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**Fast and Easy extraction combined with High Resolution-Mass
Spectrometry for Residue Analysis of two Anticonvulsants and their
transformation products in Marine Mussels**

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ABSTRACT

Environmental field studies have shown that carbamazepine (Cbz) is one of the most frequently detected human pharmaceuticals in different aquatic compartments. However, little data is available on the detection of this substance and its transformation products in aquatic organisms. This study was thus mainly carried out to optimize and validate a simple and sensitive analytical methodology for the detection, characterization and quantification of Cbz and oxcarbazepine (Ox), two anticonvulsants, and six of their main transformation products in marine mussels (*Mytilus galloprovincialis*). A modified QuEChERS extraction method followed by analysis with liquid chromatography coupled to high resolution mass spectrometry (HRMS) was used. The analyses were performed using two-stage fragmentation to reveal the different fragmentation pathways that are highly useful for the identification of isomeric compounds, a common problem when several transformation products are analyzed. The developed analytical method allowed determination of the target analytes in the lower ng/g concentration levels. The mean recovery ranged from 67-110%. The relative standard deviation was under 11% in the intra-day and 18% in the inter-day analyses, respectively. Finally, the method was applied to marine mussel samples collected from Mediterranean Sea cultures in southeastern France. Residues of the psychiatric drug Cbz were occasionally found at levels up to 3.5 ng/g dw. Lastly, in this study, other non-target compounds, such as caffeine, metoprolol, cotinine and ketoprofen, were identified in the real samples analyzed.

Keywords: pharmaceuticals; marine environment; aquatic organisms; biota; QuEChERS; metabolites;

45 1. Introduction

46 The presence of different pharmaceutical compounds in surface and marine
 47 water has been largely attributed to the low efficiencies of municipal wastewater
 48 treatment plants (WWTPs) for removing many of these compounds [1-3]. The coastline
 49 is becoming increasingly urbanized and faces a double pollution threat: by oil slicks or
 50 chemical spills, as well as, by pollution generated by inland activities which discharge
 51 their wastes into marine coastal waters via streams, rivers and wastewater. In order to
 52 improve and maintain the quality of surface water along the European coasts, the
 53 European Parliament and the Council approved Recommendation 2002/413/CE
 54 concerning the implementation of Integrated Coastal Zone Management in Europe [4].

55 The carbamazepine (Cbz), is a prescribed drug widely sold for the treatment of
 56 epilepsy and other psychotherapy applications. It has been regarded as a potential tracer
 57 in surface water due to its poor elimination during wastewater treatment and its
 58 persistency, which makes Cbz a pharmaceutical of high environmental relevance [3,5].
 59 It has frequently been detected in WWTP effluent (up to 2.1 µg/L) [2,3,6], river water
 60 (up to 1.1 µg/L) [3,7], drinking water (30 ng/L) [8] and even seawater (up to 1.1 µg/L)
 61 [7,9]. Cbz is predominantly metabolized in the liver to carbamazepine-10,11-epoxide
 62 (Epoxy), a pharmacologically active compound which is further metabolized into 10,11-
 63 dihydro-10,11-trans-dihydroxycarbamazepine (TRANS). Oxcarbazepine (Ox) is a keto
 64 analogue of Cbz which generates transformation products common of those of Cbz,
 65 such as TRANS and 10-hydroxy-10,11-dihydrocarbamazepine (10OH), its
 66 therapeutically active metabolite. In 2003, Miao and Metcalfe reported, for the first
 67 time, on the presence of Cbz transformation products in Canadian WWTP effluents and
 68 surface water [10]. Their study showed that TRANS transformation products exhibited
 69 threefold higher concentrations than Cbz itself (up to 1.3 µg/L in effluents and 2.2 ng/L
 70 in surface waters). Similar results on these by-products were recently published in
 71 French WWTPs by Leclercq *et al.* [6]. However, little information on the detection of
 72 Cbz and Ox in marine organisms and no data on its transformation products are
 73 available in literature. This issue could be partly explained by the high complexity of
 74 the matrix and the lack of suitable protocols, which include effective extraction methods
 75 and sensitive and specific analytical methods to detect these analytes at trace levels
 76 (ng/g or lower).

Most methods for the analysis of organic micropollutants from aquatic organisms are based on lipid isolation, which often involves complicated extraction and clean up procedures to generate extracts ready for analytical determination. SPME (solid-phase micro-extraction) [11], MASE (microwave-assisted solvent extraction) [12], MSPD (matrix solid phase dispersion) [13], SFE (supercritical-fluid extraction) [14], and especially PLE (pressurized liquid extraction) [15-18], have been reported. Nevertheless, most of these methods are long, tedious, time-consuming and require large volumes of organic solvents. In 2003, Anastassiades *et al.* [19] developed the approach called QuEChES (quick, easy, cheap, effective, rugged and safe). This procedure has frequently been used for the extraction of pesticides in food matrices (milk, olive oil, several fruits and vegetables) [20,21]. However, there are few reported studies related to pharmaceutical and personal care products (PPCPs) determination in fish [22] and it has not been applied for the extraction of target anticonvulsants from mussel samples.

