Talanta 106 (2013) 104–118

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Analysis of quinolone antibiotic derivatives in sewage sludge samples by liquid chromatography–tandem mass spectrometry: Comparison of the efficiency of three extraction techniques

N. Dorival-García, A. Zafra-Gómez *, F.J. Camino-Sánchez, A. Navalón, J.L. Vílchez

Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, Campus of Fuentenueva, University of Granada, Granada E-18071, Spain

ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form
28 November 2012
Accepted 30 November 2012
Available online 14 December 2012

Keywords:
Antibiotics
Quinolones
LC–MS/MS
Sewage sludge analysis
Extraction techniques
Matrix effect

ABSTRACT

This work presents a comparison of three extraction techniques –ultrasound-assisted extraction (USE), microwave-assisted extraction (MAE) and pressurized liquid extraction (PLE) – and evaluates their efficiency in the determination of quinolone antibiotics in sewage sludge samples. Extraction parameters for each technique were optimized using design of experiments, and the compounds were detected and quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS), operating in positive electrospray ionization (ESI) mode. The use of two selected reaction monitoring transitions for each compound allowed simultaneous quantification and identification in one run. Analytes were separated in less than 10 min. Marbofloxacin and cincophen were used as surrogates for amphoteric and acid quinolones, respectively. The limits of detection (LODs) were between 2 and 5 ng g^{-1}, and the limits of quantification (LOQs) were between 4 and 18 ng g^{-1} for the various analytes. The inter- and intra-day variability was $<7\%$. Due to the absence of certified reference materials (CRMs), the method was validated using matrix-matched calibration and a recovery assay with spiked samples. Recovery rates were between 97.9% and 104.8%. Statistical comparison demonstrated no significant differences between the three extraction techniques. The methods were successfully applied for the determination of quinolones in sewage sludge samples collected from different wastewater treatments plants (WWTPs) located in the province of Granada (Spain). The analytical methods developed here may be useful for the development of more in-depth studies on the occurrence and fate of these commonly used pharmaceuticals in WWTPs and in the environment.

1. Introduction

Pharmaceuticals and personal care products are used worldwide in healthcare, household products and animal husbandry. Since the last decade, growing attention has been paid to the environmental behaviour and impact of these compounds [1,2] and they are now being recognized as "emerging" pollutants because of their bioactivity, wide usage, and potential health and ecological risks [3]. Antibiotics and their metabolites are among the most commonly used drugs. The use of antibiotics remains a subject of discussion because of their potential role in the spread and maintenance of (multi-) resistance of bacterial pathogens. This poses a serious threat to public health and deserves much more attention than it has received so far. Several studies have detected many antibiotic-resistant bacteria in drinking water supplies. Moreover, trace concentrations of antibiotics in the environment can severely affect wildlife. The European Union recommends the prudent use of antimicrobial agents in medicine [4,5].

Quinolones represent an important class of antibacterial agents widely used worldwide to treat many human and animal infectious diseases and to promote animal growth when used at subtherapeutic levels. They are a broad-spectrum family of antibiotics that are active against gram-positive and gram-negative bacteria [6]. According to the European Surveillance of Antimicrobial Consumption (ESAC), consumption of quinolones exceeds the defined daily doses per 1000 inhabitants per day (DID) per country [7] being ciprofloxacin one of the most frequently prescribed medications in the world [8].

After administration, antibiotics are incompletely absorbed and portions of parent compound, conjugated forms and metabolites are excreted, flushed towards the WWTPs and, ultimately, released into the environment via WWTP effluents [9]. As a result, much of the research effort is focused on the occurrence and fate of these compounds during the wastewater treatment processes [2,10,11]. Moreover, considerable amounts are sorbed to suspended solids present in wastewater. Concentrations of these

* Corresponding author. Tel.: +34 958 24 33 26; fax: +34 958 24 33 28.
E-mail address: azafra@ugr.es (A. Zafra-Gómez).

0039-9140/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2012.11.080

compounds, in the order of milligrams per kilogram, have been reported in sewage sludge treated according to current regulations – that does not include emerging contaminants – to be used as fertilizer [12,13].

Because of their physicochemical properties, quinolones tend to accumulate in sewage sludge and persist in the environment [14]. In fact, any loss of compounds occurring during wastewater treatment is usually secondary to their sorption to sludge. Studies indicate that approximately 70% of the total quinolone derivatives that enter WWTPs can be found in sludge [15,16]. In addition to the application of sludge as fertilizer, these antibiotics may be transported to soils, and may enter surface water via runoff, leach into groundwater, or may be assimilated by vegetation or other living organisms. The development of analytical methods for the determination of these substances is therefore crucial. Nonetheless, most studies focus on their determination in aqueous matrices [17,18] and their behaviour and effect in other environments is unknown.

Although some analytical methods have already been developed for the determination of quinolones in soils and sediments [19–24], very few of them focused on the determination of these antibiotics in sewage sludge [25–28]. These methods can determine no more than five quinolones and always involve a solid-phase extraction (SPE) step after the extraction process. These procedures involve long and tedious analytical processes, and sometimes they are not effective enough to improve the analytical performance of the method. Different techniques have been used to extract pollutants from solid samples. The most commonly used technique is ultrasound-assisted extraction (USE) [25], but microwave-assisted extraction (MAE) or pressurized liquid extraction (PLE) [26–28] have also been successfully applied. These techniques provide shorter extraction times, low solvent consumption and better recoveries than classical extraction techniques. Traditionally, an extraction method is optimized by modifying one-variable-at-a-time, but this approach cannot solve the problem of dependence and interaction of multiple variables when obtaining optimal conditions [29]. Consequently, it was decided to apply the experimental design to evaluate the relative significance of variables and to determine the best conditions for the desired response.

The main objective of the present work was to develop accurate, selective, robust and sensitive analytical methods for the simultaneous determination of residues of 13 quinolone derivatives in sewage sludge by LC–MS/MS. The Doehlert design was used to optimize the extraction conditions for MAE. A Plackett–Burman experimental design was used to determine the significance of each one of the multiple parameters involved in PLE and then four of the most influential parameters were optimized with a Doehlert design. This approach allowed for simultaneous determination of the effects of different variables affecting the extraction efficiency. Then extraction efficiency of USE, MAE and PLE was compared. The proposed methods allow the analysis of a greater number of samples in shorter times and will facilitate the development of further studies on the occurrence, contamination pathways, fate and risk assessment of this important class of antibiotics in the environment.

2. Materials and methods

2.1. Chemicals and reagents

Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Analytical grade quinolones – pipemidic acid (PIP), enoxacin (ENO), norfloxacin (NOR), ciprofloxacin (CIP), ofloxacin (OFL), enrofloxacin (ENR), lomefloxacin (LOM), moxifloxacin (MOX), cinoxacin, (CIN), nalidixic acid (NAL), oxolinic acid (OXO), flumequine, (FLU), piromidic acid (PIR) – and the surrogates – marbofloxacin (MAR) and 2-phenyl-4-quinoline carboxylic acid (cincophen, CIC) – were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual standard solutions of compounds (200 μg mL^{-1}) were prepared in a water/methanol mixture (1:4) and stored at −20 °C. These solutions were prepared fresh monthly. Working standard mixtures were prepared by diluting each stock solution in methanol or in the initial mobile phase (*i.e.* mobile phase composition at the beginning of chromatographic gradient conditions: 90% of formic acid solution 0.2% (v/v) and 10% of methanol) immediately before use. All solutions were stored in dark glass bottles to avoid photodegradation. PIP, ENR, OFL, OXO, and FLU underwent significant degradation in stock standard solutions after 5 months.

LC–MS grade water and methanol, acetonitrile, sodium hydroxide, ammonia (> 25%) and formic acid (98%) – used for the preparation of standards, mobile phases and pH adjustments – were purchased from Fluka (St. Louis, MO, USA). Disodium hydrogen phosphate and citric acid, for the preparation of McIlvaine buffer solution [30] were obtained from Panreac (Barcelona, Spain). Acetonitrile, hexane, acetone and ethyl acetate were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation and software

A Branson digital Sonifier®unit model S-450D (Danbury, CT, USA), operated with a standard 12.7 mm titanium disruptor horn, a flat and replaceable 12.7 mm titanium tip and a temperature probe was used for USE. A Milestone ETHOS SEL extraction Labstation (Sheldon, CT, USA), operated at 2455 MHz with a maximum delivered power of 1000 W was used for MAE. Time, temperature and microwave power control were adjusted and controlled throughout the process using the easy WAVE 3 software, version 3.2.1.0. An optical fibre temperature sensor was used to monitor the temperature. A Dionex Accelerated Solvent Extractor, ASE® 200 (Sunnyvale, CA, USA) equipped with a solvent controller was used for PLE. The cell tray holds 24 sample cells and 4 rinse tubes.

The detection and quantification of the analytes was performed using an Agilent 1200 series LC system (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with a binary pump, a vacuum membrane degasser, a thermostated column compartment, and an automatic autosampler. The LC system was coupled to an API 2000 (Applied Biosystems, Foster City, CA, USA) triple quadrupole mass spectrometer system that can use atmospheric pressurized chemical ionization (APCI) or electrospray ionization (ESI) interfaces. Analyst software version 1.5.2 was used for instrument control, data acquisition and result processing. A Crison 2000 digital pH-meter with a combined glass–Ag/AgCl (KCl 3 M) electrode (Crison Instruments S.A, Barcelona, Spain) was used for pH measurements. A vortex-mixer (Yellow line, Wilmington, NC, USA), a Hettich Universal 32 centrifuge (Tuttlingen, Germany), and a Memmert oven (Schwabach, Germany) were also used. Statgraphics Plus version 5.0 software (Manugistics Inc., Rockville, MD, USA, 2000) was used for statistical treatment of data.

2.3. Sample collection and storage

Samples were collected from three WWTPs located in the province of Granada (Spain). The samples were stored in amber glass bottles and 1% (v/v) formaldehyde was added to reduce the biological activity. Once in the laboratory, samples were centrifuged at 3634 × *g* for 15 min and the solid components recovered, dried in a heater at 60 °C to constant weight and finely ground

(≤ 1.41 mm). The samples were stored in the dark at 4 °C until analysis.

2.4. Preparation of fortified samples

Due to the absence of certified reference materials (CRMs), blank samples for recovery studies were spiked at different concentrations by adding 0.5 mL of a methanolic standard solution containing the different analytes under study to 0.5 g of dry sewage sludge sample. This volume allows the analytes to come in contact with the whole sample. To ensure sorption equilibrium, the mixtures were shaken on a vortex mixer for 10 min and were then left to stand for 24 h at room temperature in the dark before analysis.

For the optimization of the extraction parameters, the procedures were also performed by extraction of spiked blank samples in triplicate with the target antibiotics (200 ng g^{-1}) and the corresponding blank sample in parallel at all the evaluated extraction conditions.

