Multi-residue extraction to determine organic pollutants in mussel haemolymph

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Abbreviations:

4-MeO-PCP	4-methoxyphencyclidine
A-3	ACN extraction 3:1 ACN:haemolymph
A-4.5	ACN extraction 4.5:1 ACN:haemolymph
E%	Efficiency
EP	Emerging pollutant
Inter-R	Reproducibility
Intra-R	Repeatability
ME	Matrix effect
MeOH	Methanol
M-4	Methanol extraction 4:1 MeOH:haemolymph
M-6	Methanol extraction 6:1 MeOH:haemolymph
MRM	Multiple reaction monitoring mode
PFAS	Perfluoroalkyl substance
PBS	Perfluorobutanesulfonate
PFDA	Perfluorodecanoic acid
PFOS	Perfluorooctanesulfonate
PFOA	Perfluorooctanoic acid
PFPeA	Perfluoropentanoic acid
PCP	Personal care product
R%	Absolute recoveries
RR%	Relative recoveries

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Abstract

This study assesses the extraction of 11 pharmaceuticals, 5 pesticides, 5 perfluoroalkyl substances and 2 illicit drugs in haemolymph from *(Mytilus Galloprovincialis*). Four extraction procedures using Phree™ Phospholipid Removal cartridges were tested using different volumes of methanol (400 µl and 600 µl) and acetonitrile (300 µl and 450 µl). The pollutants were determined by HPLC-MS/MS. The use of methanol gave several problems during the extraction procedure, such as longer times and sample loss. Three methods (acetonitrile 300 µl and 450 µl; and methanol 600 µl) were validated. Recoveries at three concentration levels (5, 50 and 100 ng/mL) ranged 35.1-129.0% and 29.3-133.0% for acetonitrile 300 µl and 450 µl, respectively, while recoveries for methanol 600 µl ranged 52.2-166.0%. Limits of detection were <10 ng/mL for most analytes using any of the methods. Methanol 600 µl was the only method capable to extract the illicit drug 4-MeO-PCP and provided better peak shape and higher signal-noise ratio. When applied to non-spiked samples from local markets salicylic acid and diclofenac were detected at 33.50-97.79 ng/mL and 28.30-30.31 ng/mL respectively. Up to our knowledge, there are no method to determine organic contaminants in haemolymph and this is the first application of Phree™ cartridges for mussel haemolymph extraction.

Keywords: assessment, illicit drugs, pesticides, perfluoroalkyl substances, pharmaceuticals, solid phase extraction.

1. INTRODUCTION

Emerging pollutants (EPs) are toxic, persistent and ubiquitous in the aquatic environment, because of uncontrolled discharges, wastewater treatment plant effluents and/or the environmental transformation of several precursors into EPs [1-3]. Due to their persistence and/or continuous release, aquatic biota is long term exposed to these bioaccumulable and biomagnificable compounds [4, 5]. Exposure of biota occurs via water, sediment, suspended solids, or the intake of other biota (food chains) and/or microplastics as reported in different studies [6], several adverse effects have been confirmed [7]. The occurrence of EPs has been reported in mussel [8], eel [6], seafood and other fish [9] used for human consumption. The health risk from eating this kind of food has been estimated in several studies [10, 11].

Biota studies analysed the species as a whole [12] or divided into their different organs and tissues [13, 14]. This latter offers additional information on absorption, distribution, metabolism, target organs, accumulation and excretion. The presence and concentrations of EPs in fish tissues and filter-feeding organisms [15] still need further study since they play a vital role in biodiversity and water depuration.

Multi-residue extraction methods have been developed to save resources and time by extracting the greatest possible variety of compounds in order to provide a whole picture of the EPs present in the sample, even if they are from different families [4]. However, the complexity of biota matrices constrains the development of such methods, since they involve the challenge of extracting pollutants with a wide polarity range, obtaining clean extracts and good recoveries. The universal method for tissues is solvent extraction where the compounds are extracted by adding an organic solvent and applying energy (i.e. manual agitation, ultrasound, pressure, temperature, microwave or vacuum). For aqueous matrices and for the clean-up of the tissue extracts the most used approach is solid phase

extraction (SPE). Different cartridges have been reported depending on the target compounds, but the common are the HLB (Hydrophilic-Lipophilic-Balanced) [14].