Mussel *M. galloprovincialis* is a common filter feeder that occurs along the European sea coasts. This species is an excellent sentinel for monitoring of organic micro-contaminants from environmental waters, because the mussels can bioaccumulate substances through their gills (dissolved substances) and/or digestive tract (substances sorbed on particles). In a recently publication, Gómez *et al.* [23] reported the bioconcentration of two pharmaceuticals (benzodiazepines) and two personal care products (UV filters) in such marine organisms.

Accordingly, our aim was to develop and validate a simple, rapid and sensitive analytical strategy for detection, characterization and quantification of two anticonvulsants (Cbx and Ox) and six of their main transformation products in mussels by accurate mass measurements in MS and MS/MS modes. For that, an easy QuEChERS extraction method followed by analysis with a liquid chromatography coupled to full scan high resolution-mass spectrometry (LC-Orbitrap-MS) system was developed and the procedure is described in this study. The effects of several parameters were investigated and reported. The more demanding requirements regarding mass spectrometric confirmation currently set by EU regulations (Commission Decision 2002/657/EC and SANCO/10684/2009 Guideline) were taken into account when confirming and quantifying the target compounds [24,25]. We reported results obtained during the optimization of the QuEChERS extraction method, evaluating the influence

of several sorbents in the clean-up step while comparing with other conventional procedures used for the analysis of mussel samples. Moreover, retrospective analysis has been applied to identify other non-target compounds (not initially included in the method) by manual processing of previously recorded and stored spectral data. Finally, another innovative aspect of the present study concerns the use of marine organisms as a tool for exposition assessment of micro-pollutants in aquatic environments.

2. Experimental

2.1. Chemicals and reagents

A comprehensive overview of the reagents used in this study has been included as supplementary data.

2.2. Sample preparation

2.2.1. *QuEChERS extraction and d-SPE clean-up*

Freeze-dried mussels ($2 \text{ g} \pm 0.01$) were weighed in a 50 mL polypropylene centrifuge tube and then 100 μL of a 2 mg/L methanolic surrogate standard solution was added (Cbz-d8 and Epoxy-d10). Next, the mussels were rehydrated by adding 10 mL of ultrapure water, and the mixture was vortexed for 30 sec. The tubes were then manually and vigorously shaken for 1 min, after the addition 10 mL of AcN. Then 4 g Na_2SO_4 (anh), 1 g NaCl, 1 g $\text{Na}_3\text{Cit} \cdot 2\text{H}_2\text{O}$ and 0.5 g $\text{Na}_2\text{HCit} \cdot 3\text{H}_2\text{O}$ were added directly into each tube and the mixture was immediately vigorously shaken (manually) to avoid salt agglomeration for 1 min more. A centrifugation (3500 rpm, 5 min) was performed, and the mixture was then allowed to stand for 5 min, 2.5 mL of the upper AcN layer was transferred into a 15 mL polypropylene tube, containing clean-up sorbent (750 mg Na_2SO_4 , 125 mg PSA, 125 mg C18), and 50 μL of formic acid was added and shaken for 1 min. After a second centrifugation step (5000 rpm, 5 min), 1 mL of mixture was evaporated to dryness at 35°C under a nitrogen stream. The residue was reconstituted in 1 mL of AcN/water (1:9, v/v). Finally, the sample was centrifuged at 10.000 rpm for 10 min to separate the residual lipids and the extract was filtered directly into an analysis vial using a 0.45 μm PTEF syringe filter

2.2.2. *PLE extraction and clean-up*

This was performed on a Dionex ASE 350 (Dionex, Sunnyvale, CA) system. Several experimental extraction variables were optimized (data not included). Finally, the best results were obtained when two GlassFiber filters (Dionex) , 7 g of Florisil (60-