2.5. Basic procedures

2.5.1. Ultrasound-assisted extraction

Dried sewage sludge samples were accurately weighted (0.5 g) into stainless steel capsules and 5 mL of the extraction buffer solution (MeOH/McIlvaine buffer, 50:50 (v/v), pH=3) and the surrogates were added. The capsules were vortexed for 2 min and sonicated for 15 min at 75% amplitude. Four samples could be simultaneously extracted. Two extraction cycles were required. The combined extracts were centrifuged for 30 min at 3634 × *g*. The supernatant was then decanted into an amber glass vial and evaporated to dryness. The initial mobile phase (250 μL) was added to re-dissolve the residues. The obtained extract was centrifuged again at 3634 × *g* for 30 min, and directly injected into the LC system.

2.5.2. Microwave-assisted extraction

Sewage sludge samples (0.5 g) were placed in the microwave vessels and 10 mL of extraction solution (MeOH/McIlvaine's buffer, 50:50; v/v, pH=3) and the surrogates were added. Ten vessels were processed simultaneously. The extraction process lasted 17 min (5 min for holding) and was performed at 87 °C and 1000 W. Only one extraction cycle was required. After microwave irradiation, an airflow cooled the vessels (< 45 °C). The extracts were centrifuged for 30 min at 3634 × *g* and the supernatants decanted into a glass vial and evaporated to dryness. The residues were dissolved in 250 μL of initial mobile phase, centrifuged at 3634 × *g* for 30 min and directly injected into the LC system.

2.5.3. Pressurized liquid extraction

The samples (0.5 g) and the surrogates were transferred into 11 mL stainless steel extraction cells of the extractor. The operating conditions were as follows: extraction temperature, 86 °C; extraction pressure, 1000 psi; preheating time, 1 min; static extraction time, 5 min; number of extraction cycles, 5; solvent flush, 30% of the cell volume and nitrogen purge, 90 s. The extraction solvent was a mixture MeOH/McIlvaine buffer (50:50; v/v, pH=3). Final extraction volume was approximately 15 mL. The extracts were evaporated to dryness at 50 °C under a stream of nitrogen and 250 μL of the initial mobile phase were added to dissolve the residues. The extracts were centrifuged for 30 min at 3634 × *g* and the supernatants were directly injected into the LC.

2.5.4. Liquid chromatographic–mass spectrometric analysis

Chromatographic separations were achieved on a Kinetex C_{18} column (100 mm × 2.1 mm i.d., 2.6 μm particle size) and a C_{18} guard column, both supplied by Phenomenex (Torrance, CA, USA). The flow rate was 400 μL min^{-1}. The column was maintained at 45 °C and the injection volume was 5 μL. A gradient mobile phase consisting of 0.2% (v/v) formic acid aqueous solution (solvent A) and methanol (solvent B) was used. Gradient conditions were as follows: 90% solvent A was linearly decreased to 30% over 5.0 min and to 0% over 0.5 min and held for 2.5 min to clean the column using 100% organic mobile phase. After returning to the starting conditions in 0.5 min, the column was equilibrated for 4.5 min before the next injection. Total run time was 13 min.

The mass spectrometer (MS) was operated with electrospray ionization (ESI) in positive ion mode. Both Q1 and Q3 resolutions were set to unit mass. Mass spectrometric conditions were optimized for each compound by continuous infusion of the standard solutions (10 μg mL^{-1}), using the $[M+H]^+$ adduct as precursor ion. The ion source temperature was maintained at 400 °C. The IonSpray voltage was set at +5 kV. Nitrogen (curtain gas) was used at 55 psi, and air (nebulizing and desolving gas) was used at 50 and 60 psi, respectively. The collision gas was air at 6 psi. Additional parameters optimized are shown in Table 1.

The two most intense SRM transitions (one used for quantification and the other for confirmation) were selected. For quantification, the most abundant transition was selected to obtain the maximum sensitivity. In terms of sensitivity, the most influential parameters were declustering potential (DP) and collision energy (CE).

3. Results and discussion

3.1. Liquid chromatography–tandem mass spectrometry analysis

The optimization of the chromatographic separation and the signal intensity was carried out using a 100 ng g^{-1} standard mixture of compounds. The following columns were tested: Gemini C_{18} liquid chromatography column (100 mm × 2 mm i.d., 3 μm particle size); Kinetex C_{18} column (100 mm × 2.1 mm i.d., 2.6 μm particle size) from Phenomenex (Torrance, CA, USA); and Acquity UPLC column (100 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (Mildford, MA, USA). Although similar resolution was achieved on the three columns, Acquity UPLC column generated pressures close to the maximum allowed by the chromatographic system, and separation times were longer on Gemini C_{18}. Consequently, the Kinetex C_{18} column was selected for our study.

The main variables affecting the chromatographic separation and the signal intensity were studied. Methanol, acetonitrile and acetonitrile–methanol mixtures were evaluated as organic mobile phases (solvent B) and methanol was selected. Deionized water (solvent A) with different additives was studied. In this respect, the addition of organic acids improves the chromatographic separation of quinolones and the ionization in positive ESI mode, especially for amphoteric quinolones (pK_{a1} 5.5–6.0), since the cationic form is the most abundant at acid pH values [31,32]. However, the sensitivity for acidic quinolones was not significantly enhanced by acidification since the non dissociated forms are predominant due to the absence of the piperazine ring at C-7 (pK_a 2.3–5.0) [33]. Concentrations from 0.0% to 0.3% (v/v) of formic acid used as additive were assayed. The 0.2% (v/v) concentration provided the optimal results. Higher concentrations resulted in significant sensitivity loss because, at high concentrations, formic acid may also suppress the ionization efficiency [34].

Table 1
Optimized SRM conditions for the target antibiotics.

Compound	Transitions	Identity of product ions	Dwell time (ms)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
PIP	304.1→286.2[a]	MH^+—H_2O	112.5	+50	+200	+10	+35	+35
	304.1→217.1[b]	MH^+—CO_2—Pip[c]						
MAR	363.1→345.4[a]	MH^+—H_2O	150.0	+50	+200	+10	+40	+40
	363.1→320.0[b]	MH^+—C_2H_5N						
OFL	362.2→344.2[a]	MH^+—H_2O	150.0	+60	+200	+10	+40	+35
	362.2→318.3[b]	MH^+—CO_2—Pip[c]						
ENO	321.1→303.1[a]	MH^+—H_2O	112.5	+60	+200	+10	+40	+35
	321.1→234.3[b]	MH^+—CO_2—Pip[c]						
NOR	320.1→302.3[a]	MH^+—H_2O	123.8	+60	+200	+10	+40	+35
	320.1→276.0[b]	MH^+—CO_2						
CIP	332.1→313.9[a]	MH^+—H_2O	112.5	+50	+200	+10	+40	+35
	332.1→288.1[b]	MH^+—CO_2						
ENR	360.2→342.3[a]	MH^+—H_2O	112.5	+60	+200	+10	+20	+30
	360.2→316.2[b]	MH^+—CO_2						
LOM	352.2→334.0[a]	MH^+—H_2O	112.5	+50	+200	+10	+20	+30
	352.2→265.2[b]	MH^+—CO_2—Pip[c]						
MOX	402.2→384.1[a]	MH^+—H_2O	112.5	+75	+200	+9	+40	+20
	402.2→260.2[b]							
CIN	263.1→244.9[a]	MH^+—H_2O	225.0	+25	+380	+7	+27	+33
	263.1→216.9[b]	MH^+—H_2O—N-1[d]						
OXO	262.0→244.0[a]	MH^+—H_2O	262.5	+50	+200	+10	+35	+35
	262.0→216.1[b]	MH^+—H_2O—N-1[d]						
NAL	233.4→214.9[a]	MH^+—H_2O	187.5	+30	+360	+5	+25	+30
	233.4→187.0[b]	MH^+—H_2O—N-1[d]						
FLU	262.0→244.0[a]	MH^+—H_2O	262.5	+50	+200	+10	+35	+35
	262.0→202.0[b]	MH^+—H_2O—C_3H_6						
CIC	249.9→222.3[a]		112.5	+40	+380	+9	+50	+24
	249.9→203.6[b]	MH^+—H_2O—N-1[d]						
PIR	289.1→271.1[a]	MH^+—H_2O	206.3	+20	+380	+8	+33	+38
	289.1→243.1[b]	MH^+—H_2O—N-1[d]						

DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

[a] SRM transition used for quantification.
[b] SRM transition used for confirmation.
[c] Loss of CO_2 followed by rupture of piperazine ring (Pip).
[d] Loss of water followed by loss of an alkyl group attached to N-1.

The effect of column temperature, flow rate and injection volume were also studied. Flow rates from 0.1 to 0.5 mL min^{-1} (maximum recommended for ESI interface) were tested. This parameter significantly improved the resolution, peak shape, intensity of the response and retention times. 0.4 mL min^{-1} provided optimal values. Lower flow rates showed poor resolution and decrease in peak separation, while greater flow rates showed less eluent vaporization capacity in the interface. Following column specifications, temperatures from 30 to 50 °C were investigated, and 45 °C provided the highest resolution, greatest number of separated peaks and strongest response. Finally, in order to enhance the signal intensity, injections volumes between 5 and 40 μL (maximum allowed by the chromatographic system) were evaluated. An injection volume of 5 μL was selected because volumes $\geq$ 10 μL resulted in excessive peak broadening.

3.2. Electrospray mass spectral behaviour and MS/MS reactions of the studied quinolones

For all the target antibiotics, except for cincophen, the major product ion corresponded to the loss of H_2O from the carboxylic group. For most of the amphoteric quinolones, the second major fragment was produced by the loss of CO_2. Neutral loss of 87 amu that corresponds to rupture of the piperazine ring was also observed in these quinolones. Second product ion of marbofloxacin was characterized by the direct loss of a fragment of the piperazine ring (C_2H_5N). Acid quinolones produced fewer product ions due to the lack of the reactive C-7 substituent (piperazine ring) [32]. H_2O loss from the carboxyl group at C-3, followed by the loss of R_1 (as C_2H_4) from the ethyl groups at N-1 was characteristic as the second ion product in most of the acidic quinolones. Flumequine exhibited a neutral loss of 42 amu from $[M–H—H_2O]^+$ to produce the ion at m/z 202, probably corresponding to loss of $CH_3–CH=CH_2$ from the ring structure between N-1 and C-8. Table 1 shows the product ions selected.

3.3. Optimization of ultrasound-assisted extraction

In addition to the strong interactions (hydrophobic and electrostatic) of quinolones with organic matter, main component of sewage sludge [35], they are also capable of forming stable complexes with metal cations [26,36,37]. Moreover, the behaviour of quinolones in different media is also determined by their characteristic acid–base properties. These factors are crucial for quinolones adsorption in sludge, and make necessary an exhaustive optimization of the extraction process for quantitative and selective recovery of the antibiotics from samples. pH, extraction solvent and the most influential variables affecting the USE procedure were optimized. Samples (0.5 g) spiked at 200 ng g^{-1} were used for optimization, because these concentration level has been previously found in sludge samples [26,38]. The highest recommended amplitude (75%) as initial working conditions was selected. The extraction procedure involved two cycles of 20 min. The extraction volume was to 5 mL per cycle to avoid solvent saturation. The experimental values of the optimization are shown in the Supplementary materials.