SPE clean-up has been used in methods screening pharmaceuticals [14], personal care product (PCPs) [16], perfluoroalkyl substances (PFASs) [9], organophosphate flame retardants [17] or illicit drugs [18] in biota matrices. This makes SPE a clean-up suitable for multi-residue extraction procedures but sometimes, SPE sorbents fail to eliminate interferences from the matrix. The last trend within this field is the development of specific sorbent variations, like the Phree™ and Captiva ND (to separate phospholipids and proteins that favour the elimination of lipids) [19], Ostro™ (to eliminate phospholipids and proteins) [20] or EMR- Lipids (to remove lipids). The application of these new sorbents is still incipient given their recent placement on the market, even though they offer a very promising solution to problems as the high content of lipids in biota matrices. Phree™ cartridges have been tested in the extraction of blood and/or human serum [21], and recently as a clean-up of fish muscle [22] and mussels showing promising results in liquid samples.

Among the bivalve mollusks, mussels have been used as sentinel organisms in biomonitoring programs, such as Med Pol, UNEP Mediterranean Biomonitoring Program or the OSPAR Convention [23, 24]. The haemolymph of these bivalve mollusks (the invertebrate equivalent of mammalian blood) is an attractive bio-fluid in contact with different tissues to assess biomarker responses to contamination, because it can report about the functional status of the organs which are perfused but lacks the molecular complexity of whole organ tissues [25]. Mussel haemolymph has a very variable proportion of minerals, proteins and cells depending on the tissue and the specimen [26, 27], which make it a complex matrix. However, up to our knowledge, analytical methods to determine contaminants a haemolymph have not been reported yet.

The aim of this study was the development and validation of a method based on the use of the application of Phree™ cartridges followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) to determine 11 pharmaceuticals, 5 pesticides, 5 perfluoroalkyl substances and 2 illicit drugs in haemolymph from *(M. Galloprovincialis*). Then, this method was successfully employed for evaluating the presence of these contaminants in haemolymph of several commercial samples and of mussels that were exposed to several emerging contaminants. This study improves our knowledge of the presence, distribution and biodegradation of EPs in aquatic biota, which has particular importance for the environment and the human population.

1. MATERIAL AND METHODS

2.1 Reagents and materials

The LC grade methanol (MeOH) and acetonitrile (ACN) of a purity ≥ 99.8%, were from VWR Chemicals® (Radnor, Pennsylvania). Formic acid (CH_2O_2) was provided by ACROS ORGANICS (Geel, Belgium). Ammonium formate (NH_4HCO_2) was from Alfa Aesar (Karlsruhe, Germany).

Phree™ Phospholipid Removal Solutions 1 mL tubes were from Phenomenex® (Torrance, CA, USA). The 1 mL polypropylene syringes BD Plastipak™ and the needles 25G x 5/8" 0.5x16 mm BD Microlance™ were from BD (Madrid, Spain). The VISIPREP™ manifold was distributed by Supelco. High purity water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA). The 15 mL polypropylene centrifuge falcon tubes were from VWR International Eurolab (Barcelona, Spain). The 2 mL amber glass vials with stoppers 99 mm + Septum Sil/PTFE used to inject the samples were from Análisis Vínicos S.L. (Tomelloso, Spain), and the 250 µL polypropylene inserts were from Agilent Technologies (Santa Clara, CA, USA). The

105 STUART Sample Concentrator SBHCONC/1 with a STUART Block Heater SBH200D/3
106 set to 39 ºC was from Stuart® (Staffordshire, UK).

