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Single injection LC-MS/MS analytical method for the quantification of diverse families of micropollutants, including PFAS and organotins, in *Gammarus fossarum*

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Abstract

Since the last decades, light has been shed on the pollution of aquatic ecosystems. Considering apolar compounds in water, analysis of the dissolved phase is not sufficient due to their possible bioaccumulation. Additional analysis of sediments, biota, or sentinel species is necessary. Among sentinel species, *Gammarus fossarum* is a small shrimp of 30 mg that lives naturally in the river. Its ability to bioaccumulate makes it a good bioindicator of river pollution.

Nevertheless, micropollutants are difficult to extract from gammarids due to their small size and their high level of lipids. Extracted interferences can lead to analytical difficulties. Targeted micropollutants in this work were organotins, personal and pharmaceutical care products (PPCPs), pesticides, flame retardants, and perfluorinated compounds. A sample preparation based on QuEChERS followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was developed: different salts (acetate and citrate) and purification (addition of hexane, dispersive SPE) were tested and validated. Targeted molecules used to be analyzed by LC-MS/MS, except organotins which are principally analyzed in gas chromatography. One of the main challenges was to quantify them also in LC-MS/MS to implement an original multi-residue method. The analysis of 40 compounds was finally validated according to ICH guidelines, with LOQs ranging from 0.04 ng.g⁻¹ to 313.5 ng.g⁻¹. Regarding Perfluorooctanesulfonic acid (PFOS) and the sum of hexabromocyclododecane, LOQs reached the environmental quality standards in biota which are respectively of 9.1 and 167 ng.g⁻¹.

Finally, the method was applied to 15 real samples. Many compounds were quantified: perfluorinated compounds, drugs such as ketoprofen, and even cosmetics products such as octocrylene. This is the first study to quantify as many emerging compounds, especially organotins, in a sentinel species such as *G. fossarum*.

Keywords: Micropollutant, *Gammarus fossarum*, liquid chromatography, tandem mass spectrometry, miniaturization, PFAS

1. Introduction

Industrialization and the increasing use of chemicals to meet the needs of agriculture or domestic activities are responsible for multiple releases of chemicals into the environment. Chemical contaminants belong to many families such as pesticides, perfluorinated compounds, organotins, flame retardants, pharmaceuticals, and personal care products (PPCPs), etc. Some perfluorinated compounds such as perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are restricted by the international agreement Stockholm convention [1], and PFOA was banned from import, export, and production by the European Commission [2]. However, these compounds are released continuously in rivers due to the contamination of soils which are considered as reservoirs, with an estimation of about 1 500 to 9 000 tons of PFAS [3]. Pesticide pollution is a well-known problem and some of them have been reported in rivers, like the Seine for example, since almost 1995 [4]. Wastewater treatments cannot eliminate them and they are finally released into rivers and may reach drinking waters [5,6]. Organotins are synthetic molecules principally used for the antifouling painting of boats. These compounds are also reported in the Rotterdam Convention since 2008 as pesticides and for industrial use. Tributyltin (TBT) is the major molecule of the family; it is known for its toxic effect on aquatic organisms [7]. In 1996, Ruiz et al. reported the impact of TBT in coastal environments like Arcachon Bay [8]. France was the first country to limit TBT in antifouling painting in 1982.

Over the years, maximum levels in water were set. An Environmental Quality Standard (EQS) is defined as “the concentration of pollutants or a group of pollutants in water, sediments or biota that should not be exceeded to protect human health and the environment”. For years, water was the only matrix to be monitored and to have EQS. In 2008, Directive 2008/105/CE indicated that several compounds could not be monitored only by analyzing surface water and recommended analysis of biota [9]. Finally in 2013 [10], the Water Frame Directive (WFD) included EQS in biota (EQS_{biota}) for hydrophobic molecules difficult to monitor in water, such as fluoranthene, mercury, PFOS, hexabromocyclododecane (HBCDD), heptachlore, dioxine, etc. The so-called sentinel species such as mollusks, fish, or shrimps can be used for biomonitoring as they may accumulate micropollutants, especially ones with a $\log K_{ow} > 3$. *Gammarus fossarum* is one of the most commonly used sentinel species. This freshwater amphipod crustacean lives in pure water like rivers and feeds mainly on decaying dead leaves. Currently, the analysis of micropollutants in gammarids is not well documented. Only a few papers referred to their use for the biomonitoring of waters [11,12]. However, their pertinence for biomonitoring was already reported in 2013 [13].

Extraction and instrumental analysis of traces of micropollutants in biota such as gammarids remain challenging. The extraction represents a key point in the procedure, needed to isolate and enrich relevant compounds in biota. Several extraction techniques were used, mainly liquid-liquid extraction (LLE), solid

phase extraction (SPE), or QuEChERS extraction (Quick, Easy, Cheap, Effective, Rugged, and Safe). At the beginning of the century, QuEChERS extraction was developed for pesticide analysis in fruits and vegetables [14] and has rapidly settled into the technique of choice for many environmental matrices. The procedure is based on a salting-out liquid-liquid extraction followed by a dispersive Solid Phase Extraction (dSPE) purification step. This additional purification is crucial for complex matrices such as biota, because of the possible co-extraction of lipids and other components detrimental to the instrumental analysis. The dSPE phase is often composed of $MgSO_4$, PSA (primary secondary amines), and/or C18 sorbents, and can be adapted by adding some graphitized carbon black to remove pigments or polar compounds. QuEChERS procedure was deployed in two major recognized methods: the American method [15] using $MgSO_4$ and sodium acetate salts and the European method [16] based on $MgSO_4$, NaCl, and citrate buffer. QuEChERS was adapted to various contaminant families or matrices such as organotin in bottom sediments [17], pesticides in honey [18], or various emerging compounds in benthic invertebrates [19]. Pesticides remained mainly extracted with QuEChERS salts followed by dSPE purification in fish muscles [20] as well as hexabromocyclododecane with modified QuEChERS extraction with ethyl acetate [21].

One of the disadvantages of these techniques is the quantity of matrix recommended: 10 or 5 g for QuEChERS, which is very high for biota. For example, 10 g of *G. fossarum* matrix represent more than 300 invertebrates and is difficult to sample. Because of the use of a small-sized sentinel species, the quantity of matrix must be adapted as well as the extraction. Dufour et al. [22] developed a miniaturized extraction for pesticides in biotic matrices and reported similar performances compared to a protocol that used a higher mass of sample. Furthermore, the reduction of solvent used for extraction is part of the green chemistry approach.

Concerning purification, SPE and dSPE are known to drastically reduce matrix effects. A recent study mentioned the use of dSPE for analyzing PPCPs, with a new specific Enhanced Matrix Removal (EMR-lipid) for purification in *Anguilla Anguilla* [23]. This new technology is based on size exclusion and hydrophobic interactions to remove lipids. Molecular Imprinted Polymers (MIPs) are based on solid phases designed to retain specific compounds, such as tributyltin [24]. This last solution is efficient but has disadvantages: time-consuming, high cost, and difficult to apply for multi-residue analysis.