100 mesh, Sigma Aldrich), another GlassFiber filter and 50 g of glass beads (1 mm diameter, Assistent) were placed in the stainless-steel extraction cells (66 mL). $3 \text{ g} \pm 0.1$ of homogenized mussel enriched with standard was next placed in the cells, mixed with glass beads and finally covered with 15 g of glass beads. Extraction was carried out with water/acetone (3/2, v/v). The extraction conditions were as follows: cell heating time (5 min), static time (10 min), pressure (1500 psi), temperature (80°C), purging time (200 s), flushing volume (60%) and cycles (2). After extraction, the acetone content was evaporated using a rotary evaporator at 35°C. The remaining aqueous matrix was diluted with 100 mL of water and filtered with a throw Glass Fiber filter (Whatman). This matrix was cleaned by SPE using Oasis HLB extraction cartridges preconditioned with 2 mL of MeOH and 2 mL of H₂O. The extracts were passed through cartridges and then dried. Elution was carried out with 6 mL of MeOH (method 1) or 2 mL MeOH + 4 mL EtAc (method 2). The eluate was then evaporated to dryness at 40°C, under a nitrogen stream (Turbovap). The residue was dissolved in 1 mL of AcN/water (1:9). After homogenization, centrifugation was carried out at 12.000 rpm for 8 min. The final extract was transferred into vial after filtration using a 0.45 μm PTEF filter. (Note: a dilution 1:1 was necessary to apply the extracts obtained using method 1 before injection into the LC-MS).

160 2.3. LC-MS analysis

A detailed discussion of analytical method and validation study developed in this work has been included as supplementary data. In summary, the analyses were run on an Exactive mass spectrometer (Thermo Fisher Scientific, USA) equipped with a heated electrospray ionization probe (HESI) source in positive ion mode. Data were acquired by continuously alternating scan events: one without and one with fragmentation. Several instrumental settings were tested to maximize the analyte signals: mass range, resolution, automatic gain control (AGC) target, tube lens, heated capillary temperature, capillary voltage and gas flow rate. The optimal conditions were as follows: HCD (10 eV), resolving power (50.000), AGC (5x10⁵) and scan range (100-400 m/z) in both scan events. The identification criteria applied to the target analytes were: (i) retention times and (ii) 2 diagnostic ions (the protonated molecular ion and one product ion) together a mass accuracy < 5 ppm. Thus, an appropriate detection according with the requirements established for HRMS analysis was achieved [24,25].

For LC separation, a ZORBAX-XDB-C18 analytical column (100 mm length x 2.1 mm I.D and 1.8 μm particle size) from Agilent Technologies was used. Different chromatographic conditions were evaluated in order to achieve the best analytical results (flow rate, gradient time, mobile phase additives and two analytical columns). The best separation was achieved using a 200 $\mu\text{L}/\text{min}$ flow rate and AcN and water with 0.1% formic acid in both mobile phases. The linear gradient was set at 10% to 100% AcN for 10 min, and then maintained for 5 min. The re-equilibration time was 10 min.

3. Results and discussion

3.1. LC-MS analysis

Several chromatographic conditions were tested to achieve optimal resolution and peak shape. The major advantage of using a 1.8 μm particle column is the increased column efficiency, resulting in narrow peaks, increased S/N ratios and the separation of isomeric compounds. The increased resolution power as compared to columns of higher particle size (3.5 μm , XTerra-C18) was noteworthy and there was an almost twofold greater peak width achieved when using 3.5 μm instead of 1.8 μm . A ZORBAX-XDB-C18 column with a 1.8 μm particle size was therefore the final analytical column selected (see supplementary data "Figure S1"). On the basis of our experience and previously published studies, AcN and two additives for the aqueous mobile phases (formic acid 0.1% and a ammonium formate 10 mM/formic acid 0.1% buffer) were assayed. Only Cbz showed a sensitivity improvement when ammonium formate was used, while the results obtained for all of the target compounds were better with formic acid. In view of the results, AcN and water containing 0.1% formic acid in both phases was the condition selected for the analysis (see supplementary data "Figure S2").

The ionization source working parameters for the target compounds were sequentially optimized by analysing a standard mixture at 10 ng/g. An increase in the sensitivity and narrow peak resolution was achieved using a 65 V capillary voltage, 300°C temperature and 4 kV spray voltage. The tube lens voltage depends directly on the molecular structure, and therefore different values were fully evaluated (90, 120 and 150 eV), on the basis of previous experience and published literature⁹. The best results were obtained when a tube lens of 90 eV was applied. Continuing with the optimization, AGC is a crucial parameter, because substantial data quality variations may occur when the ion population is not accurately maintained. Previous studies have reported that the ion density in the trap must be kept as low as possible to ensure the best resolution and

mass accuracy, without a significant sensitivity loss [26]. Three AGC values were tested: 3×10^6 (high dynamic), 1×10^6 (balance scan) and 5×10^5 (ultimate scan). The absolute abundances and S/N ratios were similar in the ultimate and balance scans for most of the compounds studied. However, with high dynamic scans, a decrease in the peak intensity was observed for all analytes (see supplementary data “Figure S3”).