3.3.1. Effect of extraction solvent and pH

All quinolones are acids due to the presence of a carboxylic group at C-3 position, however, depending on the presence or absence of substitution of piperazinyl group at C-7 position,

quinolones are divided in two groups, amphoteric (with cationic, zwitterionic and acid forms), and acids (with neutral or anionic forms). The reported *p*Ka for acid quinolones ranges from 6.0 to 6.9. For amphoteric quinolones, pKa_1 and pKa_2 were between 5.5 and 6.6, and between 7.2 and 8.9 [39]. The acid quinolones analysed here were FLU, OXO, NAL, CIN and PIR; and the amphoteric quinolones were CIP, ENR, NOR, PIP, OFL, LOM, MOX and ENO.

In order to select the extraction solvent, methanol, acetonitrile, acetone and ethyl acetate were evaluated. Since the cationic form of amphoteric quinolones is predominant at low pH values, electrostatic repulsion between quinolones and sludge surface may partly account for the higher extraction efficiency at acidic pHs [26]. Then 10% formic acid (v/v) was added to each solvent [40]. For the majority of compounds, methanol and acetone offered similar recovery, and ethyl acetate was the least effective. Methanol was selected, as it was also the organic solvent of the mobile phase for the chromatographic separation. It was observed that the recoveries with all solvents were lower than 70% for all the analytes, except for PIR (90%). Therefore, it was necessary to improve solvent efficiency and to study the influence of pH.

Following previous studies [25], we evaluated a mixture 1:1 of aqueous buffer and methanol for the determination of the optimal extraction pH. McIlvaine's buffer solution [30] was selected because of its wide range of pH values (2 to 8) and a NaOH solution was used for pH values of 10 and 11. The use a methanol/aqueous buffer solution mixture increased recoveries between 20% and 40% for amphoteric quinolones, achieving recoveries between 80% and 90%. For acid quinolones, the recoveries only improved between 9% and 15%, with recoveries between 65% and 85%, except for PIR (95%). Amphoteric quinolones exhibited lower recoveries above pH 5 and acid quinolones did not show a clear behaviour pattern with pH variations. A pH value of 3 was selected as optimal, because it improved the analysis of most of the quinolones under study, especially those that are commonly found in environmental samples.

Finally, the effect of the aqueous/organic solvent ratio was also studied. Different buffer/methanol mixtures (10:90; 30:70; 50:50; 70:30; and 90:10; v/v) were studied and it was confirmed that the highest recoveries were obtained with a 50:50 (v/v) ratio.

3.3.2. *Effect of the number of extraction cycles and extraction time*

To investigate the effects of extraction time, times from 5 to 20 min were assayed. Times >20 min were not evaluated, because longer times cause system overheating. A period of 15 min per cycle was selected as compromise extraction time. To determine the number of cycles, extractions with 1 to 3 cycles were carried out. Two cycles were necessary for quantitative recovery of compounds with the selected extraction times.

The results obtained for USE optimization confirm the strong interactions between these antibiotics and sludge matrix, since the conditions required were two extraction cycles, 30 min of total extraction time at the maximum ultrasonic amplitude (75%). Despite the drastic conditions that the USE required, the mean recovery was approximately 80%.

Finally, to avoid bonding of antibiotics with metallic ions present in sludge matrix, the effect of Na_2EDTA as a chelating agent was also evaluated. Na_2EDTA (0.25 g) was added to 0.5 g of sludge before the extraction but no effect on recovery rates was observed.

3.4. *Optimization of pressurized liquid extraction*

For PLE optimization, the aqueous buffer solution/methanol mixture was used as extraction solvent. A Plackett–Burman (PB) design was used in the preliminary optimization process to

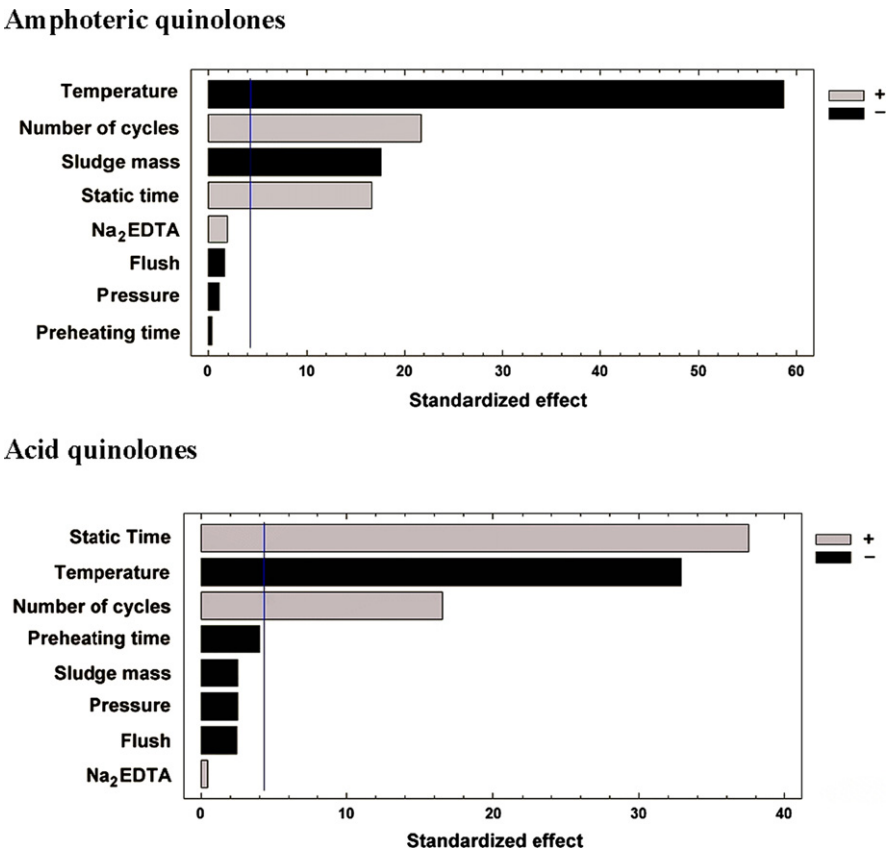

Fig. 1. Standardized main effect Pareto charts for the Plackett–Burman design.

determine the most influential factors in PLE, given the multiple parameters involved in this extraction technique. PB design was selected, among other screening methods, to minimize experimental runs and to determine the significant variables in PLE extraction efficiency. Then ANOVA was employed for the determination of significant variables. The experimental PB design resulted in 24 experiments and three replicates for the central point. Variables were evaluated at low and high level. The central point was evaluated at intermediate level. The following variables were analysed: sample weight (0.5 and 2.0 g), temperature (60 and 160 °C), pressure (600 and 1500 psi), Na_2EDTA concentration (0 and 1 g/g sample), static time (4 and 12 min), number of extraction cycles (1 and 5), preheating time (0 and 2 min) and flush (30% and 150%). Samples spiked with 200 ng g^{-1} of antibiotics were used for optimization. These conditions were proposed following the methodology presented in previous studies [9,26]. Purge time was not included for optimization because it does not significantly affect recoveries [41], and it was set at 90 s. The fitness of the model was determined, and statistically significant effects of the variables were screened using a Student's *t*-test for ANOVA. Variables having a confidence greater than 95.0% were considered to have a significant effect on the extraction efficiency. Fig. 1 shows the statistically significant effect of each variable included in the PB design, presented as the sum of recoveries of each one, considering the differences between amphoteric and acid quinolones.

A minimum *t* value – represented by the vertical line in Fig. 1 – was obtained. Variables with higher *t* values were considered as statistically significant factors. Temperature was one of the most statistically significant parameters for both groups of quinolones, followed by static time and number of extraction cycles. Sample weight only affected amphoteric quinolones. Pressure, flush, preheating time and presence of Na_2EDTA did not have a significant influence on the extraction efficiency of both groups of

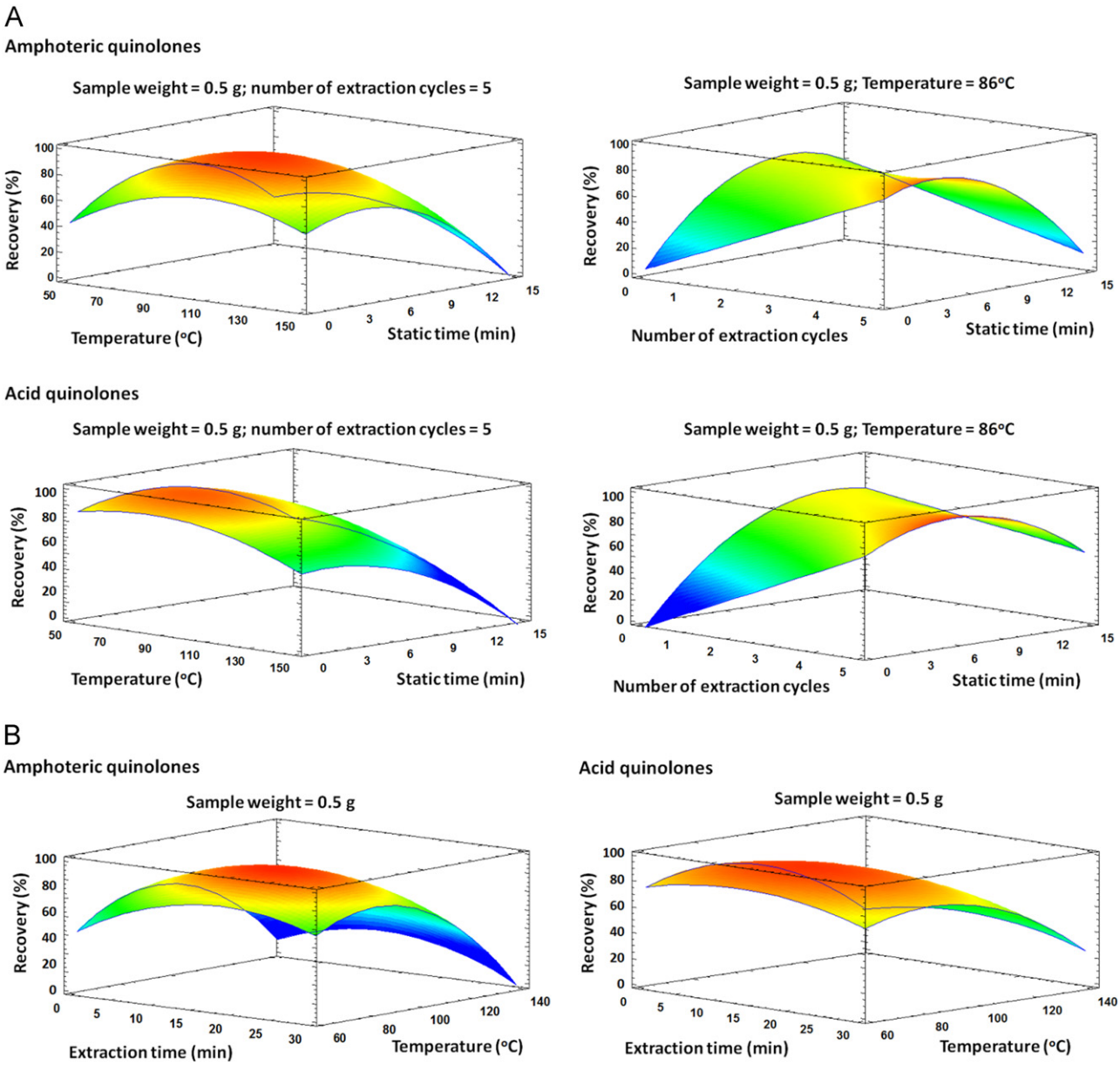

Fig. 2. Optimization of most influential variables on extraction efficiency in PLE (A) and MAE (B). Representative response surfaces for studied antibiotics were divided in two groups of quinolones according to Doehlert experimental design. Sludge samples were spiked with 200 ng g^{-1} of each antibiotic.

quinolones. Consequently, these factors were set during the rest of the optimization process. The central point values were chosen for pressure (1000 psi) and preheating time (1 min). However, the use of the minimum flush setting (30%) was enough to clean the system and avoid extreme dilutions of the extracts and to handle large volumes.