The objective of this study was to develop a single quantitative method adapted to the analysis of molecules with various physicochemical properties belonging to different families, namely pesticides, perfluorinated compounds, PPCPs, flame retardants and even organotins in *G. fossarum*. The targeted compounds (Table 1) were chosen because they are not yet monitored but are considered of interest according to the French

Water Agency. Among them, two have an EQS in biota: sum of HBCDD (167 $\mu\text{g.kg}^{-1}$ of fresh weight) and PFOS (9.1 $\mu\text{g.kg}^{-1}$ of fresh weight). These chemicals were analyzed by liquid chromatography (LC) coupled to quadrupole tandem mass spectrometry (MS/MS). This coupling allows both high sensitivity, reproducibility, selectivity, and robustness for the analysis of mid-polar to polar compounds. The extraction method was based on QuEChERS. To the best of our knowledge, this is the first time that a single extraction was optimized to such a wide range of micropollutant families in *G. fossarum*. In addition, the originality of the method comes from a unique LC-MS/MS injection for all the compounds, including organotins.

2. Experimental

2.1. Materials and reagents

Analytical standards related to their CAS number and supplier are reported in Table S1. The ULC-MS grade solvents methanol (MeOH), acetonitrile (MeCN), and water, and hexane (purity 99%) were purchased from Biosolve (Dieuze, France). Formic acid with a purity of 99% was purchased from Carlo Erba (Val de Reuil, France). Ammonium formate with purity higher than 99.995% was from Sigma Aldrich. QuEChERS (citrate buffer) and dSPE salts (MgSO_4 , PSA), according to EN Method 15662, were from Restek (Bellefonte, USA). Salt dSPE PSA/GCB (MgSO_4 , PSA, GCB) was purchased from Phenomenex (Torrance, USA).

Stock solutions of standards were prepared at a concentration of 1000 $\mu\text{g.mL}^{-1}$ in MeOH and stored at -20°C . Working solutions were prepared by successive dilution of stock solutions. A solution of PFUDA C13 (Perfluoro-n-[1.2- $^{13}\text{C}_2$]undecanoic acid) at 0.5 $\mu\text{g.mL}^{-1}$ in MeOH was prepared.

2.2. Sample collection

Blank matrix (i.e. matrix free of the target compounds) is essential for analytical method development. To this aim, organisms from a reference population (Gammaref®) were sampled in outdoor rearing tanks by BIOMAE in Chateau-Gaillard, France. They were transported in water to their laboratory then stabilized under controlled conditions in aquariums with borehole water, thermoregulated at $12^\circ\text{C} \pm 1^\circ\text{C}$ and fed *ad libitum* with *Alnus glutinosa* leaves. After at least seven days of stabling, organisms were sorted to keep only adult males whose average weight per individual was comprised between 22 and 32 mg fresh weight. This matrix was stored at -20°C and was considered blank for further developments and matrix-matched calibration.

Application of the developed method was done with gammarids that were placed in river waters, upstream/downstream wastewater treatment, or industrial discharge, on Water Agency monitoring station spread across France for 14 days in May and November 2022.

2.3. Optimized analytical protocol

2.3.1. Sample extraction

The optimized sample preparation was as follows: 0.125 g of gammarids were grinded in a tube with 1 stainless steel ball and 200 μL of water ULC/MS grade. A volume of 20 μL of mixture of internal standards was added, then 250 μL of MeCN 1% HCOOH and 250 μL of hexane. These additions were followed by 1 min of vortex to homogenize. Then, 100 mg of QuEChERS salts (61.5 mg MgSO_4 , 15.5 mg NaCl, 23 mg citrate buffer) and 50 mg of ammonium acetate were added and the mixture was submitted to a vortex 30 s, then submitted to ultrasounds for 20 min, and finally centrifuged 10 min at -10°C to fix the fatty fraction. After centrifugation, the supernatant was transferred to a dSPE tube containing 82 mg of MgSO_4 , 14 mg of PSA, and 4 mg of GCB. This mixture was submitted to vortex for one min, and then to centrifugation for 10 min at -10°C . Finally, 150 μL of the MeCN phase was transferred into a vial and analyzed by LC-MS/MS.

2.3.2. LC-MS/MS analysis

LC-MS/MS analyses were performed using high-performance liquid chromatography LC30AD pumps coupled to a triple quadrupole mass spectrometer LCMS8060NX, both from Shimadzu (Kyoto, Japan). A Kinetex XB-C18 reversed phase (100 x 2.1 mm, 2.6 μm) from Phenomenex was used for chromatographic separation. Contaminants were analyzed using a single method with mobile phases constituted of 0.005% HCOOH and 10 mM of ammonium formate in water (A) and MeOH (B). Column oven was set at 40 $^\circ\text{C}$.

Separations were made with a gradient starting at 40% of (B) increasing to 50% in 1 min and kept for 2 min, then increased to 100% of (B) in 6 min and kept for 3 min to finally return to initial condition in 0.1 min for 3 min. The total duration of the gradient was 15 min. the injection volume was 5 μL in MeCN and the flow rate was set at 0.2 $\text{mL}\cdot\text{min}^{-1}$.

All compounds were analyzed in multiple reaction monitoring (MRM) mode (Table 1). The ionization was operated with electrospray ionization (ESI) in both positive and negative polarity switching modes, which required one single injection. The optimization of the detection (collision energy, Q1 and Q3 voltage) was performed automatically with the LabSolutions software. Molecules were infused individually at a concentration of 100 $\mu\text{g}\cdot\text{mL}^{-1}$ in $\text{H}_2\text{O}/\text{MeOH}$ (50/50). Data were processed with LabSolutions Insight 2.7. The MRM transitions for each compound are reported in Table 1. Dwell time was optimized manually (Sup. S1).

2.4. Method validation

2.4.1. Strategy

Sample extraction and LC-MS/MS analysis were validated according to International Conference on Harmonisation (ICH) guidelines [25] over three days. Each day a full range from the limit of quantification (LOQ) to 50 \times LOQs in the matrix was extracted to study linearity. LOQs were evaluated by considering a signal-to-noise ratio of 10.

Intra-day precision represents repeatability with the same conditions. This criterion was evaluated in triplicate at three concentrations: low level (LOQ), middle level (10 × LOQ) and high level (40 × LOQ). Inter-day precision was determined by variation over the three days of validation: different bottles of solvent for the mobile phase were used each day of validation to add a possible variation. These two types of precision were calculated as the relative standard deviation (RSD %). According to ICH guidelines, they must be less than 20%. To be validated, accuracy and repeatability must be comprised between 80 and 120%.

2.4.2. Quantification and matrix effects

Quantification was performed using matrix-matched calibration, except for perfluorinated compounds, which were quantified by internal calibration. Five or six calibration points were used depending of each compound. For each matrix-matched calibration point, 0.125 g of the blank matrix, which represents about four or five gammarids, were weighted and spiked with concentrations comprised between LOQ and 50 × LOQ and prepared as mentioned in the *Sample extraction* section. All the compounds were quantified with a linear calibration curve and a weighting method of 1/C. Perfluorinated compounds were quantified with an internal standard (PFUnA C13), with an addition of 20 µL of a 500 ng.mL⁻¹ solution, at the beginning of the extraction.

Matrix effects were calculated by the following ratio:

$$\text{Matrix effect(\%)} = \left(\frac{A_{\text{Spiked}}}{A_{\text{Solvent}}} - 1 \right) \times 100$$

with A_{spiked} the compound area in the extract spiked after extraction and A_{solvent} the compound area in solvent.

