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Quantification of emerging micropollutants in an amphipod crustacean by nanoliquid chromatography coupled to mass spectrometry using multiple reaction monitoring cubed mode

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ABSTRACT

An innovative analytical method has been developed to quantify the bioaccumulation in an amphipod crustacean (*Gammarus fossarum*) of three micropollutants regarded as anthropic-pollution markers: carbamazepine, oxazepam, and testosterone. A liquid-liquid extraction assisted by salts, known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) was miniaturised and optimised, so it could be adapted to the low mass samples (approximatively 5 mg dry weight). For this same reason and in order to obtain good sensitivity, ultra-trace analyses were carried out by means of nanoliquid chromatography. A preconcentration system by on-column trapping was optimised to increase the injection volume. In order to improve both sensitivity and selectivity, the multiple reaction monitoring cubed mode analyses (MRM^3) were carried out, validated and compared to the classic MRM. To the best of our knowledge, this is the first time that MRM^3 is coupled to nanoliquid chromatography for the analysis and detection of organic micropollutants <300 Da. The optimised extraction method exhibited recoveries superior to 80%. The limits of quantification of the target compounds were 0.3, 0.7 and 4.7 ng/g (wet weight) for oxazepam, carbamazepine and testosterone, respectively and the limits of detection were 0.1, 0.3 and 2.2 ng/g (wet weight), respectively. The intra- and inter-day precisions were inferior to 7.7% and 10.9%, respectively, for the three levels of concentration tested. The analytical strategy developed allowed to obtain limits of quantification lower than 1 ng/g (wet weight) and to establish the kinetic bioconcentration of contaminants within *G. fossarum*.

1. Introduction

During the last few years, a great attention has been paid to the occurrence of the so-called emerging pollutants in the environment [1–3]. This is the case of pharmaceuticals, which are released in the aquatic ecosystem mainly on the discharge of wastewater treatments plant (WWTP) effluents or directly by industry or direct human activities. Although they have been present for a long time in the aquatic environment, their impacts, like bioaccumulation and toxicity on the organisms of the first trophic levels are still little-known, due mainly to the lack of adequate methodologies to perform the analysis of small organisms. To assess these impacts, biomonitoring studies have been carried out on invertebrates such as bivalves, amphipods or aquatic larvae which are able to bioaccumulate contaminants [4–6]. The development of such studies requires knowledge about the kinetics of accumulation in the studied organisms. For instance, up to date, it was not possible to assess bioaccumulation knowledge at the scale of a single organism because the analytical strategies available were not sensitive enough to quantify the pollutants in a few or only one crustacean of low mass (<30 mg wet weight) [7–11]. That is why the analytical method described in this study was developed at an individual scale. For the extraction of pharmaceuticals in biota, studies reported classic solid-liquid extractions using organic solvents [12,13], sonication [14–16], micro-wave assisted extraction [17,18], pressurized liquid extraction [19], and pulverized liquid extraction [11]. The purification was mainly performed by solid phase extraction (SPE) [11,15,18]. The main drawbacks of these extraction and purification methodologies are the long times required and a large amount of organic solvent used. In 2003, Anas-

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tassiades et al. developed a new extraction methodology named QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) which included both the extraction and purification steps [20]. The extraction consists of a liquid-liquid extraction assisted by salts, and purification is performed by a dispersive phase sorbent. In the first step, the analytes are extracted from the matrix using an organic solvent which must be highly polar and miscible in water so that an addition of salts leads to a phase separation and the transfer of the analytes into the organic phase. In the second step, a dispersive sorbent is added and retain coextractants. Although QuEChERS was initially developed for the analysis of pesticides in vegetables, the method was successfully applied to various complex matrices including animal tissues [21,22], honeybees [23], fishes [24,25], earthworms [26], and crustaceans [27,28].

Regarding the analysis of pharmaceuticals in biota, recent studies have been carried out by high performance liquid chromatography (HPLC) [11–13,16,21–23,26] or by gas chromatography (GC) [25] both coupled to tandem mass spectrometry (MS/MS), as it is at present the most sensitive and selective technique for this type of analysis. Some recent studies also used the analysis by ultra high performance liquid chromatography UHPLC, thereby reducing analysis time while maintaining good performance in terms of sensitivity and resolution [14,15,19,29]. However, all these analyses were performed on samples of several hundred milligrams and even masses greater than 1 g. However, for the analysis of macroinvertebrate such as *Gammarus fossarum,* only a few milligrams of solid matrix were available to quantify compounds at trace levels, and the use of nanoliquid chromatography (nanoLC) becomes indispensable. NanoLC was presented for the first time in 1988 by Karlson and Novotny [30], who used a capillary separation column with an internal diameter (id) of less than 100 μm. Sensitivity increases with the square of the inner diameter reduction factor [31]. Therefore, switching from a typical id of 4.6 mm–100 μm, sensitivity rises approximately 2000-fold. In addition, the inner diameter reduction leads to perform analyses using much lower flow rates, which is advantageous since it has been demonstrated that flow reduction increases the electrospray ionisation recovery when detection is performed by mass spectrometry [32], increasing the sensitivity. Therefore, nanoLC is a particularly suitable technique when samples are available in very small quantities such as benthic invertebrates. To date, nanoLC has been used mainly in proteomic analysis in blood plasma and urine or other biological matrices [33–36]. To the authors' knowledge, there are no works reporting the nanoLC analysis of emerging compounds in the environment or aquatic biota except for the studies published in our laboratory [27,28].