3.4.1. Optimization of the PLE variables by response surface methodology (RSM)

RSM was applied to evaluate the effects of the selected process variables – according to results of PB design – and their interaction on response variables. A multivariate Doehlert experimental design was used [42]. This design is very useful for second-order models, offering more advantages and more efficacy than central composite and Box–Behnken designs. The design involves fewer experiments, which are more efficient and can move through the experimental domain. Different criteria can be used to assign the factors. As a general rule, it is preferable to choose the variable with the stronger effect as the factor with the highest number of levels in order to obtain maximum information of the system.

The matrix for 4 factors consisted of 21 experiments, including three central points. Seven levels for temperature (from 60 to 150 °C) and static time (from 1 to 13 min), and five levels for number of cycles (from 1 to 5), and three levels for sample weight (from 0.5 to 2 g) were considered. Fig. 2A shows the response surfaces for the sum of recoveries for each one of the two groups of quinolones:amphoteric and acid quinolones.

Data were evaluated by ANOVA, in order to identify the effect of variables on the recovery of each compound. ANOVA test gave a determination coefficient (R^2) over 0.949 and 0.977, for amphoteric and acid quinolones, respectively. Since the P value for the lack-of-fit test is >0.05 in all cases, the model appears to be satisfactory for the obtained data at the 95.0% confidence level.

Temperature and static time, particularly their quadratic terms, were the most influential parameters. In general, temperatures over 100 °C were detrimental to quinolones recoveries, probably due to thermal degradation processes [43]. Likewise, at higher extraction temperatures, it was observed that darker extracts were obtained and, consequently, a loss of selectivity of the method due to the extraction of matrix components [27]. In the static method, the long exposure to solvent allows the matrix to swell, thus improving the penetration of solvent into the sample interstices and the contact of the solvent with the analytes. However, due to the significant interactions between temperature and static time, static times >6 min had a negative effect, probably due to degradation of analytes [44]. As compromise solution, temperature was set at 86 °C and static time at 5 min.

The number of cycles was also an important parameter, with a linear and positive effect. With each new cycle, the introduction of fresh solvent maintains a favourable solvent/sample equilibrium and, hence, improving partitioning into the liquid phase and increasing recoveries [26]. A total of five cycles was established as optimum value.

It was also confirmed that sample weight can only affect amphoteric quinolones, having a linear negative effect, since the larger sample amounts are analysed, the higher amounts of matrix components are extracted. This probably results in solvent saturation, thus, reducing the extraction efficiency. Consequently, 0.5 g of sludge was chosen as optimal sample weight. Under these conditions, mean recoveries were 94.6% and 90.4% for amphoteric and acid quinolones, respectively.

3.5. Microwave-assisted extraction optimization

A multivariate Doehlert experimental design was also used. The Doehlert matrix was established for three factors and consisted of thirteen experiments, including three central points. The temperature was studied at seven levels (from 60 to 130 °C), irradiation time at five levels (from 5 to 30 min), and sample weight at three levels (from 0.5 to 2 g). The same aqueous buffer/methanol mixture was used as extraction solvent. In order to simplify the procedure, only one extraction cycle was performed and 10 mL used as extraction volume (minimum possible volume for our microwave unit). The microwave power was fixed at 1000 W to reach the highest temperatures. Fig. 2B shows the

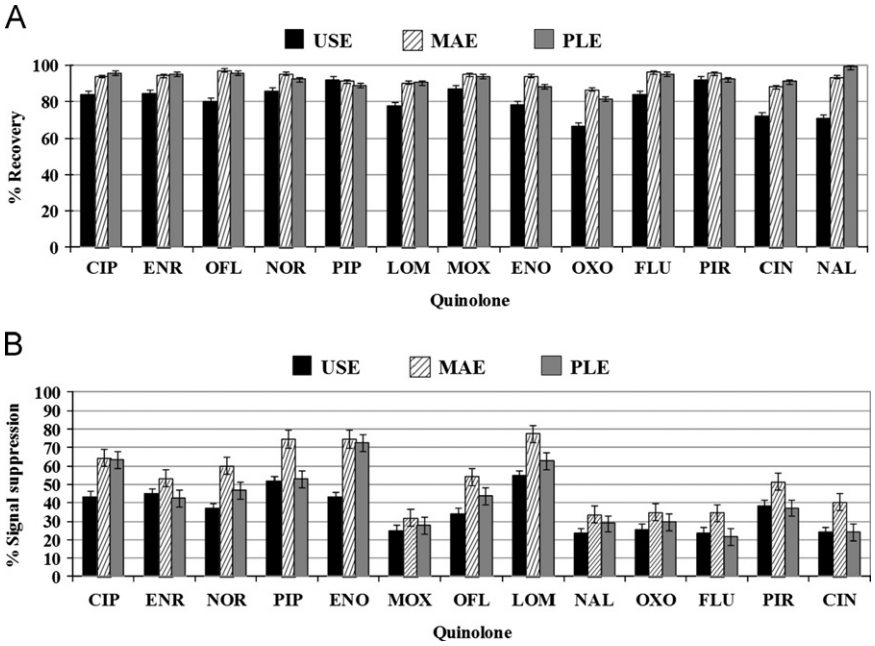

Fig. 3. (A) Antibiotics extraction efficiencies by the three evaluated techniques. LSD values are given in parentheses: CIP (1.97); ENR (1.45); OFL (1.51); NOR (1.86); PIP (2.26); LOM (2.13); MOX (1.71); ENO (2.86); OXO (2.15); FLU (3.48); PIR (2.75); CIN (1.27); NAL (1.38). (B) Percentages of MS ion suppression in sewage sludge extracts obtained from the evaluated extraction techniques. LSD values are given in parentheses: CIP (1.58); ENR (1.27), OFL (3.84); NOR (1.65); PIP (3.88); LOM (3.68); MOX (4.57); ENO (3.18); OXO (3.98); FLU (3.72); PIR (1.24); CIN (2.50); NAL (2.75). $n=6$ and significance level of 95.0% was selected for all cases.

Table 2
Analytical and statistical parameters.

Parameter[a]	USE												
	CIP	ENR	NOR	PIP	ENO	MOX	NAL	OXO	FLU	PIR	OFL	LOM	CIN
n	42	42	42	42	42	42	42	42	42	42	42	42	42
b (g ng^{-1})	2.4×10^{-2}	2.5×10^{-2}	1.6×10^{-2}	1.4×10^{-2}	1.8×10^{-2}	2.6×10^{-2}	2.3×10^{-2}	1.5×10^{-3}	1.4×10^{-2}	8.7×10^{-2}	1.1×10^{-2}	8.8×10^{-3}	7.7×10^{-3}
s_b (g ng^{-1})	9.7×10^{-5}	1.0×10^{-4}	6.5×10^{-5}	9.1×10^{-5}	7.1×10^{-5}	1.3×10^{-4}	5.3×10^{-5}	8.7×10^{-6}	9.6×10^{-5}	1.9×10^{-4}	3.3×10^{-5}	3.7×10^{-5}	3.8×10^{-5}
$s_{y/x}$	0.185	0.176	0.113	0.159	0.123	0.234	0.092	0.015	0.166	0.328	0.058	0.064	0.066
% R^2	99.93	99.94	99.93	99.83	99.94	99.89	99.98	99.87	99.82	99.98	99.97	99.93	99.90
LOD (ng g^{-1})	4	3	3	5	3	4	2	5	5	2	2	3	4
LOQ (ng g^{-1})	12	11	11	18	10	14	6	15	18	6	8	11	13
LDR (ng g^{-1})	LOQ—800												
Parameter[a]	MAE												
N	42	42	42	42	42	42	42	42	42	42	42	42	42
b (g ng^{-1})	3.6×10^{-2}	4.7×10^{-2}	3.6×10^{-2}	1.6×10^{-2}	3.0×10^{-2}	6.9×10^{-2}	6.3×10^{-2}	4.4×10^{-3}	5.5×10^{-2}	1.5×10^{-1}	1.4×10^{-2}	1.5×10^{-2}	4.2×10^{-2}
s_b (g ng^{-1})	1.6×10^{-4}	1.2×10^{-4}	1.5×10^{-4}	6.3×10^{-5}	1.3×10^{-4}	1.6×10^{-4}	2.4×10^{-4}	1.4×10^{-5}	2.2×10^{-4}	6.9×10^{-4}	6.5×10^{-5}	8.8×10^{-5}	1.6×10^{-4}
$s_{y/x}$	0.281	0.216	0.255	0.110	0.226	0.274	0.425	0.025	0.381	1.203	0.114	0.153	0.280
% R^2	99.92	99.97	99.94	99.94	99.93	99.98	99.94	99.96	99.94	99.92	99.92	99.87	99.94
LOD (ng g^{-1})	4	2	3	3	3	2	3	3	3	4	4	5	3
LOQ (ng g^{-1})	12	7	11	11	11	6	10	9	11	12	12	15	10
LDR (ng g^{-1})	LOQ—800												
Parameter[a]	PLE												
n	42	42	42	42	42	42	42	42	42	42	42	42	42
b (g ng^{-1})	3.6×10^{-2}	5.6×10^{-2}	3.3×10^{-2}	1.4×10^{-2}	4.1×10^{-2}	7.1×10^{-2}	8.7×10^{-2}	4.7×10^{-3}	4.4×10^{-2}	2.2×10^{-1}	2.5×10^{-2}	2.6×10^{-2}	6.5×10^{-2}
s_b (g ng^{-1})	1.3×10^{-4}	2.0×10^{-4}	9.4×10^{-5}	9.2×10^{-5}	1.7×10^{-4}	2.4×10^{-4}	2.3×10^{-4}	2.3×10^{-5}	7.3×10^{-5}	5.3×10^{-4}	1.6×10^{-4}	1.1×10^{-4}	1.6×10^{-4}
$s_{y/x}$	0.233	0.345	0.164	0.160	0.287	0.412	0.408	0.041	0.128	0.928	0.272	0.188	0.271
% R^2	99.94	99.95	99.97	99.82	99.94	99.96	99.97	99.90	99.99	99.98	99.85	99.93	99.98
LOD (ng g^{-1})	3	3	2	5	3	3	2	4	1	2	5	3	2
LOQ (ng g^{-1})	10	9	8	18	11	9	7	13	4	6	16	11	6
LDR (ng g^{-1})	LOQ—800												

b=slope; s_b=slope standard deviation; $s_{y/x}$=regression standard deviation; R^2=determination coefficient; LOD=limit of detection; LOQ=limit of quantification; LDR=linear dynamic range.