3. Results and discussion

3.1. LC-MS/MS optimization

3.1.1. Organotins

One of the main challenges in this study was to include organotins within a multi-residue method. Organotin compounds are a family of molecules composed of a tin (Sn) central element related to different groups. Organotins are mainly analyzed by gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) [26], after a sample preparation that includes ethylation, extraction, and preconcentration. Due to their cationic form, the extraction of these compounds from environmental matrices needs a time-consuming step of derivatization (Grignard derivatization for example [27]) before the analysis. Since organotins are apolar molecules, they can also be found in biota. Recently, HPLC-ICP-MS was reported as a novel solution to analyze some organotins, but results demonstrated that this method was not as sensitive as GC-MS/MS [28].

Considering LC-MS/MS, TBT is probably the easiest to analyze because of its structure: the atom of tin can form four bonds, therefore Sn is positively charged and TBT can be analyzed on its $[M]^+$ form. Triphenyltin (TPhT) shows the same ionization form with the tin element bonded to three phenyl groups. Their degradation products, monobutyltin and dibutyltin from TBT, and monophenyltin and diphenyltin from TPhT, are more difficult to analyze because of their cationic form Sn^{2+} or Sn^{3+} .

One of our objectives was to avoid a derivatization step, this means that ion pairs should be formed with tin molecules. In this way, the use of two common types of buffers in mass spectrometry was investigated to form ion pairs: ammonium formate/formic acid and ammonium acetate/acetic acid. Both salts were tested and tin compounds showed better affinity with formate buffer. Unfortunately, compounds in the butyltin family had the same retention time, and this was a recurring problem for the phenyltin family. Further investigation revealed that tributyltin and triphenyltin fragmented in-source into their degradation products, mono, and di, leading to cross-talk. Consequently, TBT and TPhT degradation products were finally removed from the development of the sample preparation, method validation, and field sample analysis stages, to avoid false positives.

3.1.2. Mobile phase

Concerning the mobile phases, MeOH and MeCN were investigated as organic solvents. MeCN reduced backpressure compared to MeOH but, due to its higher eluting power, co-elutions of the targeted compounds were more frequent. The addition of a buffer in the organic phase was necessary to ionize all compounds in a single analysis method for positive and negative ionizations. For example, perfluorinated compounds were ionized in negative mode and the presence of salts like ammonium formate was necessary for their ionization. However, ammonium formate was difficult to dissolve in MeCN. To this end, the addition of 10% water with salts in the organic phase was tried but without success: a precipitate was quickly formed in the MeCN phase. The use of MeOH was finally preferred.

During these previous tests, inhibition of signals due to the addition of acid was observed, particularly for 4-tert-octylphenol (4t-OP), as already reported in the literature [29,30]. During the chromatographic optimization, the two alkylphenolic compounds bisphenol A and 4t-OP, that are ionized in the negative mode were not detected anymore in the presence of acid. Additional experimentations were performed with different mobile phases using MeCN or MeOH as organic solvent, and with the use of salts and/or acid. The results show that the presence of a low amount of acid (0.005% HCOOH) with ammonium formate is sufficient to inhibit the signal of these molecules. As mobile phase conditions were not compatible with the analysis of bisphenol A and 4t-OP, these molecules were finally not further considered (Table 1).

3.1.3. Mass spectrometer interface parameters

Interface parameters are crucial to improve the detection of analytes. Manual adjustment of seven parameters in the ESI source was performed: interface temperature, desolvation line (DL) temperature, heat block temperature, flow rate of nebulizing gas, heating gas, and drying gas.

For each parameter (interface, desolvation, DL, and heat block) temperature, steps of 50 °C were experienced from the lowest to the highest possible. Regarding gases, the nebulizing gas flow rate can be adjusted to a maximum of 3 L.min⁻¹, so three flow rates were considered: 1, 2, and 3 L.min⁻¹. Heating gas can have a minimum value of 3 L.min⁻¹ and a maximum of 25 L.min⁻¹ while the maximum value of drying gas flow is 20 L.min⁻¹. On the other side, the addition of these two parameters cannot exceed 25 L.min⁻¹. In total, 34 tests in triplicate were made iteratively for the optimization of the interface parameters.

The optimum interface temperature was 250 °C (Table 2). A higher temperature reduced the signal area of most compounds, excepted a few such as sulfamethoxazole whose signal was better at a temperature of 400 °C. Conversely, PFUnA, PFDoA, and PFtriA had their highest area at a temperature of 100°C. The flow rate of the drying gas (DGF) was optimum at 3 L.min⁻¹, which is the lowest possible value for this type of instrument. Drying gas flow is used for desolvation before entrance in the analyzer [31]. This value can be explained by the flow rate of the method (0.2 mL.min⁻¹) which can be considered as low in liquid chromatography. The Nebulising Gas Flow (NGF) was set at 3 L.min⁻¹ and this value was optimal for the majority of compounds but the signals of some perfluorinated compounds such as PFBS, PFNS, PFDA, PFUnA, and PFtriA were higher with a NGF at 1 L.min⁻¹.

In summary, the optimized parameters represented the best conditions to improve signal intensity for the majority of compounds, with a few exceptions. For example, triclosan would need a lower source temperature. According to the literature [32,33], one of the hypotheses is that a higher temperature coupled with an increase of heating gas flow may induce an in-source fragmentation. Regarding perfluorinated compounds, a higher temperature of the heat block led to a higher sensitivity with an optimum at 200 °C. These observations confirm the complexity of the optimization of multiresidue methods and the necessity of finding compromises.

3.2. Miniaturized Extraction

This study aimed to quantify micropollutants in the small invertebrate *Gammarus fossarum*. Given the limited mass of the matrix, it was necessary to miniaturize as much as possible the extraction.

3.2.1. Influence of salts and solvent

As mentioned in the *Introduction*, QuEChERS is based on the use of salts and solvents to induce phase separation and extract compounds of interest. This method was initially developed for 10 g of matrix,

whereas we aimed to use a sample mass around 100 times smaller. The two methods principally described in the literature, *i.e.* AOAC based on acetate buffer and European Norm (EN) based on citrate buffer, were considered as a starting point with 0.125 g of gammarids spiked at 100 ng.g⁻¹ and using 250 mg of salts. Very different LC-MS/MS responses were observed depending on the chemistry of the compounds (Figure 1). Citrate salts allowed a higher signal than acetate for perfluorinated compounds composed of a sulfonic acid group and for triphenyltin. Conversely, acetate buffer was better adapted for those including a carboxylic acid like PFTriA and PFHpA. Mercaptodimethur, quinoxifen, and tributyltin were better extracted with acetate salts. These differences may also be due to the proportions between the two salts, which are not the same: AOAC salts contain more MgSO₄, while EN salts contain NaCl in addition to citrate buffer salts. The choice of salts depends on both compounds and matrix [34]. For a majority of compounds (58%) citrate buffer enabled better responses and was therefore kept for further development.