107 The analytical standards of pharmaceuticals (acetaminophen, atenolol, caffeine,
108 diclofenac, etoricoxib, ibuprofen, metformin, naproxen, salicylic acid, triclosan,
109 vildagliptin), pesticides (bentazone, chlorfenvinphos, chlorpyrifos, imazalil,
110 terbutylazine) and PFASs [perfluoropentanoic acid (PFPeA) and
111 perfluorobutanesulfonate (PFBS)] were from Sigma-Aldrich (Steinheim, Germany).
112 While perfluorooctanoic acid (PFOA), perfluorodecanoic acid (PFDA) and
113 perfluorooctanesulfonate (PFOS) were from Wellington (Ontario, Canada). Finally, illicit
114 drugs: bufotenine and 4-methoxyphencyclidine (4-MeO-PCP) were from LGC Standards
115 (Ontario, Canada).

116 The surrogate (internal) standards acetaminophen-d3, atenolol-d7 and ibuprofen-d3 were
117 from Sigma Aldrich. Caffeine-d9, chlorfenvinphos-d10 (diethyl D5), chlorpyrifos-d10
118 (diethyl D10) and vildagliptin-d3 were from LGC Standards. Diclofenac-d4 and
119 triclosan-d3 were purchased in Toronto Chemicals Research (Toronto Canada). And
120 PFOA-d4 (MPFOA), PFOS-d4 (MPFOS) and PFDA-d4 (MPFDA) were from
121 Wellington.

122 **2.2. Sampling**

123 The sample analysed was haemolymph from Mediterranean mussels (*M.*
124 *galloprovincialis*) harvested in the Mediterranean Sea next to the city of Valencia, Spain.
125 These mussels —cultivated using raft can be in contact with anthropogenic contaminants
126 due to the proximity of Valencia city— are available just between March-July and are an
127 emblematic ingredient for the local gastronomy, commonly known as “clótxinas”.
128 Mussels were purchased from three different local markets and processed when they were

still alive, the shells were filed with a steel file next to the posterior adductor muscle until opening a hole big enough to introduce the syringe needle. Then the haemolymph was extracted directly from the posterior adductor mussel using a 1 mL syringe. Haemolymph composition is different depending on the tissue and it is not clear where the fluids come from when it is extracted from the posterior adductor mussel. However, Eggermont et al. [27] suggest that this haemolymph could be from small spaces and fissures between the muscle fibres that are connected to the posterior gastro-intestinal artery. The volume extracted was between 0.2-0.5 mL depending on the specimen. For the method optimization, haemolymph from the three different markets was pooled. Then haemolymph from mussels of the same supermarket was collected and stored separately in order to test the selected method in real non-spiked samples. All the samples were stored in 15 mL falcon tubes and frozen at -20 ºC until analysis.

2.3. Sample extraction

The Phree™ cartridges were placed in a vacuum manifold, and loaded with 100 µL of haemolymph. A solution of ACN 1% formic acid spiked with the IS (300 µL) was added directly in the sample placed in the cartridge (the so called direct addition). This step is crucial to ensure a proper mixing and complete precipitation [28]. Addition of the solvent sliding down the walls of the cartridge would not provide satisfactory results. After 2 min to assure complete precipitation (Figure S-1), vacuum (254-381 mmHg) was applied to elute the remaining mix of solvent and haemolymph dropwise in 15 mL falcon tubes. The extracts were stored in vials with 250 µL polypropylene (PP) inserts and frozen at -20 ºC until analysis. Four variations of this procedure were tested.

Two procedures employed ACN 1% formic acid as solvent. In this case, the procedures were exactly as described above but one employed 300 µL (A-3) of solvent and the other 450 µL (A-4.5), obtaining a final proportion haemolymph:ACN of 1:3 and 1:4.5,

154 respectively. The concentration of the IS in the solvent was adjusted for each method,
155 ensuring a final concentration of 20 ng/mL in the final extracts (assuming recovery of the
156 100%). The other two procedures employed MeOH 1% formic acid as solvent. The
157 procedure was very similar as the described above, but in this case, the vacuum pressure
158 applied during the SPE was higher (508-635 mmHg) and amount of solvent employed
159 was 400 µL in M-4 and 600 µL in M-6 obtaining a proportion haemolymph:MeOH in the
160 extracts of 1:4 and 1:6, respectively. With both AcN and MeOH, the lowest amount of
161 solvent corresponds to that recommended by the manufacturers, and as they indicate that
162 a higher proportion of solvents can improve sometimes results, solvents with 50% more
163 organic component were tested.