[a] n=points of calibration.

response surfaces for the two groups of quinolones. Data were evaluated by ANOVA, in order to identify the effect of variables on the recovery of each compound. Therefore the fitted regression equations explain more than 98.9% and 99.7% of the total variation in the data for amphoteric and acid quinolones, respectively. Since P value for lack-of-fit test is >0.05 in all cases, the model appears to be satisfactory for the obtained data at the 95.0% confidence level.

Temperature and irradiation time, particularly their quadratic terms, showed a significant influence on extraction efficiency, especially for amphoteric quinolones, since temperatures and extraction times different from the optimal caused a decrease in recoveries of about 60% and 30%, for amphoteric and acid quinolones, respectively. The optimal temperature and irradiation time for amphoteric quinolones was 87 °C and 17 min, respectively. The sludge weight had also a negative effect on recoveries for both amphoteric and acid quinolones, which corroborates the more drastic conditions characteristic of MAE, since more matrix components are extracted with the analytes, facilitating solvent saturation and reducing extraction efficiency. Consequently, the minimum sludge weight (0.5 g) was selected as optimal value. With the selected experimental conditions, amphoteric and acid quinolones had mean recoveries of 96.7% and 93.9%, respectively.

3.6. Comparison of extraction techniques

3.6.1. Extraction efficiency

The results of the comparison of the extraction efficiency of the three techniques are shown in Fig. 3A. Mean values of relative recoveries for each target antibiotic with the evaluated extraction techniques were compared using the least significant difference (LSD) multiple range test with a 95.0% confidence level. The results showed statistically significant differences.

MAE and PLE provided the highest recoveries ($>90\%$) in comparison with USE (80%). USE was clearly not as efficient as MAE and PLE, especially for acidic quinolones. MAE was slightly better than PLE, with a maximum difference in recoveries of 6%.

3.6.2. Matrix effects

One of the major drawbacks of LC–ESI-MS/MS is the signal suppression/enhancement caused by those coeluting components in sample extracts that have similar ions in the MS/MS experiment. Coeluting components influence the ionization efficiency and affect the reproducibility, sensitivity and accuracy of the method. Humic substances in particular have been proved to cause matrix effects [35,44], which therefore have to be investigated.

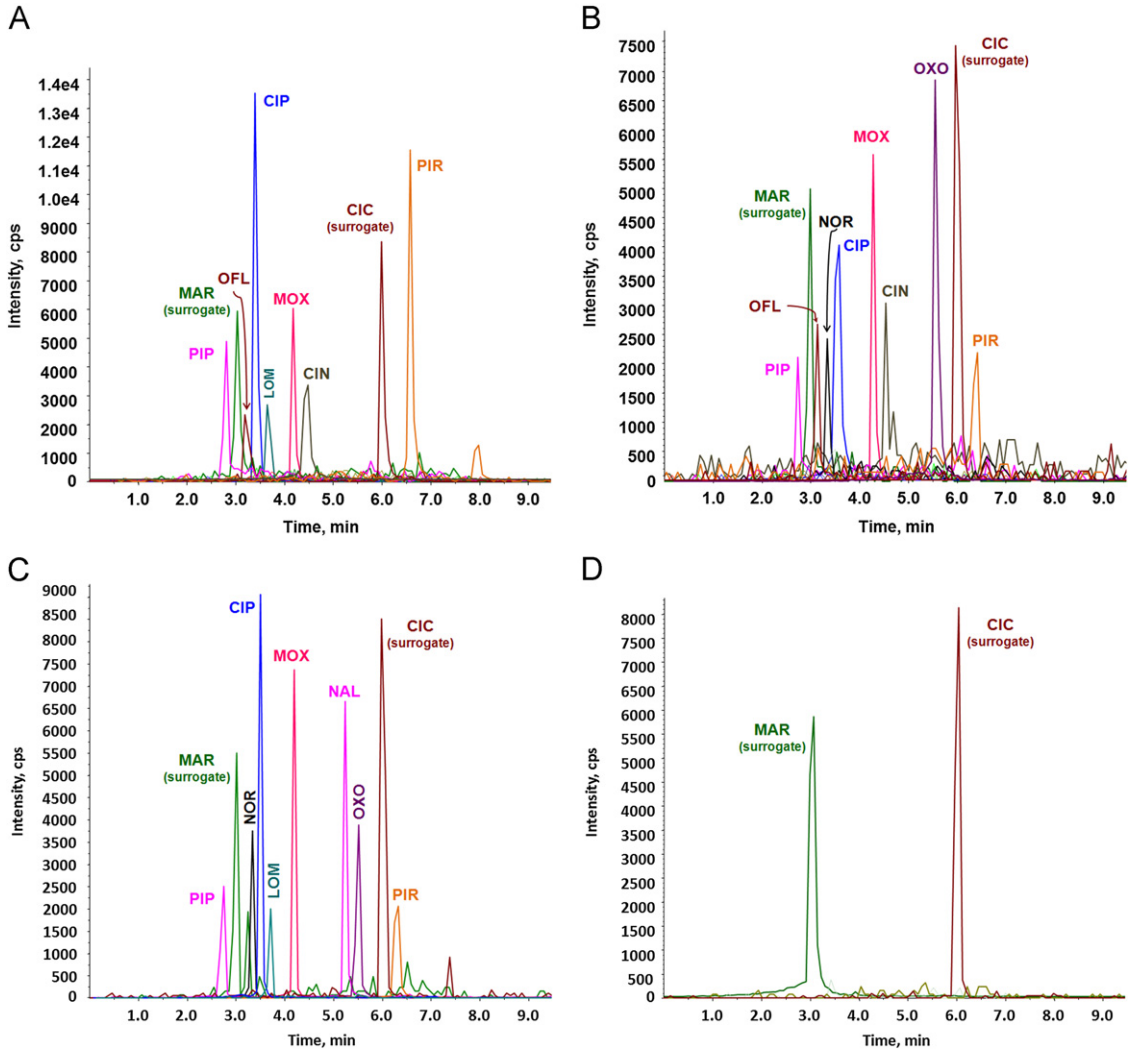

Fig. 4. SRM mode chromatograms of the extracts of real sewage sludge samples collected from different wastewater treatment plants of Granada, obtained with different extraction methods (A) USE, (B) MAE and (C) PLE. (D) Chromatogram of a non contaminated (blank) sewage sludge sample.

Table 3
Accuracy of the methods. Precision and trueness in sewage sludge samples from three different WWTPs.

	Sludge type	Parameter		USE			MAE			PLE		
				Level (ng g^{-1})			Level (ng g^{-1})			Level (ng g^{-1})		
				20	200	600	20	200	600	20	200	600
CIP	WWTP 1	Trueness	Recovery (%)[a]	99.6	99.6	100.3	99.3	99.2	101.7	98.5	99.7	100.0
		Precision	Intra-day (%)[b]	1.5	1.1	0.3	1.2	1.6	2.6	1.2	1.2	2.2
			Inter-day (%)[c]	2.6	1.0	0.5	2.4	2.5	3.4	2.0	1.6	2.5
	WWTP 2	Trueness	Recovery (%)[a]	102.1	100.7	101.2	96.7	97.5	96.0	94.9	96.8	97.3
		Precision	Intra-day (%)[b]	2.0	1.8	0.6	1.4	2.1	3.3	2.0	1.5	3.0
			Inter-day (%)[c]	2.5	2.3	0.6	2.2	2.6	4.1	2.4	2.2	3.2
	WWTP 3	Trueness	Recovery (%)[a]	98.7	100.0	99.9	97.5	99.1	99.5	97.7	97.3	98.6
		Precision	Intra-day (%)[b]	1.9	1.6	0.2	1.8	1.7	2.7	2.2	0.8	1.7
			Inter-day (%)[c]	2.9	3.0	0.4	2.6	3.0	3.6	2.7	1.9	2.8
ENR	WWTP 1	Trueness	Recovery (%)[a]	98.4	99.7	99.9	98.7	99.4	99.8	99.0	99.9	100.0
		Precision	Intra-day (%)[b]	2.6	1.6	1.5	2.6	1.5	0.9	1.1	1.1	1.2
			Inter-day (%)[c]	2.9	2.3	1.8	3.1	2.0	1.0	2.4	1.9	2.0
	WWTP 2	Trueness	Recovery (%)[a]	96.1	96.4	100.9	99.3	103.2	101.3	98.5	98.3	100.1
		Precision	Intra-day (%)[b]	2.2	1.4	1.1	2.6	1.5	0.9	1.1	0.9	1.1
			Inter-day (%)[c]	3.0	2.7	2.4	4.1	3.0	1.4	2.1	1.6	2.3
	WWTP 3	Trueness	Recovery (%)[a]	97.2	98.5	98.7	100.4	97.8	98.4	96.7	99.7	103.4
		Precision	Intra-day (%)[b]	2.6	1.9	1.5	2.1	1.8	0.6	1.7	1.4	1.6
			Inter-day (%)[c]	3.2	2.9	1.9	3.6	2.7	1.4	2.8	2.0	2.2
NOR	WWTP 1	Trueness	Recovery (%)[a]	98.2	100.8	100.1	98.4	99.8	100.1	101.1	100.3	99.2
		Precision	Intra-day (%)[b]	4.1	2.6	0.6	3.7	1.2	1.1	1.7	0.8	1.5
			Inter-day (%)[c]	5.6	2.8	1.1	6.7	1.9	2.3	1.9	1.2	2.1
	WWTP 2	Trueness	Recovery (%)[a]	96.8	99.4	97.2	98.3	102.1	99.1	99.7	100.3	101.9
		Precision	Intra-day (%)[b]	4.3	3.0	1.1	4.1	2.2	1.1	1.9	1.5	1.5
			Inter-day (%)[c]	5.1	3.7	1.6	7.3	2.5	2.2	2.1	2.5	1.8
	WWTP 3	Trueness	Recovery (%)[a]	100.3	103.6	101.8	99.1	101.8	103.8	100.8	98.6	97.5
		Precision	Intra-day (%)[b]	5.0	2.6	0.4	3.1	2.3	0.8	2.1	0.7	2.1
			Inter-day (%)[c]	6.3	3.3	1.5	6.3	2.4	1.6	2.4	2.2	2.8
PIP	WWTP 1	Trueness	Recovery (%)[a]	100.1	99.6	99.4	99.1	99.7	99.3	99.8	98.6	100.7
		Precision	Intra-day (%)[b]	3.4	2.3	1.0	4.1	0.7	2.0	1.5	2.2	3.0
			Inter-day (%)[c]	4.1	2.3	1.7	4.6	1.1	2.7	1.8	4.9	3.9
	WWTP 2	Trueness	Recovery (%)[a]	98.7	101.2	101.3	96.8	98.9	101.5	98.0	97.9	98.6
		Precision	Intra-day (%)[b]	4.0	2.4	1.0	3.4	1.2	2.1	2.0	2.3	2.7
			Inter-day (%)[c]	4.4	2.8	2.5	4.9	1.7	3.1	2.5	5.2	4.0
	WWTP 3	Trueness	Recovery (%)[a]	103.8	98.7	98.9	96.9	100.0	100.8	103.0	99.8	102.1
		Precision	Intra-day (%)[b]	4.1	2.3	0.8	4.1	0.7	1.7	1.8	2.1	3.0
			Inter-day (%)[c]	5.1	3.5	1.9	5.6	1.4	3.2	2.0	4.3	4.2
ENO	WWTP 1	Trueness	Recovery (%)[a]	97.9	99.8	99.8	99.1	99.4	99.8	98.8	101.2	99.9
		Precision	Intra-day (%)[b]	2.9	1.6	0.4	4.4	0.8	0.5	3.0	1.8	1.7
			Inter-day (%)[c]	5.5	1.6	0.9	6.3	1.1	0.8	3.1	2.9	1.8
	WWTP 2	Trueness	Recovery (%)[a]	98.6	101.6	98.9	98.5	98.7	98.2	97.6	100.1	97.2
		Precision	Intra-day (%)[b]	3.1	1.8	0.9	5.2	0.8	0.5	3.4	2.2	2.0
			Inter-day (%)[c]	5.1	2.0	1.7	5.7	1.2	1.4	4.0	3.4	1.9
	WWTP 3	Trueness	Recovery (%)[a]	96.8	98.6	100.8	97.7	98.2	99.9	98.5	104.8	99.5
		Precision	Intra-day (%)[b]	2.9	1.6	0.4	5.4	0.4	0.9	3.5	1.8	1.7
			Inter-day (%)[c]	6.0	2.2	1.5	5.8	1.7	1.2	3.5	3.2	2.2