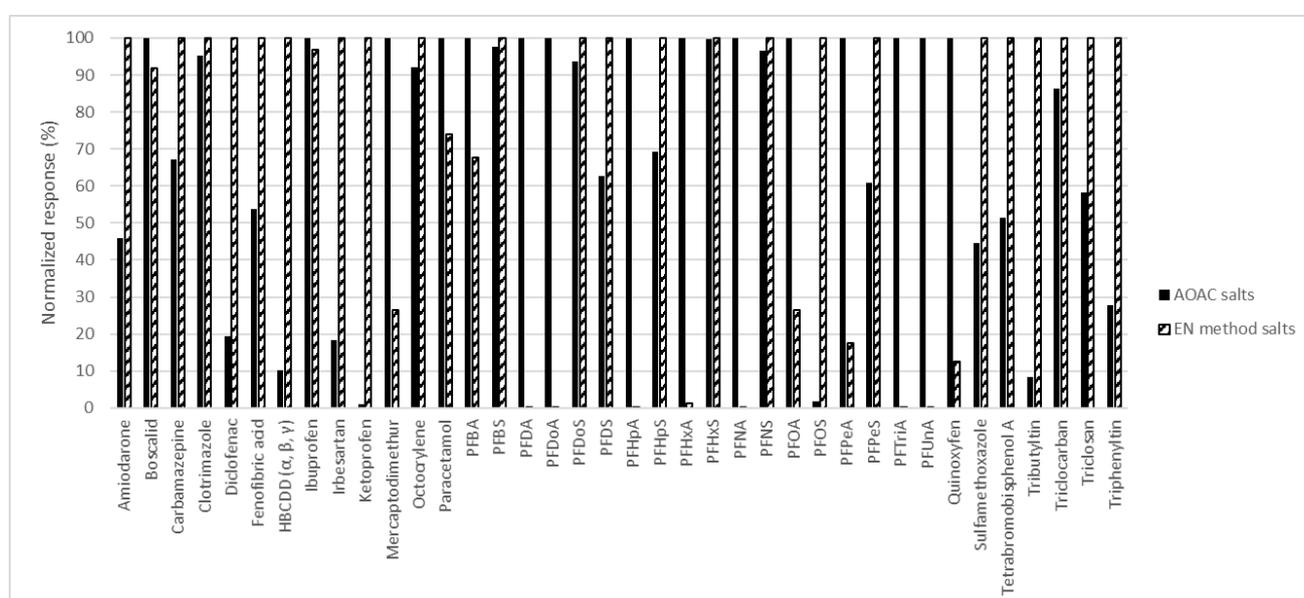


Figure 1. Comparison of normalized responses between the use of AOAC or EN method for QuEChERS salts.

Then, different amounts of QuEChERS salts were experienced. Firstly, the ratio of salt quantity to solvent volume was kept at 1, and two quantities of salts were compared: 500 mg and 250 mg. A ratio of 1 and the use of 250 mg of salts resulted in a better average response of the compounds (Figure 2) that corresponds to a lower volume of extraction: with 250 μL of solvent, the sample was concentrated by 2. Then the ratio was reduced to use 100 mg of salts. The volume of solvent was kept the same for two reasons: to vary only the amount of salts and to achieve better phase separation between water and acetonitrile. This ratio of 0.4 increased significantly compounds responses.

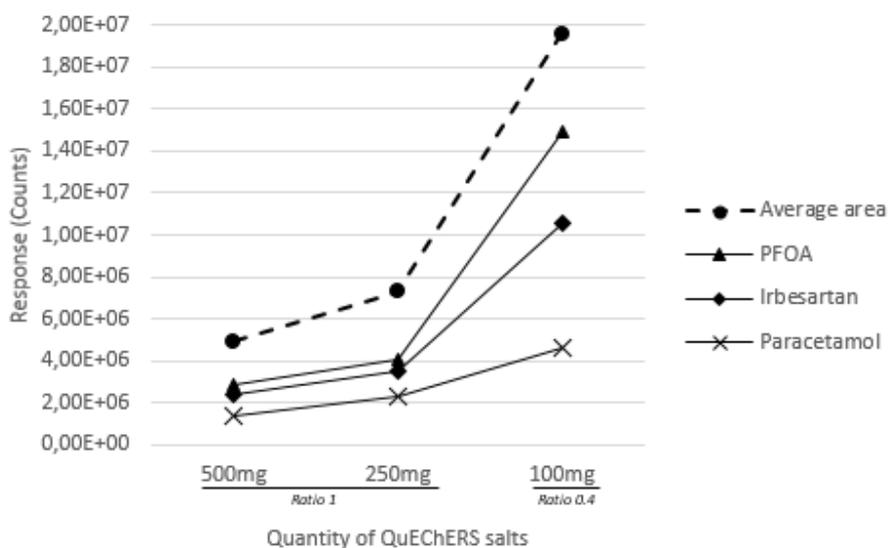


Figure 2. Influence of concentration and quantity of QuEChERS salts on compounds of interest.

The addition of salts can also influence ionization. Kucharski et al. [17] described the use of ammonium acetate to enhance ionization in ESI source because of its ease of vaporization, especially for TBT and TPhT. This application was investigated with 50 mg of $\text{NH}_4\text{CH}_3\text{COOH}$ but no significant differences were found for a majority of compounds (62%). Contrariwise, ammonium acetate enhanced response, especially for triphenyltin (Figure 3). Ammonium acetate was finally conserved.

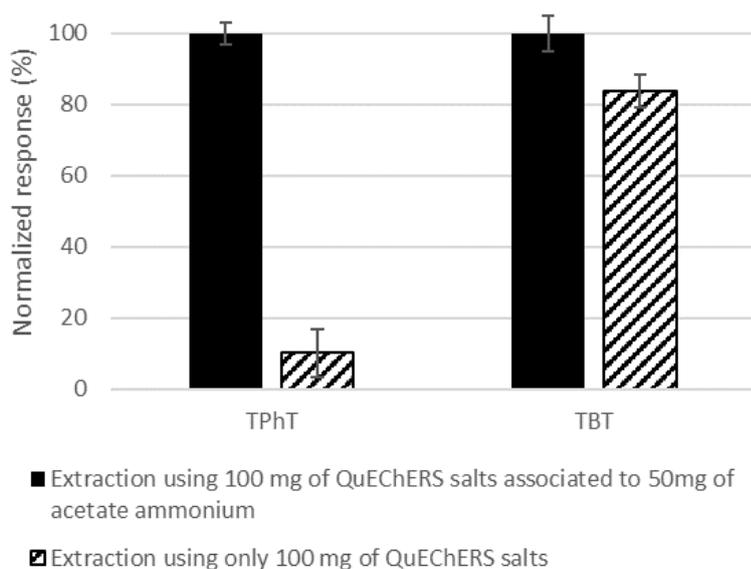


Figure 3. Influence of adding ammonium acetate with QuEChERS salts in the extraction step.

The choice of the appropriate extraction solvent is critical. In the QuEChERS method, MeCN is the solvent of choice. The addition of a non-polar organic solvent, such as hexane, can be beneficial during sample preparation of fatty matrices to remove lipids as reported in the literature [35]. Therefore, the benefit of adding 250 μ L of hexane to MeCN was evaluated. This volume of hexane was chosen to clearly observe a phase separation and maintain a ratio of 1 between the two solvents. The addition of hexane had a significant positive effect on the signal responses, allowing the areas to increase for 77% of the compounds, by reducing matrix effects. Perfluorinated compounds were the most affected with an area more than ten times higher. Hexane addition was maintained for further optimizations.

3.2.2. Purification step

G. fossarum is a complex fatty matrix and the use of an additional purification step can be necessary to remove interfering substances, even with the use of hexane during the extraction step. The multiplication of injections of the extract may cause column clogging, soiling of the mass spectrometer, and/or reduction of the lifetime of instruments. In addition, a complex matrix can introduce interferences that can cause quantification problems. It is therefore important to find a way to clean up the sample and reduce matrix effects.

