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

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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

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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