MS/MS analyses, by means of MRM scans, is the most used technique for quantification of pharmaceuticals in environmental matrices [11–14,16,19,21,23,25–29,37]. The advantage of the MRM is its great sensitivity and selectivity. Nevertheless, in the case of complex matrices, MRM is not always sufficient and false positive remain even with different chromatographic conditions. In 2009, Fortin et al. showed that sensitivity and specificity of mass spectrometry detection could be improved by adding a second fragmentation to the classic MRM process. They named this technic MRM^3 [38]. This mode takes advantage of the hybrid triple quadrupole linear ion trap (QqLIT) and allows to use the third quadrupole as a linear ion trap to select/trap/fragment a product ion into a second generation of product ions in a low pressure environment using a resonant excitation [39,40]. It results in a second transition which gives even more sensitivity and selectivity compared to MRM mode. MRM^3 was used for protein [41], peptids [42], retinoic acid [43] and drugs quantification [44]. To the best of our knowledge MRM^3 was never coupled to nanoLC with a nanospray interface for the quantification of pharmaceuticals in environmental matrices.

The aim of the present study was the development of a novel analytical methodology based on miniaturised QuEChERS extraction and followed by nanoLC coupled to mass spectrometry in MRM^3 for the quantification of carbamazepine, oxazepam, and testosterone in a complex matrix of small size such as macroinvertebrates. The method was applied to the individual analysis of the crustacean *G. fossarum* and allowed to establish the kinetics of bioconcentration of the targeted compounds.

2. Experimental section

2.1. Chemicals and reagents

Carbamazepine, oxazepam, and testosterone standards were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) with purity higher than 99%.

LC–MS grade MeOH, ACN and acetic acid were obtained from Fluka (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Ultra-pure water (18.2 MΩ cm) was obtained from a MilliQ device made by Millipore (Saint-Quentin-en-Yvelines, France). Stock solutions of individual standards were prepared at 400 mg/L in MeOH and kept at −20 °C. Working standards solution mixtures were prepared by adequate dilution of the stock solutions in MeOH.

For the QuEChERS sample preparation, citrate buffer kits were obtained from Agilent Technologies (Massy, France) and contained 308 mg of $MgSO_4$, 77 mg of NaCl, 77 mg of sodium citrate and 38.5 mg of disodium citrate sesquihydrate (pH = 5-5.5). For purification optimisation, PSA dSPE was obtained from EuroClean and contained 60 mg of $MgSO_4$ and 10 mg of PSA. PSA/C18 dSPE was obtained from Agilent and contained 60 mg of $MgSO_4$, 10 mg of PSA and 10 mg of C18. Z-Sep dSPE was obtained from Supelco and contained 60 mg of $MgSO_4$ and 20 mg of Z-sep (Zirconia-coated silica).

2.2. Sample collection and acclimatization

G. fossarum were collected in the Bourbre river, near La Tour du Pin, France. Only male adult invertebrates were selected to avoid sexual bias. They were kept at 12 ± 1 °C, an optimal temperature for gammarids, in aquaria filled with groundwater. An oxygenation system maintained oxygen saturation between 70 and 90%. The invertebrates were fed *ad libitum* with alder leaves (*Alnus glutinosa*) collected from a pristine site near La Tour du Pin. For all the tests and matrix-matched calibration, 200 clean gammarids were pooled after being freeze-dried, ground and stored at 4 °C.

2.3. Exposure

To evaluate kinetic bioconcentration, gammarids were exposed to 200 ng/L of a mixture of the three target pharmaceuticals during 14 days plus two additional days to evaluate kinetic depuration. Fifteen male individuals of *G. fossarum* were placed into 500 mL beakers filled with groundwater spiked at the working concentration. Six different beakers were set up, one for each sampling time during the accumulation, plus two beakers for the depuration evaluation. To avoid bacterial growth, adsorption and degradation issues, and to maintain the same concentration during 14 days, the exposure solutions were renewed each day with groundwater previously oxygenated during 24 h, and spiked with stock solutions. The spiked volume was calculated so that the exposure solutions contained <0.1‰ methanol (v/v). Beakers were kept at 12 ± 1 °C. Alder leaves were added to each beaker to feed gammarids *ad libitum*. At the start of the exposure experiments, exposure solutions were analysed to know the exact concentration of the solutions and three non-exposed gammarids (blanks) were analysed. Each day, gammarids were counted to evaluate mortality, and temperature