After a first purification by hexane, a dispersive solid phase extraction (dSPE) was investigated. To make the sample clearer and to reduce matrix effects, Graphitized Carbon Black (GCB) can be used for its capability to capture pigments from the extract. A small amount of this sorbent is enough to be effective but this quantity is crucial to avoid a negative impact on planar compounds [36]. In this way, a study of the quantity of the GCB phase was carried out using gammarids spiked at 100 $\text{ng}\cdot\text{g}^{-1}$. One extraction without dSPE purification and two extractions using dSPE with different amounts of GCB were performed, all in triplicate. These two salts contained the same quantity of PSA (14 mg) and MgSO_4 (82 mg) but different amounts of GCB: 1.4 and 4 mg. These amounts of GCB were chosen to introduce as little phase as possible, thus reducing the impact on planar compounds, while attempting to make the purification of the extract efficient. Recoveries and matrix effects with these three extraction conditions were compared (Fig. 4 and 5, respectively).

Overall, recoveries were better with 4 mg of GCB since more than 60% of the compounds were better extracted in this condition. Ketoprofen and tetrabromobisphenol A were negatively affected by this sorbent because of their planar structure and recovery was better without dSPE purification. But in cases where recoveries were lower, matrix effects were still greatly reduced with this condition. For example, tributyltin recovery was below 10% but matrix effects were reduced by 97% with 4 mg of GCB. To conclude, dSPE was

efficient in reducing matrix effects. Similarly, the appearance of the sample was much clearer in this condition.

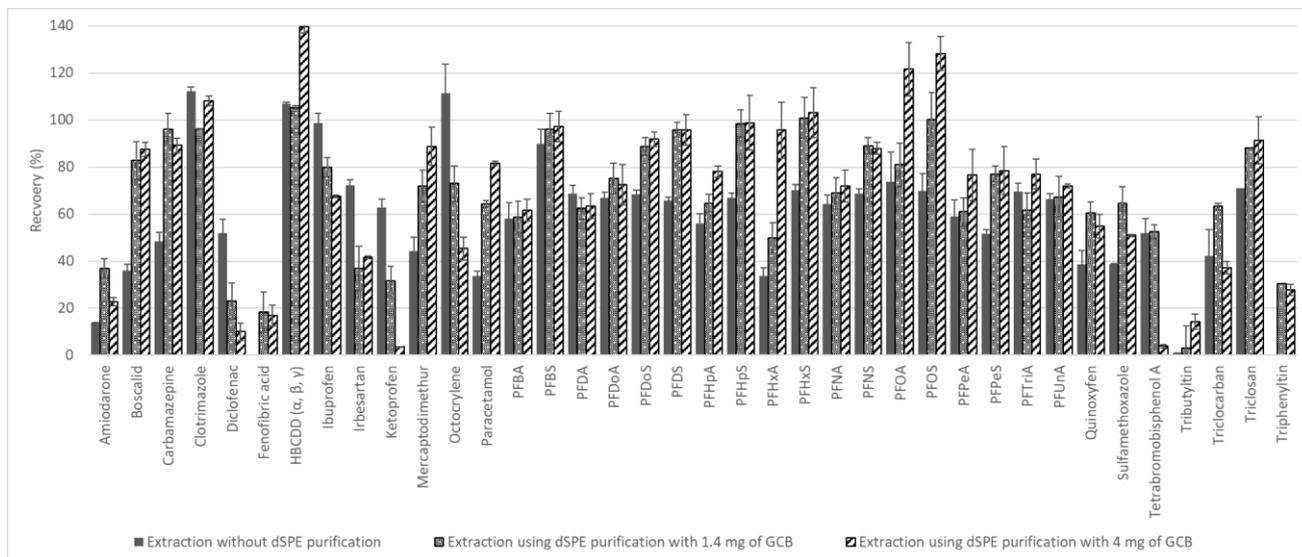


Figure 4. Impact of dSPE purification and the use of different quantity of GCB on extraction rates.

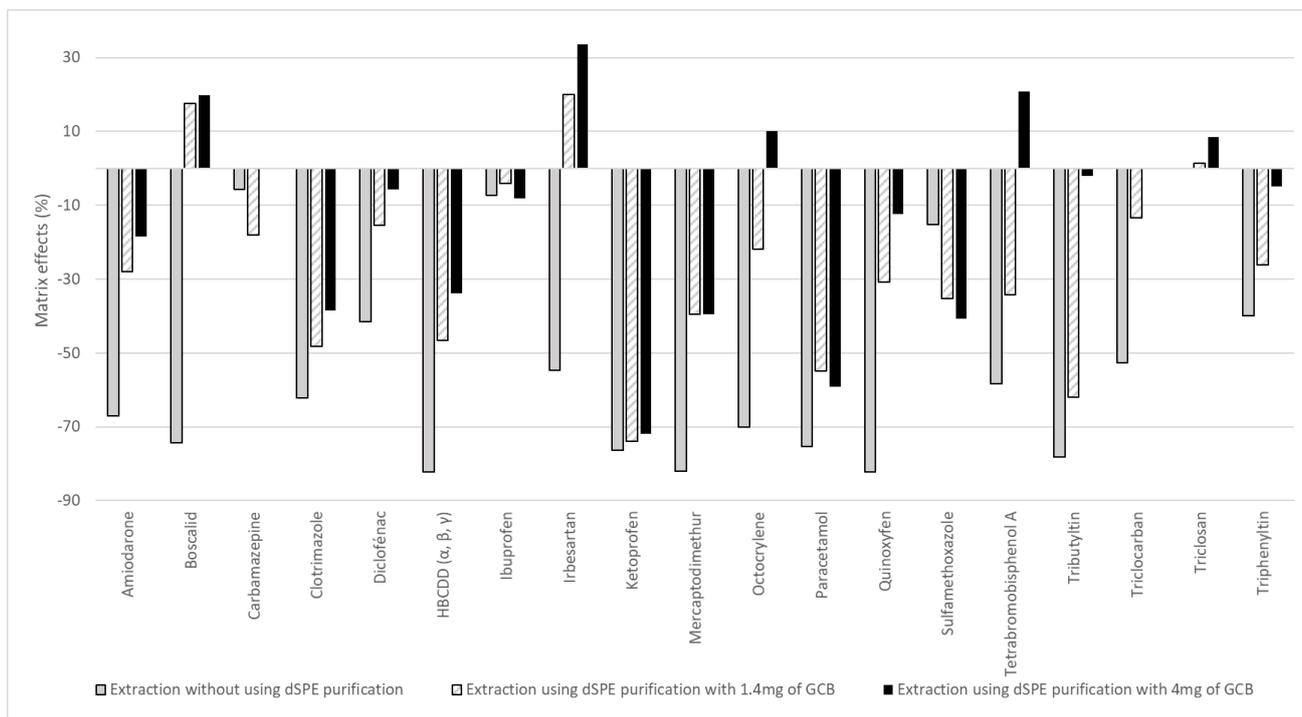


Figure 5. Impact of dSPE purification on matrix effects.

