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Multiresidue determination of 21 pharmaceuticals in crayfish (*Procambarus clarkii*) using enzymatic microwave-assisted liquid extraction and ultrahigh-performance liquid chromatography-triple quadrupole mass spectrometry analysis

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ABSTRACT

In this paper, a multiresidue enzymatic-microwave assisted extraction prior to ultrahigh performance liquid chromatography and triple quadrupole mass spectrometry analysis has been developed for the determination of 21 pharmaceuticals in crayfish (*Procambarus Clarkii*) samples. The analysed compounds corresponding to 6 therapeutic families were: fluoroquinolones (ciprofloxacin, danofloxacin, enrofloxacin, flumequine, gatifloxacin, grepafloxacin, marbofloxacin and norfloxacin); tetracyclines (chlortetracycline and oxytetracycline); sulphonamides (sulfamethoxazole, sulfadiazine, sulfamethazine, sulfamerazine); penicillins (amoxicillin); anfenicols (chloramphenicol, thiamphenicol and florfenicol); non-steroidal anti-inflammatory drugs (ibuprofen and salicylic acid) and trimethoprim an antibiotic that is frequently co-administered with sulfamethoxazole. The main factors affecting the extraction efficiency were optimized for 0.5 g of lyophilized tissue. The enzymatic microwave extraction was carried out using an extraction time of 5 min with 5 mL of an acetonitrile: water (1:1, v/v) mixture, 50 μL of Proteinase-K solution and 5 μL of formic acid at 50 W. After centrifugation, the liquid extract was evaporated and the residue was reconstituted with 1 mL of 0.1% (v/v) formic acid.

Chromatographic and MS parameters, in both positive and negative ionization modes, were also optimized. The mobile phase used consisted on a mixture of 0.1% (v/v) formic acid aqueous solution and acetonitrile in gradient elution mode at a 0.4 mL min^{-1} flow rate. The proposed method was validated and recoveries over 70% were obtained for all the analytes with detection limits in the 0.6-12 ng g^{-1} range. The proposed method was successfully applied to crayfish specimens from Doñana National Park, Spain.

1. Introduction

Pharmaceuticals are theemerging pollutants that have probably led to a greater scientific concern and social impact in recent years. The increasing food demand and therefore the number of intensive livestock activities also contribute significantly to the amount of drugs consumed. Their detection in virtually all types of inland waters has raised the interest of the scientific community, and as a result, a lot of research on their presence as well as the number of new analytical methodologies developed have increased and improved greatly in recent years, especially studies in the environmental field [1].

The evaluation of environmental and human risks caused by pharmaceuticals has been an important issue for the scientific community in recent years. The first evidence of the presence of drugs in waterways occurred in the 70 s in wastewaters from USA with the identification of clofibric acid, which is the active metabolite of several regulators of blood lipids such as clofibrate, etofilin clofibrate and etofibrate [2]. However, it was not until the early 90 s that the issue of the presence of drugs in the environment emerged strongly, as supported by the numerous articles published since

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then. Currently, more than 3000 different types of pharmaceutical active principles are used in the European Union (EU), being mainly antibiotics, β-blockers, lipid regulators, antidepressants and analgesics. It is estimated that the consumption of drugs in the EU countries reaches several tons per year, with similar data on pesticides [3]. Their presence in surface waters are due to an incomplete removal in wastewater treatment plants (WWTPs). Surface waters and groundwater levels are usually in the ng L^{-1} or even μg L^{-1} range [4,5]. Nowadays, there is a huge concern about the risks associated with direct and indirect effects on human health due to both active and passive consumption of pharmaceuticals, which has led to regulations on the use of some antibiotics in food production from animal origin and the establishment of maximum residue limits (MRLs) for those involving known risks. The European Union establishes these limits in its Commission Regulation (EU) No 37/2010 of 22 December 2009 [6].

There are many reports about the presence of pharmaceuticals in environmental water samples but analytical methods regarding their presence in aquatic organisms (marine and not marine) are less widespread, in particular, those that not only are important from an environmental point of view, but also for human consumption reasons. However, in recent years these have gained great interest, although their focus has been mainly limited to fish samples [7,8]. Other works dealing with analytical methodologies for crayfish samples are scarce. Crayfish (*Procambarus Clarkii*) is an invasive species introduced in the Doñana National Park [9], damaging agricultural infrastructures [10]. It lives in muddy bottoms of rivers, marshes and ponds, digging tunnel shelter. *P. Clarkii* has a very strong physiology, tolerates low levels of dissolved O_2 (2–14 mg L^{-1}), changes in temperature (17–22 °C) and high pollution, which makes it an appropriate species to be used as a bioindicator. P. *Clarkii* occupies a key role in the food chain of Doñana Park, transferring energy and pollutants between trophic levels [11].

Doñana Nature Reserve is a protected area over 100,000 ha. It is located in the north of the Guadalquivir estuary between the provinces of Huelva, Seville and Cádiz. Within this area, is the Doñana National Park (DNP) with 54,252 ha. It is one of the most important wetlands in Europe, integrated in the Ramsar Convention for the protection of wetlands and recognized by UNESCO first as a Biosphere Reserve and then as a World Heritage Site. It is a wildlife sanctuary with more than 800 species of plants and 400 species of animals [12]. Agricultural activities are an important driving force in the economy of the area but affect Doñana in 3 ways: extraction of groundwater for irrigation, use of surface waters for rice crops, and pollution from fertilizers and pesticides.