One of the most serious issues encountered during the analysis of complex matrix samples is the possibility of finding high amounts of co-eluting compounds, resulting in interference at the same nominal mass. For that, we checked the resolving power of the method. An extract spiked at 10 ng/g was thus analyzed at three scan rates: 1, 2 or 10 Hz, corresponding to a mass resolution of 100.000 FWHM (ultrahigh), 50.000 FWHM (high) or 10.000 FWHM (medium), respectively. Greater mass deviation was obtained when the scan was performed at 10 Hz. The best peak shape and mass assignment over the chromatographic peak was obtained at 100.000 and 50.000 FWHM. In view of the results, a scan resolution of 50.000 (high) and an AGC target of 5×10^5 (ultimate scan) were the best compromise shape and width, as well as the mass deviation over the chromatographic peak. A 100–400 m/z scan range was set (see supplementary data “Table S1”).

3.1.1. High resolution/high mass accuracy MS/MS experiments.

Three different fragmentation conditions in the HCD cell (10, 25 and 50 eV) were evaluated in order to obtain useful product ion spectra with enough fragment ions to accurately identify all the target compounds. In view of the results, an HCD experiment at 10 eV was chosen because, under this condition, sufficient fragmentation was obtained for a positive confirmation of most compounds, except transformation products AI and AO. The use of high voltages resulted in a decrease in the number and intensity of product ions or a total reduction of the precursor ions, which was avoided, as far as possible. No product ions were obtained for transformation products AI and AO at 10 eV due to the stability of these molecules and the low fragmentation energy used. However, the methodology used allowed us to obtain more information about the characteristic fragmentation pathways so as to be able to accurately identify the target isomeric compounds (Ox, Epoxy and 2OH). Three isomer compounds were characterized by the same MS/MS fragment ions. Although these compounds have the same molecular formula, they presented different molecular structures and therefore different properties (see Table 1). In the product ion mass spectrum of protonated Cbz, the only ion m/z 194 corresponds to a neutral loss of the carbamoyl group (CONH, 43

Da) from $[M+H]^+$. For Ox, a HCD of 10 eV yielded the ions of m/z 236 and 208, associated with the loss of NH_3 (17 Da) and CONH (43 Da), respectively. Two major ions at m/z 237 and 194, which correspond to losses of H_2O (18 Da) and CONH + H_2O (43 + 18 Da), respectively, were observed for a mass spectrum of 10OH. The product ions found in the 2OH spectrum were at m/z 210 and 180, corresponding to loss of CONH (43 Da) and CONH + H_2CO (43 + 30 Da). More complex product ion mass spectra were obtained for the protonated molecules of TRANS and Epoxy. Similar product ions were found for both transformation products (180, 210 and 236 m/z), except for the ion m/z 254 for 10OH. A fragmentation pathway for TRANS has previously been proposed by Miao and Metcalfe [10]. In this pathway, the TRANS molecule generated ion m/z 253 by loss of H_2O . Then two different fragmentation pathways were planned, which originated ions at m/z 210 and 236 by loss of HNCO (43 Da) and NH_3 (17 Da), respectively. Finally, as a last step, these intermediate molecules could yield the product ion of m/z 180 by rearrangement of the ring and loss of H_2CO (30 Da) and 2CO (56 Da), respectively. However, based on the information obtained through the use of HRMS technology, our study indicated a small difference in the fragmentation route for TRANS transformation products (see Figure 2). The molecule first generated the ion m/z 254, instead of m/z 253, by loss of NH_3 (17 Da). This gave rise to ions m/z 236 and 210 by loss of H_2O (18 Da) and CO (28 Da), consecutively. Finally, the product ion of m/z 180 was obtained by rearrangement of the ring and loss of H_2CO (30 Da), as previously suggested [10]. The ring-double-bond equivalent (RDBE) parameter is related to the degree of π -electron conjugation and was used as a tool to support the structure of the intermediates or product ions obtained in the MS/MS spectrum.

3.2. Extraction method

3.2.1. QuEChERS versus PLE technique

Table 2 summarizes the best results obtained with the two evaluated extraction techniques. The total solvent consumption was: 70 mL H_2O :acetone (3:3, v/v) + 6 mL MeOH (method 1) or 70 mL H_2O :acetone (3:3, v/v) + 2 mL MeOH + 4 mL EtAc (method 2) with PLE, while it was only 10 mL AcN with the QuEChERS procedure. The optimal sample amounts were 3 g in PLE and 2 g in QuEChERS. In a typical preparation with QuEChERS extraction, a single analyst can manually prepare 8 samples for LC-MS/MS analysis in less than 2 h. Average recoveries in the PLE method

274 ranged from 4 to 67% and 5 to 48% when using MeOH and EtAc as elution solvents,
275 respectively. Furthermore, 2OH was not recovered when EtAc was used as solvent.
276 However, the average values obtained by the QuEChERS method were over 67% for all
277 compounds, with the exception of product IM, which was excluded from the final
278 method. At last, the QuEChERS extraction method was selected based on the results
279 obtained and taking the general parameters into account, such as extraction times,
280 solvent consumption, sample amount, use of laboratory glass material, and the tedious
281 protocol associated with the PLE technique.