To further improve the extraction efficiency, acidification of the extraction solvent was investigated. Because of the matrix complexity, it may be possible that some compounds bond to the matrix by hydrophobic interactions. Adding formic acid in extraction solvent may induce acid hydrolysis and improve recovery for

substances by disrupting hydrophobic matrix-compounds interactions. Formic acid was added at 1% in MeCN leading to a pH of 4.5. Recoveries were globally better with formic acid, especially for PPCPs molecules (Figure S1). The difference was not significant for perfluorinated compounds, except for PFOA, for which acidification decreased recovery. Ketoprofen and tetrabromobisphenol recoveries increased to 45% and 70%, respectively. Diclofenac was also positively influenced by formic acid in the extraction with a recovery six times higher.

Finally, a recovery of less than 10% for tributyltin was observed. One explanation may be its presence in the hexane or in aqueous phase. The hypothesis that this molecule was in the hexane phase was investigated. Two extractions were carried out in parallel. For the first one, only MeCN was taken up after the dSPE step and put in a vial for analysis. For the second one, MeCN and hexane were taken up, evaporated to dryness, and reconstituted in the same volume as the first experiment. Taking into account the two phases (MeCN and hexane) led to an increase in recovery, especially for irbesartan and fenofibric acid. Tributyltin was also present in the hexane/MeCN phase but more moderately than the two previous molecules. The extraction rate was still below 20%, confirming that this molecule may remain trapped in the matrix. Nevertheless, as seen in *Section 3.2.1*, purification with hexane increased compound response by removing lipids. The addition of hexane was so kept in the protocol.

3.3. Method validation

Limits of quantification, dynamic range, accuracy (%), and intra-day and inter-day precision (RSD %) for all three levels are reported in Table 2. As contamination problems with PFAS were already reported, matrix blanks were periodically extracted and analyzed to ensure no contamination. LOQs ranged from 0.04 ng.g⁻¹ to 313.5 ng.g⁻¹, with a LOQ below 1 ng.g⁻¹ for 54% of the compounds. These results were coherent with the required limits in biota for PFOS and HBCDD, which are respectively 9.1 and 167 ng.g⁻¹ [10] and for which the limit of quantifications were 0.21 and 0.8 ng.g⁻¹. For the 40 compounds validated, inter- and intra-day RSDs were less than 20% and the accuracy was comprised between 80 and 120%, which is in line with the ICH guidelines.

Despite low recoveries for organotin compounds, the extraction and analysis proved to be repeatable and accurate. Due a to lack of data in *Gammarus fossarum*, it is not possible to directly compare the method developed with data from the literature. However, this development does not enable us to reach the limits of quantification currently achieved in GC-MS for the analysis of organotins in aquatic biota. For example in mussel tissues, with a sample mass of 200 mg, LOQ was 7.3 ng.g⁻¹ for TBT, using solid phase microextraction (SPME) followed by GC-MS [37]. Zhu et al. (2012) quantified TBT at 1 ng.g⁻¹ with LC-MS/MS and a sample

mass of 100 mg of frozen marine organisms [24]. It is important to note that the compared methods were specific for organotin. To reach these LOQs, the extraction protocol included a purification step with molecular imprinted polymers (MIPs) specific to the retention of organotin. Triphenyltin was validated in this work with LOQs of 41.6 ng.g⁻¹ which is in the same order of magnitude as a study in marine sediments that presents LOQs varying from 14 to 38 ng.g⁻¹ with a method dedicated to phenyltins by GC-MS [38,39]. LOQs obtained with this multi-residue method may not be as low as those of dedicated methods, but can still be used to detect levels in case of contamination.

Concerning PPCPs and PFAS, Dussault et al. [40] analyzed carbamazepine and triclosan in *Hyallolella Azteca*, a crustacean morphologically similar to gammarids. In their study, LOQs were 3.66 ng.g⁻¹ for carbamazepine and 26.65 ng.g⁻¹ for triclosan, well above the limits reached in this study in *G. fossarum*. In *Anguilla anguilla*, a LOQ of 7.6 ng.g⁻¹ was reached for paracetamol, a bit higher than this study but in the same order [23]. Berlioz-Barbier et al. (2014) also analyzed molecules in *Gammarus fossarum*, with nano-liquid chromatography coupled with MS [11]. Achieved LOQs were 0.5 ng.g⁻¹ for carbamazepine and 2.9 ng.g⁻¹ for PFOS and PFOA, which are five to ten times higher than in our study. In contrast, LOQs of 2.7 ng.g⁻¹ for diclofenac, 5 ng.g⁻¹ for ketoprofen, and 15.9 ng.g⁻¹ for ibuprofen were lower and can be explained by a better ionization in nanoLC.

Matrix effects are different according to the matrix, the ionization, or the efficiency of the extraction steps [41]. The evaluation of matrix effects is crucial to avoid quantification errors during validation and further analyses. Matrix effects can affect compounds negatively (lowering the signal) or positively (exalting). Twenty compounds were negatively impacted with matrix effects between -96% and -7% (Table 3). Most PPCPs were negatively impacted, except fenofibric acid, irbesartan, ketoprofen, and triclosan. Concerning organotins and hexabromocyclododecane, matrix effects led also to signal suppression. Conversely, 25 compounds were positively impacted with matrix effects between 1% and 497%. Perfluorinated compounds were all positively impacted. Whether the effect was positive or negative, matrix effects were relatively repeatable over the three different concentration levels.

3.4. Application to *In situ* experiments

The developed method was applied to 15 gammarid samples engaged during two different campaigns in May and November 2022. Thirteen samples were taken from rivers monitored by the Rhône-Méditerranée-Corse water agency and two samples were from upstream and downstream of industrial pharmaceutical sites.

A third of the molecules included in the current method were quantified at least once (Table 4). Among them, 70% were perfluorinated compounds, confirming the current concern about these pollutants [42].

PFDA was the most quantified compound. This molecule is used in industry as a fluorosurfactant [43]. Many other PFAS were quantified like PFOS, PFOA, PFNA, PFDoA, and PFUnA. PFOS was quantified at the highest concentration. Maximum values of 15.7 and 9.68 ng.g⁻¹ were found in both campaigns, exceeding the limit set in the biota of 9.1 ng.g⁻¹.

Boscalid is a fungicide particularly toxic for aquatic organisms [44]. It was quantified once in this study with a concentration of 0.07 ng.g⁻¹ in May, which corresponds to the agricultural field treatment season.

Some drug residues were also observed: carbamazepine, irbesartan, and amiodarone, which are respectively anticonvulsant, antihypertensive, and antiarrhythmic drugs. These results reflect drug consumptions used to treat common illnesses in France.

Octocrylene is a UVB solar filter principally found in cosmetics and solar creams. This molecule was not detected in May, but it was quantified with a frequency of almost 90% in November, with a maximum concentration of 5.98 ng.g⁻¹. Octocrylene detection is probably a consequence of the widespread consumption of UV-filtering sunscreens during summer, which induces its presence in rivers and then an accumulation in aquatic organisms. Looking at the samples placed before and after the pharmaceutical industry, only one molecule, octocrylene, was quantified after the industry. Octocrylene pollution is a known problem already reported in sediments, particularly in the Mediterranean coast [45].

4. Conclusion

This article presents the development of a multi-residue method to analyze micropollutants from various families in *Gammarus fossarum* using a single LC-MS/MS injection. One of the analytical challenges was to include organotins, usually analyzed by GC-MS.