In general, multiresidue analysis has been focused on different kinds of environmental waters [13,14] but has also been applied to biological matrices as aquatic plants [15] and several kinds of foods [16,17]. The multiresidue analysis of pharmaceuticals in fish and related organisms (shellfish, molluscs, bivalves...) is scarcer [7,8,18–21]. From our knowledge, no method have been previously developed for multiresidue pharmaceutical analysis in crayfish. Reported studies on Doñana Park only involve water and sediments analysis of a low number of analytes [22–24].

The objective of this work was to develop and validate an affordable multiresidue method for the simultaneous determination of several selected pharmaceuticals in crayfish samples (*P. Clarkii*) from Doñana National Park. Up to 21 pharmaceutical active principles corresponding to 6 therapeutic families were analysed: fluoroquinolones (ciprofloxacin, danofloxacin, enrofloxacin, flumequine, gatifloxacin, grepafloxacin, marbofloxacin and norfloxacin); tetracyclines (chlortetracycline and oxytetracycline); sulphonamides (sulfamethoxazole, sulfadiazine, sulfamethazine, sulfamerazine); penicillins (amoxicillin); anfenicols (chloramphenicol, thiamphenicol and florfenicol); non-steroidal anti-inflammatory drugs (ibuprofen and salicylic acid) and trimethoprim an antibiotic that is frequently co-administered with sulfamethoxazole.

2. Material and methods

2.1. Chemical and reagents

Ciprofloxacin (CPR), danofloxacin (DNF), enrofloxacin (ENR), flumequine (FMQ), gatifloxacin (GTF), grepafloxacin (GPF), marbofloxacin (MRB), norfloxacin (NRF), chlortetracycline (CLT), oxytetracycline (OXT), sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfamethazine (SMT), sulfamerazine (SMR), trimethoprim (TMP), ibuprofen (IBU), salicylic acid (SAC), amoxicillin (AMX), chloramphenicol (CLF), thiamphenicol (TIF) and florfenicol (FLF) (97–99.9% purity) were purchased from Fluka- Sigma-Aldrich, S.A. (Madrid, Spain). Methanol (LC–MS grade), acetonitrile (LC–MS grade), formic acid (98–100% purity), all analytical grade, were purchased from VWR (Barcelona, Spain). Ultrapure deionized water was obtained from a Milli-Q plus water system Millipore (Billerica, MA, USA). Proteinase-K 20.2 mg mL^{-1} recombinant PCR grade was obtained from Roche Diagnosis (Barcelona, Spain).

Standard solutions of pharmaceuticals were prepared daily by adequate dilutions in ultrapure water from (400 mg L^{-1}) stock solutions, except for CPR (100 mg L^{-1}) and CLT and OXT (100 mg L^{-1}). Stocks solutions were prepared by dissolving the drugs in methanol (DNF, ENR, FMQ, GTF, GPF, MRB, NRF, SMX, TMP, SDI, SMT, SMR, AMX, IBU and SAC), water (CLT, OXT, CLF, TIF and FLF) or methanol:water (80:20, v/v) mixture (CPR). All stock solutions were stable for at least two months at 4 °C except CPR, CLT and OXT that were kept at room temperature and prepared daily.

2.2. Instrumentation

Chromatographic separation was performed using an Agilent 1290 Infinity UPLC system coupled to an Applied Biosystems 3200 QTRAP LC/MS/MS instrument (ABSciex, Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQLIT) mass spectrometer equipped with an electrospray ion source (TurboSpray ®). Separations were carried out using a reversed-phase Zorbax Eclipse XDB-C18 analytical column (150 mm × 4.6 mm, particle size 3 μm)) preceded by a guard-column Zorbax ODS (4.6 mm × 12.5 mm, particle size 5 μm) (Agilent technologies Spain S.L).

2.3. LC and MS/MS conditions

The LC separations were performed using a mobile phase consisting of a mixture of 0.1% (v/v) formic acid aqueous solution (component A) and acetonitrile (component B). Two different gradient elution programs at a 0.4 mL min^{-1} flow rate were used to achieve the separations for positive and negative ionization modes, respectively.

To obtain the chromatogram in positive ionization mode, a linear elution gradient was applied from 0% to 30% B in 12 min, then % B increased linearly to 100% B in 5 min more and finally, an isocratic step from 17 to 21 min was applied. The mobile phase returned to initial conditions and 5 min were waited before the next injection.

For negative ionization mode, a linear elution gradient from 10% to 30% B was applied in 15 min followed by an increase to 100% B in 6 min more, returning then to initial conditions after 21 min and 5 min were waited before the next injection.

Multiple Reaction Monitoring (MRM) where the parent ions and fragment ions were monitored at Q1 and Q3, respectively, was applied. $[M+H]^+$ and $[M-H]^-$ were selected as precursor ions for positive and negative ionization modes, respectively. Nitrogen at 4 psi was used as collision gas. The ion source and curtain gases