Table 3 (*continued*)

	Sludge type	Parameter		USE			MAE			PLE		
				Level (ng g^{-1})			Level (ng g^{-1})			Level (ng g^{-1})		
				20	200	600	20	200	600	20	200	600
MOX	WWTP 1	Trueness	Recovery (%)[a]	99.5	98.9	100.1	99.1	99.6	99.6	99.2	100.9	99.4
		Precision	Intra-day (%)[b]	1.4	1.1	0.5	2.3	1.4	1.3	1.4	4.9	1.1
			Inter-day (%)[c]	2.9	1.5	0.5	3.2	1.6	1.8	2.4	5.0	1.5
	WWTP 2	Trueness	Recovery (%)[a]	101.3	96.6	100.6	100.8	99.0	99.2	99.7	103.0	97.8
		Precision	Intra-day (%)[b]	1.4	1.7	0.6	3.0	1.8	2.0	1.8	3.8	1.4
			Inter-day (%)[c]	2.7	2.1	1.5	4.3	2.3	2.3	2.6	4.9	2.1
	WWTP 3	Trueness	Recovery (%)[a]	98.7	101.3	100.8	100.1	99.1	101.2	98.1	99.9	101.9
		Precision	Intra-day (%)[b]	2.4	2.1	1.1	2.1	1.4	1.1	1.9	5.2	1.2
			Inter-day (%)[c]	3.1	2.8	1.3	3.5	2.0	2.1	2.8	6.0	1.6
NAL	WWTP 1	Trueness	Recovery (%)[a]	98.7	98.2	99.6	100.5	98.2	99.2	99.1	99.4	100.9
		Precision	Intra-day (%)[b]	1.6	1.0	0.6	4.7	1.7	1.9	2.1	1.8	1.3
			Inter-day (%)[c]	3.7	1.4	1.0	6.1	3.4	2.7	3.1	2.2	1.5
	WWTP 2	Trueness	Recovery (%)[a]	97.9	99.4	100.9	98.9	99.3	99.0	99.0	98.7	101.0
		Precision	Intra-day (%)[b]	1.7	1.0	1.0	5.1	1.4	2.5	2.1	1.8	2.0
			Inter-day (%)[c]	3.6	2.0	1.6	6.7	4.6	2.9	3.3	2.6	2.3
	WWTP 3	Trueness	Recovery (%)[a]	98.0	97.9	98.1	101.1	101.8	98.9	103.3	99.6	103.2
		Precision	Intra-day (%)[b]	2.4	1.7	0.4	4.6	1.7	1.8	1.9	2.1	1.5
			Inter-day (%)[c]	3.9	2.2	1.4	5.7	4.0	3.3	3.5	2.5	1.6
OXO	WWTP 1	Trueness	Recovery (%)[a]	99.3	98.9	100.2	99.1	104.8	99.3	98.7	99.8	100.1
		Precision	Intra-day (%)[b]	3.2	1.3	1.0	2.8	1.0	0.5	3.8	2.4	1.6
			Inter-day (%)[c]	3.3	1.9	1.3	3.7	1.6	1.3	3.9	3.3	2.4
	WWTP 2	Trueness	Recovery (%)[a]	98.7	98.5	98.8	98.4	98.0	100.0	100.2	99.5	99.8
		Precision	Intra-day (%)[b]	3.2	1.9	2.2	3.5	1.0	0.7	4.2	2.4	1.6
			Inter-day (%)[c]	4.4	2.1	2.8	4.0	1.7	1.0	4.8	3.7	2.1
	WWTP 3	Trueness	Recovery (%)[a]	103.6	98.0	102.1	97.1	100.5	101.0	98.1	99.6	100.5
		Precision	Intra-day (%)[b]	3.5	1.3	1.0	2.7	1.2	0.5	3.0	2.2	1.5
			Inter-day (%)[c]	4.2	2.3	1.9	3.5	2.3	1.5	3.6	3.6	3.0
FLU	WWTP 1	Trueness	Recovery (%)[a]	102.7	100.5	99.3	99.6	102.0	99.7	99.9	99.8	100.1
		Precision	Intra-day (%)[b]	2.4	1.6	0.6	2.3	2.1	2.1	1.6	1.3	0.7
			Inter-day (%)[c]	3.0	3.6	1.0	3.9	3.3	3.6	2.2	1.6	0.9
	WWTP 2	Trueness	Recovery (%)[a]	103.0	97.4	100.1	101.7	99.9	100.0	97.7	99.0	100.6
		Precision	Intra-day (%)[b]	2.6	1.5	1.1	2.2	2.6	2.4	2.4	1.5	1.0
			Inter-day (%)[c]	3.4	2.2	1.4	3.3	3.1	3.2	2.7	2.2	1.7
	WWTP 3	Trueness	Recovery (%)[a]	99.7	97.9	100.1	98.7	99.4	98.3	100.0	99.9	100.2
		Precision	Intra-day (%)[b]	2.4	1.9	0.6	1.8	3.1	2.1	1.6	1.2	1.0
			Inter-day (%)[c]	3.3	3.3	1.5	2.8	3.5	3.0	2.8	2.5	1.4
PIR	WWTP 1	Trueness	Recovery (%)[a]	100.1	101.0	100.3	99.5	99.4	100.3	100.5	100.1	99.9
		Precision	Intra-day (%)[b]	2.4	0.4	0.7	3.3	1.0	1.7	1.5	1.2	0.6
			Inter-day (%)[c]	3.1	0.6	0.7	3.5	2.1	2.3	2.3	1.3	0.9
	WWTP 2	Trueness	Recovery (%)[a]	98.4	100.0	100.6	97.2	100.0	99.4	99.3	100.5	100.4
		Precision	Intra-day (%)[b]	3.1	0.4	0.3	3.7	2.5	2.7	1.2	2.0	1.4
			Inter-day (%)[c]	3.4	1.4	0.9	4.2	3.0	3.2	2.4	2.4	2.2
	WWTP 3	Trueness	Recovery (%)[a]	97.5	101.0	101.0	99.6	98.2	104.7	98.4	97.6	99.3
		Precision	Intra-day (%)[b]	2.4	0.4	0.7	3.3	2.7	3.3	1.5	2.1	0.6
			Inter-day (%)[c]	4.4	0.9	1.0	3.6	3.5	4.1	2.2	2.6	1.1
OFL	WWTP 1	Trueness	Recovery (%)[a]	100.5	99.9	100.1	100.3	99.8	100.2	99.4	98.6	101.1
		Precision	Intra-day (%)[b]	2.3	1.7	0.9	2.4	1.6	1.4	1.7	1.9	1.4
			Inter-day (%)[c]	2.7	2.9	1.0	3.0	1.9	2.5	3.4	3.9	1.7
	WWTP 2	Trueness	Recovery (%)[a]	97.4	97.0	100.8	98.9	100.4	104.5	98.1	97.7	103.8