As the mass of a gammarid is in the order of 20 mg, it was important to develop a miniaturized extraction method. Based on the QuEChERS method, the optimized extraction method comprised a salting-out liquid-liquid extraction with citrate salts and the addition of hexane followed by a GCB dSPE clean-up.

The full analytical method was validated for 40 compounds, with an accuracy between 80 and 120%. LOQs were from 0.04 to 230 ng.g⁻¹, which is consistent with environmental concentrations and environmental quality standards. To our knowledge, this is the first analytical method capable of analyzing so many molecules with different physicochemical properties in gammarids.

Finally, the method was applied to gammarids exposed in French rivers. Sixteen of the targeted molecules were quantified at concentrations between 0.04 and 15.7 ng.g⁻¹. Eleven of these compounds were perfluorinated and the remaining were PPCPs. This study confirms the importance of biomonitoring using sentinel species and highlights a large contamination by perfluorides and drug residues, reflecting consumption habits and industry waste disposal finally released in water.

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	Compounds	Ionisation	Quantifier	CE (V)	Quantitative	CE (V)	Retention time (min)
Pharmaceuticals and Personal Care Products, and Pesticides	<i>4-tert-octylphenol</i>	-	205.0>117.0	39	205.0>133.0	22	11.37
	Amiodarone	+	646.0>276.0	-39	646.0>572.9	-28	11.53
	<i>Bisphenol A</i>	-	227.0>212.0	18	227.0>133.2	25	9.00
	Boscalid	+	342.8>307.0	-19	342.8>272.1	-31	9.80
	Carbamazepine	+	237.3>194.0	-20	237.3>193.0 237.3>192.1	-35 -25	7.94
	Clotrimazole	+	277.0>165.0	-40	277.0>166.1 277.1>242.0	-25 -25	11.04
	Diclofenac	+	297.0>214.0	-40	297.0>216.0 297.0>180.1	-35 -55	10.33
	Fenofibric acid	+	318.9>233.0	-15	318.9>139.0 318.9>111.0	-30 -52	9.79
	Ibuprofen	-	205.3>161.1	10	205.3>162.1	11	11.16
	Irbesartan	+	429.5>207.0	-30	429.5>208.0 429.5>195.1	-25 -24	9.69
	Ketoprofen	+	255.2>209.2	-14	255.2>105.0 255.2>77.1	-22 -40	9.08
	Methiocarb (Mercaptodimethur)	+	226.0>169.0	-10	226.0>161.5	-16	9.69
	Octocrylene	+	362.3>250.0	-12	362.2>231.9	-24	11.99
	Paracetamol	+	152.0>93.0	-24	152.0>110.1 152.0>65.1	-20 -31	2.51
	Quinoxifen	+	309.5>198.9	-33	309.5>215.7	-35	11.70
	Sulfamethoxazole	+	254.3>92.0	-30	254.3>65.0 254.3>108.0	-37 -25	3.24
	Tetrabromobisphenol A	-	542.7>417.7	42	542.7>447.8 542.7>445.8	32 33	11.27
	Triclocarban	+	316.6>127.0	-33	316.6>93.0 316.6>164.0	-37 -20	11.17
Triclosan	-	286.6>35.1	10	288.5>35.0 288.8>35.1	10 9	11.24	
Perfluorinated compounds	PFBA	-	213.0>169.1	11			4.12
	PFBS	-	298.9>80.0	34	298.9>99.0	28	6.27
	PFDA	-	513.0>469.0	12	513.0>219.0	19	10.64
	PFDoA	-	613.0>569.0	13	613.0>169.1	26	11.13
	PFDoS	-	698.9>80.0	55	698.9>98.9 698.9>230	53 49	11.28
	PFDS	-	599.0>80.0	55	599.0>98.9	49	10.84
	PFHpA	-	363.0>319.0	11	363.0>169.1	18	9.42
	PFHpS	-	449.0>80.0	48	449.0>98.9	37	9.88
	PFHxA	-	313.1>269.1	10	313.1>119.0	21	8.52
	PFHxS	-	399.0>80.0	45	399.0>99.0	35	9.38
	PFNA	-	463.0>419.0	12	463.0>219.1	18	10.32
	PFNS	-	549.0>80.0	55	549.0>99.0	45	10.57
	PFOA	-	413.0>369.0	12	413.0>169.1	19	9.94
	PFOS	-	499.0>80.0	55	499.0>98.8	39	10.26
	PFPeA	-	262.9>219.0	9			6.02
	PFPeS	-	349.0>80.0	39	349.0>98.9	33	8.57
	PFTrDA	-	663.0>619.0	14	663.0>169.1	29	11.35
	PFUdA C13	-	565.0>520.0	12			10.89
PFUdA	-	563.0>519.0	12	563.0>269.1	19	10.90	
Organotins	Monobutyltin	+	563.0>519.0	12	563.0>269.1	19	10.90
	Dibutyltin	+	267.1>179.0	-12	267.1>123.0	-25	11.16
	Tributyltin	+	324.6>179.5	-20	324.6>125.1	-32	11.16
	Tetrabutyltin	+	291.1>235.1	-9	291.1>179.0 291.1>123.0	-14 -27	11.12
	Triphenyltin	+	331.6>290.6	-7	331.5>234.5	-12	11.14
	Diphenyltin	+	350.6>196.9	-31	350.4>119.4	-59	10.20
	Monophenyltin	+	382.2>350.5	-13	382.2>196.5 382.2>119.4	-33 -67	10.18
Flame retardant	Hexabromocyclododecane (α , β , γ)	-	640.8>79.0	21	640.8>80.9	15	12.38

Table 1. Chromatographic and mass spectrometry parameters for compounds of interest (*in italics, compounds with low sensitivity*).