164 **2.4. LC-MS/MS analysis**

165 Analysis was performed via LC-MS/MS, using an Agilent 1260 UHPLC from Agilent
166 technologies coupled to an Agilent 6410 Mass Spectrometer QQQ also from Agilent
167 technologies, with electrospray ionization (ESI) in both negative and positive ionization
168 modes (nebulizer gas 15 psi, gas flow 11 L/min. ion-spray voltage 4 kV and temperature
169 300ºC) operated in multiple reaction monitoring mode (MRM). The column used for the
170 detection pesticides and etoricoxib was Luna® 3µm C18(2) 100 Å 150x2 mm and the
171 column employed for PFAS, illicit drugs and the rest of PPCPs was a Kinetex 1.7µm XB-
172 C18 100 Å 50x2.1 mm, both from Phenomenex. Yielding a total of three LC methods,
173 one in negative ionization mode using Kinetecs column and two in positive mode using
174 Kinetecs and Luna® columns respectively. When operated in positive ionization mode,
175 the mobile phases employed were (A) H_2O 0.1% formic acid and (B) MeOH 0.1% formic
176 acid. For negative ionization mode, the mobile phases employed were (A) H_2O 2.5 mM
177 NH_4F and (B) MeOH 2.5 mM NH_4F. The linear gradient was as follows: 0 min (70% A),
178 12 min (5% A), 25 min (5% A), 26 min (70% A) and 30 min (70% A) either in positive

or negative ionization mode (only the mobile phases were different). The injection volume was 5 µl and column temperature 30 ºC. MS detailed information is available in **Tables S-1 and S-2**.

2.5 Methods validation

Every batch of samples extracted included a procedural blank (non-spiked haemolymph pool). At the beginning and at the end of each analytical sequence, a seven points calibration standard set (5, 10, 25, 50, 100, 200 and 500 ng/mL) was injected. This calibration was prepared in MeOH:MilliQ 4:1 or ACN:MilliQ 3:1 depending on the solvent used to extract the samples, respectively. A 100 ng/mL spiked sample extracts was also injected every 15 samples to check possible instrumental variation. Only regression coefficients (R^2) >0.99 were accepted in the calibration curve.

Recoveries were calculated in haemolymph fortified at three different concentrations: 5, 50 and 100 ng/mL in triplicate. After LC-MS/MS, recoveries were calculated comparing the peak area of the spiked samples with the area of the 7 points of the calibration curve. For the compounds acetaminophen, atenolol, caffeine, chlorfenvinphos, chlorpyrifos, diclofenac, ibuprofen PFDA, PFOA, PFOS, triclosan and vildagliptin, the results obtained were relative recoveries (RR%) where the matrix effect (ME), and other potential inaccuracy during sample handling, were corrected using the internal standards. The other compounds were quantified with external calibration. Hence, the results were represented as efficiency (E%), if the results are affected by either recovery and matrix effects, or absolute recoveries (R%), if the matrix effect is corrected using matrix-matched standards. Both were calculated following Eq. 1:

$$RR\%\ or\ E\% = \left(\frac{Final\ concentration\ of\ the\ spiked\ sample}{EC}\right) \cdot 100 \qquad (1)$$

Where *EC* is the expected concentration in the final extract assuming a recovery of 100 %.

For the determination of ME, a batch of ACN or MeOH (depending on the extraction solvent) with the compounds mix at the same concentration as the calibration curve (5, 10, 25, 50, 100, 200 and 500 ng/mL) was prepared. For each concentration, 300 µL of its mix was placed in 15 mL falcon tubes, and blown down to dryness under a gentle stream of nitrogen, then, 300 µL of haemolymph extract were added to the falcon tubes. This extract was then vortexed 30 s, sonicated 3 min and injected. A procedural blank was also included in each batch. After LC-MS/MS analysis using external calibration, ME was calculated comparing the slope of the calibration curve in matrix and the slope of the calibration curve in CAN [19] (Eq. 2).