		Precision	Intra-day (%)[b]	2.2	2.3	0.6	3.1	2.2	1.4	1.7	1.9	2.3
			Inter-day (%)[c]	3.0	2.7	1.5	3.6	2.6	3.9	2.8	4.1	2.4
	WWTP 3	Trueness	Recovery (%)[a]	98.0	98.1	100.1	99.6	99.8	98.7	97.5	97.1	100.5
		Precision	Intra-day (%)[b]	2.3	1.8	0.9	2.4	1.9	1.9	1.6	1.6	2.1
			Inter-day (%)[c]	2.4	3.1	2.4	3.4	2.4	2.6	3.2	3.0	2.5
LOM	WWTP 1	Trueness	Recovery (%)[a]	98.0	99.0	99.8	101.6	100.2	98.8	100.1	101.4	100.9
		Precision	Intra-day (%)[b]	1.7	2.0	0.8	3.3	2.6	1.6	0.6	3.2	3.8
			Inter-day (%)[c]	2.8	2.9	1.4	5.9	3.5	2.8	1.7	3.6	4.0
	WWTP 2	Trueness	Recovery (%)[a]	98.9	101.9	100.1	97.8	100.4	101.0	100.9	102.6	98.6
		Precision	Intra-day (%)[b]	2.5	1.5	0.8	3.5	3.0	1.9	0.6	4.2	3.8
			Inter-day (%)[c]	2.7	3.0	1.1	4.3	3.6	2.6	2.4	4.6	4.8
	WWTP 3	Trueness	Recovery (%)[a]	97.6	100.9	100.4	99.0	101.0	103.5	98.9	99.4	99.5
		Precision	Intra-day (%)[b]	2.4	2.5	0.6	3.4	2.1	2.4	1.0	3.0	4.2
			Inter-day (%)[c]	3.4	3.2	1.6	4.6	4.2	3.3	2.9	3.8	5.1
CIN	WWTP 1	Trueness	Recovery (%)[a]	100.0	98.7	99.1	99.4	98.7	101.4	100.3	100.6	99.7
			Intra-day (%)[b]	1.7	2.5	0.9	3.7	3.6	1.8	0.7	1.4	1.1
			Inter-day (%)[c]	3.0	4.8	1.9	4.6	5.0	2.5	1.0	2.8	1.7
	WWTP 2	Trueness	Recovery (%)[a]	99.2	98.6	98.0	97.5	99.7	100.5	100.7	101.7	97.3
		Precision	Intra-day (%)[b]	1.7	2.5	0.9	3.7	3.6	1.8	1.0	1.1	1.1
			Inter-day (%)[c]	2.8	4.3	2.6	4.0	5.2	3.1	2.0	2.6	1.9
	WWTP 3	Trueness	Recovery (%)[a]	101.5	97.2	99.4	98.4	99.4	101.2	99.4	104.2	99.1
		Precision	Intra-day (%)[b]	2.1	2.2	1.1	4.1	4.1	2.1	0.9	1.3	1.5
			Inter-day (%)[c]	2.4	3.7	2.1	5.7	5.5	2.7	1.6	2.0	2.3

[a] Mean value of 54 determinations.
[b] RSD (%), relative standard deviation ($n=6$).
[c] RSD (%), relative standard deviation ($n=54$).

The matrix effect was evaluated by calculating the percentage of signal suppression in the extracts obtained with the three extraction techniques. The peak areas from the analysis of spiked sludge extracts were compared with the ones corresponding to the spiked solvent (mobile phase) at the same concentration levels [45]. The results of signal suppression in extracts spiked at 50 ng g^{-1} with the studied analytes are shown in Fig. 3B.

Mean values of signal suppression for each target antibiotic with the evaluated extraction techniques were compared using LSD multiple range test with a 95.0% confidence level. The results showed that in general, matrix effects were significantly different between the three techniques. The highest signal suppression was clearly observed in MAE extracts, probably due to the more drastic experimental conditions. PLE generally resulted in more matrix effects than USE. It was also noted that amphoteric quinolones exhibited higher signal suppression than acid quinolones, which showed a signal suppression of about 20%. Moxifloxacin, an amphoteric quinolone of third generation, also exhibited low signal suppression.

3.7. Method validation

A seven-point matrix-matched calibration curve was obtained for each method in the range from the limit of quantification (LOQ) to 800 ng g^{-1}. Calibration curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Marbofloxacin and cincophen were selected as surrogates for amphoteric and acidic quinolones, respectively. Marbofloxacin is used in veterinary medicine, which is unlikely to be found in urban wastewaters. Cincophen is not a quinolone, but it shows a similar structural core. Additionally, the chromatographic retention times of each group of quinolones are close to the corresponding surrogate and neither marbofloxacin nor cincophen were detected in samples. Each calibration level was made in triplicate and analysed twice. The curves for each extraction technique were compared (Student's t test) with the ones generated in solvent (mobile phase). Statistically significant differences were observed between the slopes for the matrix-matched calibration curves and their corresponding ones in the solvent for the three methods and for the thirteen analytes. This confirms that a correct quantification of the analytes in sludge samples requires the use of matrix-based calibration curves. The analytical methods were validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision). Table 2 shows the main calibration and validation parameters.

Linearity. The determination coefficient (R^2) and the lack-of-fit test (P_{lof}) were evaluated. Good linearity was observed within the concentration range (LOQ—800 ng g^{-1}) and P_{lof} values were $>5\%$ in all cases.

Selectivity. This parameter was demonstrated by LC–MS/MS analysis of blanks. The chromatogram of a blank sample and a spiked blank sample were compared with the chromatograms of the target compounds were extracted using the three extraction methods. Fig. 4 shows the characteristic SRM chromatograms of extracts from real samples obtained using USE, MAE, PLE and a not contaminated sample (blank). These data demonstrated the high selectivity of the methods.

Sensitivity. Two fundamental aspects need to be examined in the validation of any analytical method to determine whether an analyte is present in the sample: the limit of detection (LOD) and the limit of quantification (LOQ). These parameters were determined as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10 for the LOD and LOQ, respectively. Table 2 shows the values obtained.

Accuracy (*precision and trueness*).The precision of the method in terms of intra- and inter-day variability was evaluated at three

concentration levels (20, 200 and 600 ng g^{-1}) and for sewage sludge samples collected from three different WWTPs of the province of Granada, Spain. Precision (expressed as relative standard deviation, RSD [%]) was determined from triplicate spiked sludge samples during the same day (repeatability) and in nine different days (reproducibility). The values obtained are also summarized in Table 3. RSD values fell between 0.3% and 6.7%. The data indicate that the methods are highly reproducible, and there were not significant differences between the recoveries in the case of the sludge samples from the three WWTPs.

Additionally, due to the absence of CRMs, recovery assays were carried out to validate the methods in terms of trueness. Blank spiked samples previously analysed to ensure they did not contain the compounds of interest or that these were below the LOD of the method were used. Trueness was evaluated by determining the recovery of known amounts of tested compounds in sludge samples. As shown in Table 3, the recoveries were close to 100% (97.9% to 104.8%) in all cases.

3.8. Comparison of methods

Quinolones concentrations were determined in different sewage samples using UAE, MAE and PLE with the aim of comparing statistically the efficiency of these extraction techniques. Regression curves were used for comparing the analytical methods [46]. Although none of the studied extraction techniques is considered as a reference method, this regression study was still used, since these graphs provide valuable information on the nature of any differences among the methods. The results are presented in Table 4.

A bias less than 1.5%, excellent correlation coefficients, and insignificant intercepts were obtained at both concentration ranges. This proves that the variation in measurements was mainly due to random measurement errors. A difference plot between the analysed sludge samples was also calculated, helping to determine the agreement or disagreement between methods. The differences between concentrations in every split sample were plotted against the means of each pair. This plot allows us to investigate any possible relationship between the measurement error and the true value. The true value is not known, so that the mean of the two measurements is the best estimate. The lack of agreement between methods is measured by the deviation of the points from the horizontal nil-bias line. In general, there was a narrow scatter around the zero line of no difference, with a homogeneous distribution above and below the zero line, suggesting the absence of significant proportional systematic errors between the evaluated methods. An example of a difference plot is shown in Fig. 5.

From this graph it was also possible to calculate the limits of agreement, or the differences of the measured concentration from one method below and above in relation to other. In the example showed in Fig. 5 for ciprofloxacin, it could be observed that limits of agreement for the three methods are quite similar and small enough to be acceptable and confident that any extraction method can be used interchangeably. The short 95% confidence intervals for these limits are evidence of few variations of the differences. A similar behaviour was observed for the other analysed antibiotics. A maximum difference of 1 ng g^{-1} was also observed for MOX, PIR and NAL, followed by OFL, NOR and PIP, with a maximum difference of 2 ng g^{-1}, for the low concentration range. These limits of agreement were also similar among the three extraction methods.

Table 4
Method comparison results by linear regression.

Analyte (Concentration range)	Compared methods (x vs. y)	Slope	Intercept	n	r	Bias (%)
CIP (20–95 ng g^{-1})	USE vs. MAE	1.001	−0,093	40	0.994	0.10
	USE vs. PLE	1.005	−0,242	40	0.993	−0.20
	PLE vs. MAE	0.995	0.167	40	0.993	0.40
CIP (183–834 ng g^{-1})	USE vs. MAE	0.998	−0,620	22	0.999	−0.30
	USE vs. PLE	0.999	−0,227	22	0.999	−0.20
	PLE vs. MAE	0.999	−0,376	22	0.999	−0.20
NOR (25–115 ng g^{-1})	USE vs. MAE	0.982	0,618	15	0.998	−0.60
	USE vs. PLE	0.994	0.772	15	0.996	0.90
	PLE vs. MAE	0.986	0.009	15	0.998	−1.40
PIP (13–130 ng g^{-1})	MAE vs. USE	0.999	−0.109	20	0.998	−0.93
	USE vs. PLE	0.994	0.645	20	0.998	0.93
	MAE vs. PLE	0.994	0.445	20	0.999	0.61
PIP (169–600 ng g^{-1})	MAE vs. USE	0.998	0.586	15	0.998	0.23
	USE vs. PLE	1.002	0.105	15	0.997	0.26
	MAE vs. PLE	0.999	0.656	15	0.997	0.27
MOX (15–83 ng g^{-1})	USE vs. MAE	1.001	−0.036	36	0.994	0.23
	USE vs. PLE	0.992	0.275	36	0.997	−0.19
	MAE vs. PLE	0.991	0.338	36	0.991	−0.30
MOX (197–741 ng g^{-1})	USE vs. MAE	1.001	−0.624	14	0.996	−0.22
	USE vs. PLE	0.995	−0.314	14	0.996	−0.31
	MAE vs. PLE	0.998	−0.378	14	0.997	0.24
OFL (15–129 ng g^{-1})	USE vs. MAE	0.996	0.049	25	0.995	−0.30
	USE vs. PLE	0.998	0.183	25	0.992	0.47
	MAE vs. PLE	1.003	0.137	25	0.996	0.37
PIR (13–61 ng g^{-1})	USE *vs.* MAE	0.991	0.373	13	0.995	0.40
	USE vs. PLE	0.993	0.401	13	0.995	0.60
	PLE vs. MAE	0.998	−0.017	13	0.994	−0.34
NAL (12–44 ng g^{-1})	USE vs. MAE	1.010	0.027	12	0.994	1.1
	USE vs. PLE	1.002	0.044	12	0.998	0.40
	PLE vs. MAE	1.008	−0.015	12	0.995	0.60