282 3.2.2. Optimization of the QuEChERS procedure

283 Because the extraction quality depends on the sample homogenization step,
284 mussel samples were freeze-dried with a lyophilizer. Moreover, as suggested in the EN
285 15662 standard, a minimum water percentage is necessary to get good recovery in the
286 salting-out extractions [27]. For this reason, 10 mL of water was initially added to each
287 sample prior to QuEChERS extraction and then the sample was vortexed for 30 sec.
288 Alternatively, in this study, MgSO₄ (anh) was substituted for Na₂SO₄ (anh), and then
289 the samples were allowed to stand for 5 min after centrifugation so as to efficiently
290 absorb the water. Our first attempts were focused on d-SPE optimization for the
291 development of a rapid and easy sample preparation protocol. Mussels are fat or lipid
292 containing matrices (about 15%) and, although fats are not very soluble in AcN, a
293 certain quantity of them will be co-extracted, so they have to be removed prior to the
294 final determination step [20]. For that, different clean-up sorbent materials (Z-Sep-Plus,
295 Z-Sep/C18 and PSA/C18) used to enhance matrix interference removal were evaluated.
296 Z-Sep material contain zirconium atoms, which act as a Lewis acid, while the phosphate
297 groups in phospholipids act as a strong Lewis base, strongly binding with zirconium
298 atoms. On the other hand, until now, PSA and C18 phases have mainly been used for
299 the clean-up step in the QuEChERS method. As seen in Table 2, comparable recoveries
300 from spiked mussel samples were observed for all target compounds when a percentage
301 of 2% formic acid was added. The addition of 2% formic acid was a critical step. Many
302 sample preparation techniques for biological matrices use acid to disrupt compound-
303 protein binding, which directly affects recovery and matrix effect. No significant
304 differences were observed between Z-Sep+ and Z-Sep/C18. However, the mixture
305 PSA/C18/Na₂SO₄ provided the best results in comparison to these new sorbent
306 materials for TRANS and 10OH transformation products, possibly due to the fact that
307 they allow the elimination of a greater amount of matrix co-extractive interference (see

308 supplementary data “Figure S4”). Only IM could not be recovered when formic acid
309 was added. Therefore, it was excluded from the final method. The influence of the
310 extraction time was also studied. Satisfactory recovery values were obtained using a
311 time of 1 min (similar to the original QuEChERS method) [19]. Comparable results
312 were obtained when the extraction time was increased to 2 min. Finally, the volume
313 transferred into a d-SPE tube for the clean-up step was assessed too. When 5 mL of the
314 AcN layer was transferred, mean recovery values were lower than obtained with 2.5
315 mL, possibly due to the greater amount of interference from the matrix (data not
316 included).

317 **3.3. Analytical performance**

318 A rigorous validation procedure according to SANCO/10684/2009 and
319 ISO/17025 Guidelines was used to ensure high quality analytical measurements [25,27].
320 The mean recovery data and deviations obtained, which highlights the precision of the
321 extraction method, are given in Table 1. Satisfactory recoveries were achieved
322 combined QuEChERS extraction with the mixture PSA/C18/Na₂SO₄ for the clean-up
323 step. The average recovery for both spike levels (10 and 50 ng/g dw) were higher than
324 75%, for all target compounds included in the study, with the exception of Cbz (67%).
325 The linearity of the analytical response for all the studied compounds within the studied
326 range of three orders of magnitude was very good, with correlation coefficients higher
327 than 0.997 in all cases. The matrix effect was studied by comparison of the slopes of the
328 calibration curves in solvent and in matrix. When the percentage of the difference
329 between these slopes is positive, then there is signal enhancement, whereas a negative
330 value indicates signal suppression. Table 1 shows the percentage of enhancement or
331 signal suppression found in the evaluated matrix. According to our results, no
332 compound generated a relevant matrix effect higher than 50%, being AO the analyte
333 that showed the greatest signal suppression effects of -40%. Thus, 4 compounds showed
334 no matrix effect (<20%, because this variation is close to the repeatability values), and 3
335 analytes presented a medium effect (50-20%). To minimize matrix interference, matrix-
336 matched calibration curves were used to compensate for the matrix effect and avoid any
337 under/over estimation during quantification. R.S.D values for the intra-day analysis
338 (repeatability) ranged between 5% and 11%, while they were 7% to 18% for the inter-
339 day analysis. This demonstrates the repeatability of the method and therefore its
340 effectiveness for quantitative purposes. The reporting levels of the studied compounds