$$ME = \left(\frac{Slope\ of\ calibration\ curve\ in\ matrix}{Slope\ of\ calibration\ curve\ in\ solvent}\right) \cdot 100 - 100 \quad (2)$$

The E% of the compounds without internal standard was corrected using the ME to obtain R% using Eq. 3.

$$R\% = \frac{EE\% \cdot (100 - ME)}{100} \quad (3)$$

Sensitivity was established as method limits of detection (LODs) and method limits of quantification (LOQs) (Table 1) by analysing the extractions fortified at 5 ng/mL used for the recoveries described above. The extracts (performed in triplicate) were injected in duplicate (n=6). LODs were set as three times the standard deviation (SD) of their signal and LOQs were set as 10 times the SD. Precision was evaluated in terms of repeatability (Intra-R) and reproducibility (Inter-R). Intra-R was calculated as the SD of the signal

223 divided by its mean (% RSDs) of the six injections used for the determination of LODs
224 and LOQs injected in a row. Inter-R was determined injecting one replicate of the extracts
225 fortified at 50 ng/mL also used for the recoveries described above in three different days
226 (n=3). Then Inter-R was also calculated as the SD of the signal divided by its mean (%
227 RSDs).

228 ## 3. RESULTS AND DISCUSSION

229 ### 3.1. Extraction procedure and analysis considerations

230 The solvents (ACN and MeOH) employed for the extraction were those recommended
231 by the manufacturer, who also recommended vacuum negative pressure ranges of 127-
232 254 and 381-508 mmHg for ACN and MeOH, respectively, even though it is also
233 suggested that higher pressures may be required [28]. Vacuum pressures of 254-381 and
234 508-635 mmHg for ACN and MeOH, respectively, were needed in the present work.

235 The manufacturer did not specify the time required for precipitation when using SPE
236 cartridges. After 30 s of adding the solvent the precipitation was apparently complete
237 (Figure S-1). However, since the manufacturer recommends 2 min for complete
238 precipitation when using Phree™ in 96-well plate format [28], which have higher bed
239 volume. Then, the cartridges were left 2 min to ensure complete precipitation.

240 When employing MeOH as solvent, the cartridge elution was very slow even using the
241 highest pressures that the manifold achieves (close to 762 mmHg). Due to the volatility
242 of the solvent and the high vacuum used, the sample is below vapour pressure of the
243 solvent and this favour solvent evaporation during the procedure, achieving low volumes
244 of extract (around 100-150 µL and 200-250 µL for M-4 and M-6 respectively).
245 Occasionally, it was not possible to pass or percolate the samples through the cartridges
246 and/or the process was such slow that obtaining extracts was not possible because of the

complete solvent evaporation. This was especially problematic with M-4, where no extract was obtained in the 50% of the attempts (n=10) and low volumes were obtained with the other attempts. For this reason, M-4 was discarded as a valid extraction procedure.

Recovery tests at 100 µg/L for M-4 commonly showed recoveries higher than 100%. This was probably due to the evaporation of solvent during the extraction and subsequent concentration of analytes in the extract. This was not so marked when using M-6.

Regarding LC-MS/MS analysis, the signal provided by the compounds was generally enhanced when MeOH was used in the analysis. **Figure S1** shows the chromatograms of the 500 ng/mL calibration point and extracts obtained with A-4.5 and M-6 with signal-noise remarkably higher when MeOH is employed. This difference was especially remarkable when working in negative mode (**Figure 1**). Furthermore, the signal enhanced using M-6 was generally followed by a lower background noise and better peak shape, especially for compounds such as diclofenac, ibuprofen, triclosan, caffeine, bentazone or vildagliptin (**Figure S-2**). Obviously, the background noise was also related to the proportion sample:solvent, being A-3 the method with less dilution factor hence the one that presented higher background noise. Up to our knowledge, Phree™ cartridges have not been employed for haemolymph before. As Phree™ cartridges were designed for plasma analysis there are several studies that employed them for this purpose [21, 29], which mainly use ACN as solvent. Hence, previous works that assess the differences regarding the use of MeOH and ACN have not been found.