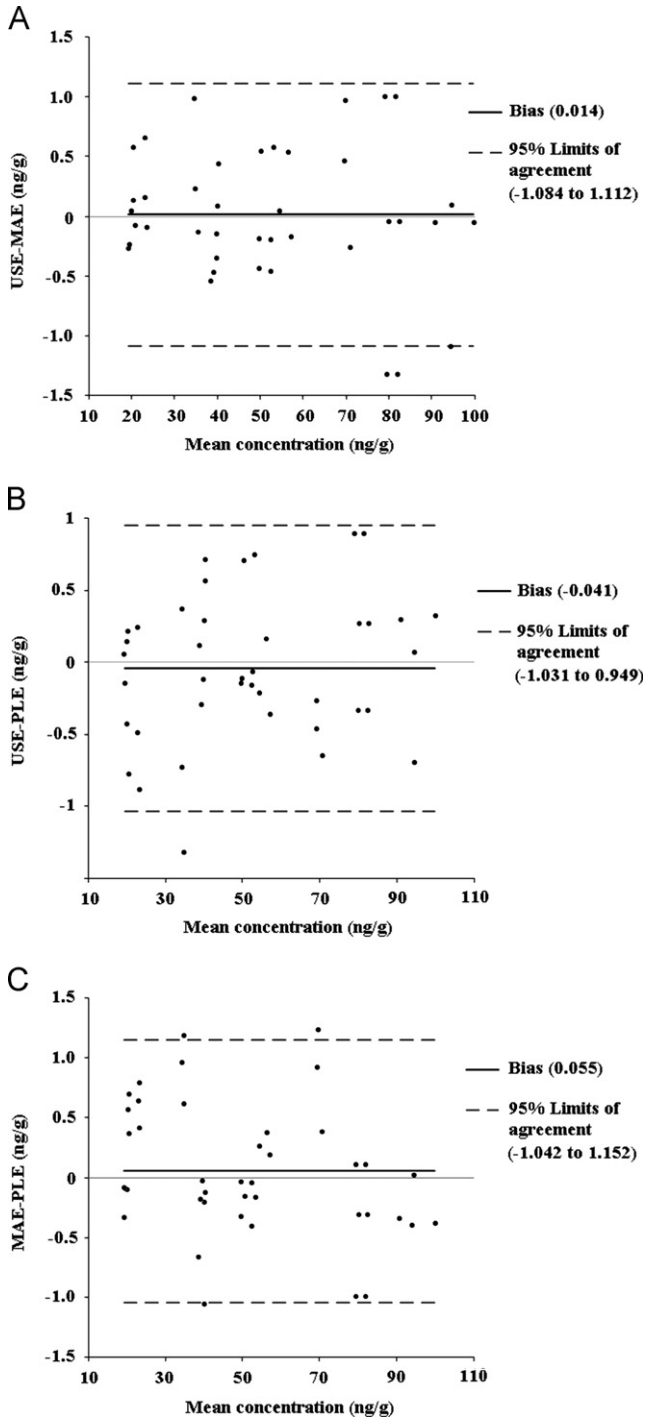

Fig. 5. Overview of difference-plots for the comparison of the extraction techniques for the determination of CIP in sludge samples in the concentration range of 20–95 ng g^{-1}.

In consequence, it can be concluded that the three methods can be used because they are completely reliable. However, the best options to be considered are MAE and PLE, because along with their higher extraction efficiency, the throughput of samples is an important advantage of these techniques, since extractions of serial samples can be completed in a fraction of the time, with a higher degree of automation compared to USE. However, we should keep in mind the use of MAE- and PLE-based techniques is also determined by cost-related issues, because of the high initial investment costs of MAE or PLE units. Nonetheless, the proposed USE procedure has the advantage of using simpler equipment and similar amounts of solvent than MAE and PLE, so that USE must also be considered as an appropriate and robust technique for the extraction of these contaminants from sewage sludge samples.

4. Conclusions

In this paper, three analytical methods based on the use of three different extraction techniques – USE, MAE and PLE – followed by LC–MS/MS analysis are proposed for the determination of 13 quinolone derivatives in sewage sludge samples. Although the extracts showed significant matrix effects, the optimization of the extraction procedures using design of experiments, together with the sensitive detection and quantification provided by LC–MS/MS, allowed the validation of the analytical performance of the methods. These methods provided low LODs (between 1 and 5 ng g^{-1}), high recoveries and good precision, which is an important achievement in comparison with other methods involving more clean-up procedures. MAE and PLE were considered as the best options for extraction, because of their higher extraction yields, easy operation, shorter analysis times and high automation degree. USE offers a simpler extraction procedure, requiring similar amounts of solvent than MAE and PLE, but technique is very tedious and requires much longer analysis times. One of the most important contributions of this work is that despite the differences in the extraction procedures, we have developed three analytical methods that exhibited good analytical parameters and no statistically significant differences in relation to sensitivity, selectivity, accuracy and precision in the determination of the selected compounds.

Acknowledgements

This study was supported by the regional Government of Andalusia (Research Contract No 3017-00) and by the Spanish Ministry of Education, Culture and Sport (Project No CTQ2011-24210). The authors are grateful to the Spanish Ministry of Foreign Affairs (Agency for International Cooperation) for the fellowship granted to N. Dorival-García.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.080.

References

[1] K. Fent, A.A. Weston, D. Caminada, Aquat. Toxicol. 76 (2006) 122–159.
[2] T.A. Ternes, A. Joss, H. Siegrist, Environ. Sci. Technol. 38 (2004) 392A–399A.
[3] C.G. Daughton, T.A. Ternes, Environ. Health Perspect. 107 (1999) 907–938.
[4] S. Kim, D.S. Aga, J. Toxicol. Environ. Health B 10 (2007) 559–573.
[5] K. Kümmerer, J. Antimicrob. Chemother. 52 (2003) 5–7.
[6] K. Kaur, A. Kumar, A.K. Malik, B. Singh, A.L.J. Rao, Crit. Rev. Anal. Chem. 38 (2008) 2–18.
[7] M. Ferech, S. Coenen, S. Malhotra-Kumar, K. Dvorakova, E. Hendrickx, C. Suetens, H. Goossens, J. Antimicrob. Chemother. 58 (2006) 423–427.
[8] L.A. Cardoza, C.W. Knapp, C.K. Larive, J.B. Belden, M. Lydy, D.W. Graham, Water Air Soil Pollut. 161 (2004) 383–398.
[9] M.S. Díaz-Cruz, M.J. López Alda, D. Barceló, J. Chromatogr. A 1130 (2006) 72–82.
[10] X.S. Miao, F. Bishay, M. Chen, C.D. Metcalfe, Environ. Sci. Technol. 38 (2004) 3533–3541.
[11] N. Vieno, T. Tuhkanen, L. Kronberg, Water Res. 41 (2007) 1001–1012.
[12] C.A. Kinney, E.T. Furlong, S.D. Zaugg, M.R. Burkhardt, S.L. Werner, J.D. Cahill, G.R. Jorgensen, Environ. Sci. Technol. 40 (2006) 7207–7215.
[13] S.R. Smith, Philos. Trans. R. Soc. London, Ser. A 367 (2009) 4005–4041.

[14] S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Biomed. Chromatogr. 26 (2012) 33–40.
[15] B. Li, T. Zhang, Environ. Sci. Technol. 44 (2010) 3468–3473.
[16] R.H. Lindberg, K. Björklund, P. Rendahl, M.I. Johansson, M. Tysklind, B.A.V. Andersson, Water Res. 41 (2007) 613–619.
[17] Y. Xiao, H. Chang, A. Jia, J. Hu, J. Chromatogr. A 1214 (2008) 100–108.
[18] R. López-Serna, S. Pérez, A. Ginebreda, M. Petrović, D. Barceló, Talanta 83 (2010) 410–424.
[19] Y. Picó, V. Andreu, Anal. Bioanal. Chem. 387 (2007) 1287–1299.
[20] A. Speltini, M. Sturini, F. Maraschi, A. Profumo, A. Albini, Trends Anal. Chem. 30 (2011) 1337–1350.
[21] A. Karci, I. Akmehmet Balcıoğlu, Sci. Total Environ. 407 (2009) 4652–4664.
[22] M. Ötker Uslu, A. Yediler, I. Akmehmet Balcıoğlu, S. Schulte-Hostede, Water Air Soil Pollut. 190 (2008) 55–63.
[23] M. Ferdig, A. Kaleta, W. Buchberger, J. Sep. Sci. 28 (2005) 1448–1456.
[24] E. Turiel, A. Martín-Esteban, J.L. Tadeo, Anal. Chim. Acta 562 (2006) 30–35.
[25] W. Chenxi, A.L. Spongberg, J.D. Witter, Chemosphere 73 (2008) 511–518.
[26] E.M. Golet, A. Strehler, A.C. Alder, W. Giger, Anal. Chem. 74 (2002) 5455–5462.
[27] M. Lillenberg, S. Yurchenko, K. Kipper, K. Herodes, V. Pihl, K. Sepp, R. Lõhmus, L. Nei, J. Chromatogr. A 1216 (2009) 5949–5954.
[28] J. Radjenović, A. Jelić, M. Petrović, D. Barceló, Anal. Bioanal. Chem. 393 (2009) 1685–1695.
[29] A.B. Marcos, E.S. Ricardo, P.O. Eliane, Talanta 76 (2008) 965–977.
[30] T.C. McIlvaine, J. Biol. Chem. (1921) 183–186.
[31] F. Tamtam, F. Mercier, J. Eurin, M. Chevreuil, B. Le Bot, Anal. Bioanal. Chem. 393 (2009) 1709–1718.
[32] D.A. Volmer, B. Mansoori, S.J. Locke, Anal. Chem. 69 (1997) 4143–4155.
[33] J. Hu, W. Wang, Z. Zhu, H. Chang, F. Pan, B. Lin, Environ. Sci. Technol. 41 (2007) 4806–4812.
[34] B. Li, T. Zhang, Z. Xu, H.H. Ping Fang, Anal. Chim. Acta 645 (2009) 64–72.
[35] H.C. Holten Lutzhoft, W.H.J. Vaes, A.P. Freidig, B. Halling-Sorensen, J.L.M. Hermens, Environ. Sci. Technol. 34 (2000) 4989–4994.
[36] J. Tolls, Environ. Sci. Technol. 35 (2001) 3397–3406.
[37] C. Gu, K.G. Karthikeyan, Environ. Sci. Technol. 39 (2005) 9166–9173.
[38] E.M. Golet, I. Xifra, H. Siegrist, A.C. Alder, W. Giger, Environ. Sci. Technol. 37 (2003) 3243–3149.
[39] J.A. Hernández-Arteseros, J. Barbosa, R. Compañó, M.D. Prat, J. Chromatogr. A 945 (2002) 1–24.
[40] J. Xu, L. Wu, W. Chen, A.C. Chang, J. Chromatogr. A 1202 (2008) 189–195.
[41] A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, J. Chromatogr. A 1216 (2009) 5619–5625.
[42] S.L.C. Ferreira, W.L.N. do Santos, C.M. Quintella, B.B. Neto, J.M. Bosque-Sendra, Talanta 63 (2004) 1061–1067.
[43] M. Roca, M. Castillo, P. Marti, R.L. Althaus, M.P. Molina, J. Agric. Food Chem. 58 (2010) 5427–5431.
[44] M. Gros, M. Petrović, D. Barceló, Talanta 70 (2006) 678–690.
[45] A. Jelić, M. Petrović, D. Barceló, Talanta 80 (2009) 363–371.
[46] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, fifth ed., Pearson Education Limited, Essex, 2005.