341 ranged from 0.1 to 0.3 ng/g (LOD) and 0.2 to 1.0 ng/g (LOQ). However, according to
 342 the recommendations found in literature, the method detection limits (MDLs, ng/g dw)
 343 are more appropriate for establishing environmental analysis detection thresholds
 344 because they take the dilution or pre-concentration steps during sample preparation into
 345 account [28]. As shown in Table 1, MDL values for mussel extracts (0.5–1.5 ng/g) were
 346 typically higher than any of the LOD data, since a 5-fold dilution step was applied to the
 347 sample. Nevertheless, the developed analytical method allowed determination of the
 348 target analytes at concentrations lower than ng/g dw from mussel organisms exposed in
 349 marine water. The specificity of the method was assessed through the analysis of three
 350 blank mussel samples extracted by QuEChERS. No other significant peaks ($S/N > 3$)
 351 were found at the specific retention times of the target pharmaceuticals.

352 **3.4. Analysis of real samples**

353 *3.4.1. Identification and quantitation of target compound*

354 The more demanding requirements regarding mass spectrometric confirmation
 355 currently set by EU regulations (Commission Decision 2002/657/EC37) were taken into
 356 account for confirmation and quantification of the target compounds [24]. The target
 357 compounds were identified on the basis of comparisons of the retention time ($\pm 2\%$) and
 358 accurate mass (< 5 ppm) of precursor and product ions obtained from LC-Orbitrap-MS
 359 analysis of the standard compounds in matrix (see Table 1). Mass deviation values were
 360 reported using the average accurate mass measurements calculated from signal obtained
 361 to matrix-matched calibration curves. As seen, the mass accuracy was < 5 ppm in both
 362 MS and MS/MS mode. The applicability of the proposed method was assessed for the
 363 analysis of real marine mussel samples collected from Mediterranean Sea cultures
 364 located in southeastern France (see supplementary data, section 1.2. Sample Collection).

365 With regard to marine waters, Togola and Budzinski reported the presence of
 366 Cbz in the Mediterranean Sea at concentrations of approximately 10–40 ng/L [29]. More
 367 recently, higher concentrations were noted by Wille *et al.* in 2010 in a Belgian coastal
 368 region (up to 732 ng/L) [9]. Concerning the results obtained for target compounds in
 369 marine mussels collected from the Mediterranean Sea ($n=10$), the psychiatric drug Cbz
 370 was only found in two samples at concentrations above its MQL (1.5 ng/g). In the
 371 sample collected in the vicinity of an emissary area, Cbz was detected at levels of up to
 372 3.5 ng/g dw, while in the lagoon sample, the concentration was below the MDL (0.5
 373 ng/g). Another positive sample purchased in a local supermarket at 1.5 ng/g dw was

found. The results obtained are in agreement with other studies in fish. For the first time, Ramirez *et al.* detected the antiepileptic drug Cbz at a mean concentration of 1.1 ng/g in wet weight from fish muscle samples collected from two streams located in Texas [28]. Finally, the results supports the hypothesis of low or no effective removal of this pharmaceutical in conventional treatment plants, in line with previous studies [1-3]. However, none of the transformation products were detected in these tests. In any case, recovery of the surrogate standards was above 70%, for both Cbz-d8 and Epoxy-d10. These standards allowed us to verify that extraction method performance and analysis were satisfactory. An example of identification of the targeted compound Cbz in a marine mussel sample can see in supplementary data "Figure S5". In full MS spectrum, the measured mass for Cbz is shown at m/z 237.10221 which matches the theoretical mass 237.10220 with an error of -0.1 ppm. The additional acquisition in full MS/MS mode provided a more comprehensive identification of this anticonvulsant drug as well as its structural characterization found for the characteristic fragment ion at m/z 194.09647 with a mass deviation of 0.2 ppm. Good mass accuracies were obtained in the MS and MS/MS scans, which ensured correct identification in line with the other evaluated parameters: retention time, empirical formula and RDBE.

3.4.2. Identification of non-target compounds

To carry out the identification of non-target compounds (analytes not included in the analytical method), samples were re-processed. The MS and MS/MS full-scan acquisitions (100-400 m/z) allowed us to carry out a retrospective analysis of the real samples analyzed. A high number of false positive results have very often been reported in full-scan MS studies [30]. However, MS and MS/MS accurate-mass full scans and isotope patterns could be used as an additional criterion in further retrospective analyses. They allowed us obtain the elemental composition of parent and fragment ions, for the identification of new compounds or degradation products, while minimizing the number of the false positive results.