3.2. Method validation

Method validation was performed for the methods A-3, A-4.5, M-6, M-4 was discarded due to extraction issues with the pressure explained before.

3.2.1. Sensitivity and precision

Regarding precision and following the European Commission Guidelines [30], Intra-R (**Table 1**) was satisfactory (<20%) except for etoricoxib when using A-3. Inter-R was satisfactory (<30%) except for metformin for M-6 and bufotenine for both A-3 and A-4.5. In general, precision results were slightly better for M-6 than both ACN extractions (**Figure 2**).

LODs showed a range of 0.44-9.28, 0.79-12.30 and 0.30-10.70 ng/mL for A-3, A-4.5 and M-6, respectively, except for salicylic acid. (15.30 ng/mL) in A-3 and bentazone (19.10 ng/mL), metformin (16.30 ng/mL) and triclosan (22.00 ng/mL) in M-6 (**Table 1**). LODs of M-6 had ranges similar to A-3 and A-4.5 ACN despite the dilution factor of the sample (7, 4, and 5.5, respectively). This is due to the higher signal-noise ratio, as described before. However, as shown in **Figure 2,** A-3 showed slight higher sensitivity when compared with the other methods.

The background noise was too high for the proper calculation of LODs, LOQs and Intra-R of bentazone and 4-MeO-PCP using the samples spiked at 5 ng/mL (except for 4-MeO-PCP in M-6). Therefore, they were calculated using the lowest point of the linearity (5 ng/mL in solvent). In a similar way, low recoveries for 4-MeO-PCP in the samples spiked at 50 ng/mL avoided the correct calculation of Inter-R for A-3 and A-4.5.

3.2.1. Matrix effect

Several compounds presented strong ME (**Fig. 3 and Table S-3**), the 52%, 61% and 39% of compounds had a ME $\geq \pm 30\%$ for the methods A-3, A-4.5 and M-6, respectively. Signal suppression was predominant for M-6. While signal enhancement was predominant for A-4.5, including remarkably strong signal enhance for salicylic acid (+200.0%), diclofenac (+160.0%), PFBS (+117.0%) and imazalil (+107.0%). On the

other hand, A-3 presented mixed results including very strong signal enhanced for PFBS (+143.0%). Despite the results in **Figure 3** are heterogeneous, overall A-3 and M-6 were the methods with weakest matrix effects.

Most of the compounds with signal enhance were acids while the opposite behaviour was predominant for basic compounds (especially in A-4.5). This is in accordance with previous studies, where basic compounds commonly showed signal suppression in biological samples [31, 32]. However, other studies suggest that signal enhancement or suppression due to matrix effect is unpredictable and unique for each analysis [33, 34].

Recoveries were determined at three concentration levels: 5, 50 and 100 ng/mL (**Table 2**). Recoveries ranging 70-120% were considered acceptable (following the European Commission Guidelines [30]). A-3 was able to satisfactory recover 15 at 50 and 100 ng/mL, while A-4.5 was able to recover 17 and 12 compounds, respectively. M-6 was the only method that recovered properly the dissociative anaesthetic drug 4-MeO-PCP, with 20 and 14 compounds within the accepted range at 50 and 100 ng/mL, respectively. And the three methods were able to extract 14 compounds at 5/ng/mL, where bentazone was not recovered in any of the three methods.

Summarizing, the three methods provided recoveries within the acceptable range (70-120 %) for the majority of the compounds. As can be seen in Figure 2 and Table 2, an average of 15 compounds (corresponding to 65%) using A3, 16 compounds (69 %) using A4.5 and M6 showed recoveries within that range.

Haemolymph analyses in bivalves are usually employed to assess the effects of the organic compounds, such as DNA damage [31], alteration of the immune parameters [32], or the analysis of other pollutants related biomarkers [35, 36]. However, up to our knowledge, studies about methodology development or occurrence of organic pollutants

in mussel haemolymph have not been found. Attending to other aquatic biota, the occurrence of the pharmaceutical fluoxetine has been determined in crab haemolymph [18, 25, 35], however it is not a target compound of the present study and the extraction procedure was substantially different. The only study found using Phree™ cartridges in aquatic biota, employed them as a purification step (after a ACN solvent extraction) for the extraction of 41 antibiotics from fish muscle, with recoveries ranging 99.8-112% and providing a remarkable improvement of the sensitivity [22].