	Compounds	Dynamic range	LOQ (ng.g ⁻¹ ww)	Low level			Middle level			High level		
				Accuracy	Intraday precision (RSD %)	Interday precision (RSD %)	Accuracy	Intraday precision (RSD %)	Interday precision (RSD %)	Accuracy	Intraday precision (RSD %)	Interday precision (RSD %)
Pharmaceuticals and Personal Care Products, and Pesticides	Amiodarone	LOQ to 50LOQ	2.0	97.2	5.2	12.2	94.4	8.1	1.3	84.8	3.0	8.2
	Boscalid		0.4	85.6	1.8	9.7	110.3	1.5	1.6	102.0	1.4	2.2
	Carbamazepine		0.1	113.3	5.1	6.5	98.7	1.6	4.2	83.5	1.1	8.4
	Clotrimazole		0.1	103.3	5.6	18.0	104.0	2.5	1.1	106.3	1.7	4.7
	Diclofenac		100.0	115.4	2.7	2.8	101.3	0.3	1.1	77.0	1.3	1.6
	Fenofibric acid		20.0	119.0	2.7	14.4	81.4	5.5	2.0	94.2	3.1	17.3
	Ibuprofen		20.0	109.7	16.3	1.9	107.5	8.7	5.4	116.6	9.0	9.7
	Irbesartan		1.0	112.7	4.2	15.3	95.1	2.4	4.9	91.4	0.9	14.1
	Ketoprofen		12.5	81.0	7.6	24.7	107.0	4.7	2.4	96.1	5.6	17.7
	Methiocarb		0.3	100.0	3.7	5.9	101.6	1.5	1.0	82.8	1.2	8.0
	Octocrylene		0.3	102.6	9.4	0.9	83.1	11.0	11.9	99.0	2.8	3.8
	Paracetamol		5.3	82.7	4.4	6.0	93.5	2.9	1.4	96.8	1.0	6.1
	Quinoxifen		0.4	80.2	15.9	26.0	105.4	8.1	3.7	88.0	3.9	15.1
	Sulfamethoxazole		2.0	112.2	2.2	13.0	95.0	2.9	4.8	81.8	1.9	1.1
	Tetrabromobisphenol A		10.0	93.7	6.3	10.6	105.9	6.8	8.4	82.3	2.8	12.3
	Triclocarban		10.0	109.2	9.2	6.8	102.7	4.5	1.9	101.9	6.7	5.2
Triclosan	10.0	83.8	9.8	13.2	110.4	4.5	5.5	81.9	2.1	15.8		
Perfluorinated compounds	PFBA	LOQ to 50LOQ	0.9	80.6	5.3	2.2	89.6	3.3	11.3	92.8	2.1	16.5
	PFBS		1.3	85.1	10.1	3.3	119.3	4.5	0.9	95.0	8.6	11.3
	PFDA		0.04	108.3	13.3	5.2	101.5	3.4	2.1	117.9	1.7	2.6
	PFDoA		0.3	90.0	14.8	8.3	96.0	11.6	0.6	86.7	4.1	13.4
	PFDoS		0.5	84.0	4.8	2.9	104.2	3.2	3.6	97.3	4.8	5.0
	PFDS		0.2	93.0	14.2	8.5	104.1	4.3	6.9	99.7	4.3	3.2
	PFHpA		0.6	112.5	8.2	4.0	104.3	3.1	2.0	117.9	2.4	5.5
	PFHpS		0.09	92.6	6.9	0.0	89.2	2.0	4.5	92.2	9.5	3.7
	PFHxA		0.5	92.2	9.6	3.3	105.7	1.5	1.1	93.9	5.1	14.5
	PFHxS		0.2	98.4	2.8	12.4	89.7	5.4	5.0	88.6	8.1	10.8
	PFNA		0.07	81.0	10.2	14.9	115.7	3.3	2.7	118.1	4.0	3.0
	PFNS		0.1	87.2	13.5	6.5	100.3	6.8	4.0	93.7	5.0	1.7
	PFOA		0.3	96.9	11.2	1.5	113.4	3.6	2.7	96.0	2.5	2.3
	PFOS		0.2	88.9	11.2	5.2	107.0	2.5	2.5	98.9	3.0	1.4
	PFPeA		1.0	83.8	6.8	4.2	80.7	2.7	1.1	89.1	6.9	8.2
	PFPeS		0.5	100.7	4.9	15.3	109.8	10.1	6.5	97.7	7.3	1.3
PFTriA	0.2	81.7	3.5	4.2	85.4	3.4	5.6	85.9	1.2	16.2		
PFUnA	0.2	88.3	14.2	0.0	106.8	1.2	0.7	105.1	1.8	7.9		
Organotin	TBT	LOQ to 40LOQ	229.9	95.7	25.9	16.9	104.1	7.3	19.4	106.2	5.7	7.9
	TPHT	LOQ to 50LOQ	41.6	92.3	8.9	9.0	108.3	5.8	3.3	105.5	5.3	3.0
Flame retardant	Hexabromocyclododecane (sum of α , β , γ)	LOQ to 50LOQ	0.8	106.3	4.7	3.0	101.0	5.6	10.6	84.1	2.3	14.9

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Table 2. Dynamic range, LOQ and for each level: accuracy (%), intra- and inter-day precision (%).

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4

	Compounds	Matrix effects (%)		
		Low level	Middle level	High level
Pharmaceuticals and Personal Care Products, and Pesticides	Amiodarone	-42	-54	-41
	Boscalid	-9	-24	-5
	Carbamazepine	9	-9	6
	Clotrimazole	-13	-33	-27
	Diclofenac	-59	-33	-6
	Fenofibric acid	27	12	16
	Ibuprofen	168	87	59
	Irbesartan	169	147	125
	Ketoprofen	16	33	44
	Methiocarb	1	-26	-15
	Octocrylene	-37	-61	-55
	Paracétamol	-69	-75	-67
	Quinoxifen	-55	-63	-55
	Sulfamethoxazole	-28	-27	-7
	Tetrabromobisphenol A	-55	-51	-32
Triclocarban	-21	-55	-52	
Triclosan	118	67	71	
Perfluorinated compounds	PFBA	55	55	75
	PFBS	54	57	92
	PFDA	136	3	1
	PFDoA	196	169	210
	PFDoS	39	70	91
	PFDS	122	50	48
	PFHpA	84	64	60
	PFHpS	81	25	58
	PFHxA	95	83	95
	PFHxS	56	7	21
	PFNA	497	226	139
	PFNS	84	61	69
	PFOA	181	45	60
	PFOS	152	32	31
	PFPeA	71	114	124
	PFPeS	36	23	40
PFTriA	88	85	84	
PFUnA	57	72	88	
Organotin	TBT	78	68	137
	TPhT	-40	-68	-55
Flame retardant	Hexabromocyclododecane (sum of α , β , γ)	-54	-60	-45

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Table 3. Matrix effects at the three levels of concentration.

	May 2022 (n = 4)			November 2022 (n = 9)			Upstream	Downstream
	Quantification rate (%)	Concentration (ng.g ⁻¹)		Quantification rate (%)	Concentration (ng.g ⁻¹)			
		Min	Max		Min	Max		
Amiodarone	N.D.	N.D.	N.D.	11	2.9	2.9	N.D.	N.D.
Boscalid	25	0.7	0.7	N.D.	N.D.	N.D.	N.D.	N.D.
Carbamazepine	50	0.4	0.9	44	0.1	0.7	N.D.	N.D.
Irbesartan	N.D.	N.D.	N.D.	33	1.0	1.9	N.D.	N.D.
Octocrylene	N.D.	N.D.	N.D.	89	0.7	5.9	N.D.	0.72
PFBA	N.D.	N.D.	N.D.	11	0.9	0.9	N.D.	N.D.
PFDA	100	1.7	4.0	100	0.04	0.8	N.D.	N.D.
PFDoA	100	1.0	3.5	78	0.3	1.5	N.D.	N.D.
PFHpA	25	0.1	0.1	N.D.	N.D.	N.D.	N.D.	N.D.
PFHpS	50	0.2	0.2	0	N.D.	N.D.	N.D.	N.D.
PFHxS	75	0.3	0.7	0	N.D.	N.D.	N.D.	N.D.
PFNA	100	0.2	1.7	78	0.1	1.4	N.D.	N.D.
PFOA	100	1.6	2.7	78	0.9	3.2	N.D.	N.D.
PFOS	100	3.8	15.7	89	0.3	9.7	N.D.	N.D.
PFTrIA	75	0.2	1.1	11	0.2	0.2	N.D.	N.D.
PFUnA	100	0.3	1.1	56	0.2	0.6	N.D.	N.D.

7

8 Table 4. Summary of quantification rates in % and concentrations of targeted compounds in ng.g⁻¹ (N.D.: Not
9 Detected) in gammarids sampled in rivers in May and November 2022 and upstream and downstream of
10 industrial pharmaceutical sites