The fragmentation in this system is generated without any precursor ion selection. Therefore, the ability to link fragment ions to a particular precursor ion will depend on chromatographic resolution or software algorithms for peak deconvolution or both. For this study, data processing was carried out using ToxID 2.1.2 software (Thermo Fisher Scientific) to identify non-target analytes in the samples. With this tool, analyte detection is fully automated by the software and based on the presence of the exact mass of ± 5 ppm and within a given time window of ± 30 s. A database of 78

PPCPs was created by our group as an input text file for ToxID screening, and it basically contains a list of analyte names, retention times and molecular formulas. After processing, it generates a file which includes information such as the analyte name, expected and detected retention times, mass accuracy, intensity, adducts and fragment ions found. Then, each result reported as positive by the software is confirmed by manual verification of the mass spectrum (characteristic fragment ions) obtained for each compound in MS/MS mode at 10 eV, in order to limit false positive identifications. Finally, the methodology proposed allowed us to detect other non-target substances by retrospective data analysis. "Table S2" in supplementary data summarized the screening results for non-target compounds from the mussel samples analyzed (n=10), including the accurate mass values obtained in the worst cases (<3 ppm in all cases). Four drugs tested positive on the basis of the criteria described above: caffeine (stimulant), cotinine (stimulant, metabolite of nicotine), metoprolol (β -blocker) and ketoprofen (analgesic). Regarding the mass deviations, values below 3 ppm in MS and MS/MS acquisitions were obtained for all analytes, indicating a high mass accuracy. Of these, cotinine was the most frequently detected substance in the real mussel samples analyzed (n=8), followed by caffeine (n=6). These chemicals have been reported as being anthropogenic markers of water contamination caused by human activities due to their constant presence in different aquatic environments [31]. The greatest number of chemicals was identified at sampling points corresponding with lagoon (#6) and emissary (#7). These points correspond to zones with a substantial harbour activity and a submarine outfall (industrial/urban discharges), respectively.

Conclusions

The analytical method developed based on HRMS permitted us to obtain different fragmentation pathways, which proved very useful for suitable identification of isomeric compounds (2OH, Epoxy and Ox), which is a common problem when several transformation products are analyzed. The performance of the two extraction techniques (PLE and QuEChERS) was compared. An improved in the results in relation to accuracy and sensitivity was obtained using QuEChERS extraction. The major advantages of the protocol proposed in this study were the short extraction and clean-up times, combined with a highly selective and sensitive final detection system. The methodology proposed enabled the detection of target pharmaceuticals and their transformation products in marine mussels (*M. galloprovincialis*) at low ng/g

442 concentration levels. Furthermore, the method allowed us to detect other non-target
443 substances by retrospective data analysis. Thus, two stimulants, caffeine and a nicotine
444 metabolite (cotinine), were frequently detected, while two pharmaceuticals (metoprolol
445 and ketoprofen) were only occasionally detected.

446 In conclusion, as a preliminary study, marine mussels have proven to be a useful
447 tool for monitoring of pharmaceuticals from marine water, thus facilitating detection of
448 trace environmental contaminants, despite their low concentration in the medium.
449 Although the initial results were positive, future experiments should be conducted on
450 the bioaccumulation of such pharmaceuticals in marine organisms in order to assess
451 their bioconcentration factors. The present findings should also be useful for further
452 research within the PEPSEA project, to evaluate their possible impact on marine
453 ecosystems and how they can be affected by urban coastal activities in the
454 Mediterranean Basin.

455

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464

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 566 their transformation products. A pilot study of their removal from a sewage treatment
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571 FIGURES

572 **Figure 1.** Scheme of the QuEChERS procedure used for the extraction of Cbz, Ox and
 573 their major transformation products from marine mussels.

574

575 **Figure 2.** Product ion mass spectrum obtained for Epoxy and TRANS at 10 eV, and
 576 proposed fragmentation pathway for TRANS.

- Analysis by LC- HRMS permitted us to obtain different fragmentation pathways.
- The performance of the two extraction techniques (ASE and QuEChERS) was compared.
- The best results were achieved using QuEChERS + PSA/C18/Na₂SO₄ (clean-up).
- No significant differences were observed between Z-Sep+ and Z-Sep/C18.
- Marine mussels proven to be a useful tool for monitoring of drugs from seawater.

Figure 1. Scheme of the QuEChERS procedure used for the extraction of Cbz, Ox and their major metabolites from marine mussels.

Figure 2. Product ion mass spectrum obtained for Epoxy and TRANS at 10 eV, and proposed fragmentation pathway for TRANS.

Table 1. Analytical performance and MS - MS/MS data for each target compound studied by LC-Orbitrap-MS method.

