pH5 and pH8 computed with the ChemAxon LogD Predictor website (https://disco.chemaxon.com/calculators/demo/plugins/logd/)