3.3 Application to non-spiked samples

Haemolymph samples from three different local markets were analysed in order to test the efficacy of the methods in real samples. The haemolymph from five mussels of each market was pulled, and then extracted by triplicate using the procedures A-4.5 and M-6. When extracted with A-4.5, results showed concentrations ranging 33.50-97.80 ng/mL for salicylic acid in two of the markets. On the other hand, results of extractions using M-6 showed concentrations between 45.29-66.82 ng/mL for salicylic acid, and 28.30-30.31 ng/mL for diclofenac in two and three of the markets samples, respectively.

The methods A-4.5 and M-6 were also tested using samples from a bioaccumulation study were *M. Galloprovincialis* were exposed to different emerging pollutants during 28 days at a concentration of 10 ng/mL in water. The haemolymph samples analysed correspond to the 14^{th} day of exposure. When extracted using A-4.5 results showed concentrations of 8.04-85.60 ng/mL for acetaminophen, diclofenac, metformin, naproxen, PFOA, and terbuthylazine, while chlorfenvinphos, etoricoxib, naproxen, PFPeA, PFDA, PFBS and PFOS showed values below the LODs or LOQs. On the other hand, the extracts of M-6 showed concentrations of 1.14-96.30 ng/mL for diclofenac, etoricoxib, ibuprofen, imazalil, metformin, PFPeA, PFOS, salicylic acid and terbutylazine, while chlorfenvinphos, chlorpyrifos, naproxen, PFOA PFDA, PFBS and vildagliptin presented

values below the LODs or LOQs. It is important to mention that M-6 generally detected more compounds at higher concentrations as is the case of chlorpyrifos, ibuprofen, imazalil, salicylic acid, and vildagliptin not detected using A-4.5. However, acetaminophen was only detected when using A-4.5.

4. CONCLUSIONS

The methods assessed are able to satisfactorily extract a wide range of organic compounds from mussel haemolymph. The method M-6 extracted all the target compounds with 20 of them ranging recoveries between 73.6-114.0%. M-6 also achieved the best precision and overall recoveries. On the other hand, A-3 provided the weakest ME and lowest LODs. Since strong ME were noticed, the use of ISs for every compound in future studies will likely improve this results. The use of MeOH as extract solvent involved improvements in the chromatographic signal-noise ratio. However, the use of MeOH also entailed slower extraction procedures and, in some cases, the clogging of the cartridges. Further research is needed to solve this extraction issues.

The proposed methods allowed the determination of organic pollutants in haemolymph from mussels purchased in local markets. The pharmaceuticals salicylic acid and diclofenac were detected with concentrations of 33.50-97.79 ng/mL and 28.30–30.31ng/mL, respectively. Furthermore, the methods were tested using samples from a bioaccumulation study. Where M-6 detected 9 compounds with concentrations ranging 1.14-96.30 ng/mL and A-4.5 detected only 6 compounds with concentrations ranging 8.04-85.60 ng/mL.

The results of the present study show that, despite the procedural issues, M-6 was the best method for the multi-residue extraction of organic pollutants in haemolymph.

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6 Conflict of interest statement

All authors declare no conflict of interest.

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Table 1: Validation of the selected methods in terms of sensitivity (LODs, LOQs), Intra-R and Inter-R.

Table 2: Absolute and relative (for compounds with internal standard) recoveries for the three methods at spiked concentrations of 100, 50 and 5 ng/mL.

Figure 1: Chromatograms of the compounds analysed in negative mode from the samples spiked at 100 ng/mL after extraction with A-4.5 (black) and M-6 (red). Ibuprofen and PFOA peaks are overlaped.

Figure 2: Comparison of the parameters: Intra-R, LODs, ME and recoveries between the methods. The graphics show the number of compounds that fulfil the different ranges of values for the different parameters.

Figure 3: ME of the validated methods for each compound.