Compound tr (min)	MS					MS/MS				Matrix Effect	Inter/intra -day (%)	Rec. (%, $\pm \sigma$)	LOQ (ng/g)	LOD (ng/g)	MDL (ng/g)
	Theo. Mass	Formula [M+H] ⁺	RDBE	Exp. Mass ¹	Δ^2 (ppm)	Theo. Mass	Formula	Ex. Mass ¹	Δ^2 (ppm)						
AI 4.7 min	180.0808	C ₁₃ H ₁₀ N	9.5	180.0809	0.5	-	-	-	-	-26	8/7	85 $\pm$ 8	0.4	0.1	0.5
TRANS 5.4 min	271.1077	C ₁₅ H ₁₅ N ₂ O ₃	9.5	271.1081	1.2	254.0812 210.0913 236.0706	C ₁₅ H ₁₂ NO ₃ C ₁₄ H ₁₂ NO C ₁₅ H ₁₀ NO ₂	254.0815 210.0916 236.0709	1.5 1.2 1.3	-17	5/9	93 $\pm$ 9	0.2	0.1	0.5
10OH 5.8 min	255.1128	C ₁₅ H ₁₅ N ₂ O ₂	9.5	255.1129	0.5	237.1022 194.0966	C ₁₅ H ₁₃ N ₂ O C ₁₄ H ₁₂ N	237.1025 194.0967	1.0 1.2	-18	9/10	110 $\pm$ 2	0.8	0.3	1.5
2OH 6.1 min	253.0971	C ₁₅ H ₁₃ N ₂ O ₂	10.5	253.0972	0.3	210.0913 180.0807	C ₁₄ H ₁₂ NO C ₁₃ H ₁₀ N	210.0914 180.0808	0.6 0.8	-24	9/17	104 $\pm$ 10	1.0	0.3	1.5
EPOXY 6.4 min	253.0971	C ₁₅ H ₁₃ N ₂ O ₂	10.5	253.0972	0.5	210.0913 180.0807 236.0706	C ₁₄ H ₁₂ NO C ₁₃ H ₁₀ N C ₁₅ H ₁₀ NO ₂	210.0915 180.0808 236.0717	1.1 0.7 2.0	1	11/14	75 $\pm$ 9	0.6	0.2	1.0
OX 6.6 min	253.0971	C ₁₅ H ₁₃ N ₂ O ₂	10.5	253.0970	-0.6	236.0706 208.0756	C ₁₅ H ₁₀ NO ₂ C ₁₄ H ₁₀ NO	236.0706 208.0758	0.2 0.8	-22	9/18	90 $\pm$ 6	0.6	0.2	1.0
AO 6.8 min	196.0756	C ₁₃ H ₁₀ NO	9.5	196.0758	0.6	-	-	-	-	-40	11/10	78 $\pm$ 2	0.2	0.1	0.5
CBZ 7.1 min	237.1022	C ₁₅ H ₁₃ N ₂ O	10.5	237.1023	0.3	194.0966	C ₁₄ H ₁₂ N	194.0965	0.7	-17	8/9	67 $\pm$ 6	0.3	0.1	0.5

Theo. Mass: theoretical mass (m/z); **Exp. Mass:** experimental mass (m/z); **Δ :** mass deviation (ppm). **Inter/intra-day:** repeatability/reproducibility of the instrumental method (R.S.D, %); R.S.D: relative standard deviation; **σ :** dispersion from the average or expected value; **Rec:** recovery average values obtained at two spiked levels (10 and 50 ng/g, dw); **LOQ:** limit of quantification (ng/g dw); **LOD:** limit of detection (ng/g dw); **MDL:** method detection limit (ng/g dw); ^{1,2}: Average accurate mass measurements were calculated from signals obtained from the matrix-matched calibration curves.

Table 2. Average recoveries and comparison different clean-up approaches in mussels spiked at 10 ng/g and 100 ng/g, by QuEChERS and PLE extraction, respectively (n=5).

Clean-up (QuEchERS) / Elution solvent (PLE)	QuEChERS				PLE	
	Z-Sep+ 50 μ L FA*	Z-Sep+ 50 μ L FA*	Z-Sep/C18 50 μ L FA*	PSA/C18 50 μ L FA*	MeOH	EtAc
Sample amount (g)	2				3	
Solvent total volume (mL)	10 mL AcN				70 mL H ₂ O:acetone + 6 mL MeOH	70 mL H ₂ O:acetone + 2 mL MeOH + 4 mL or EtAc
Total time of extraction (h)	~1.5 (per 8 samples)				~2.5 (per sample)	
Recovery (%)						
AI	82	80	70	80	4	5
TRANS	64	-	60	84	59	5
10OH	73	35	65	107	65	48
2OH	93	13	66	94	67	-
Epoxy	65	20	58	71	54	26
Ox	82	30	62	88	n.i	n.i
AO	68	75	74	77	56	11
Cbz	60	70	58	67	46	46
IM	-	75	-	-	-	-

*FA: formic acid; n.i: analyte not included in the study;