



HAL
open science

Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels

M.J. Martinez Bueno, Clotilde Boillot, H el ene Fenet, Serge Chiron, Claude Casellas, Elena Gomez

► To cite this version:

M.J. Martinez Bueno, Clotilde Boillot, H el ene Fenet, Serge Chiron, Claude Casellas, et al.. Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels. *Journal of Chromatography A*, 2013, 1305, pp.27-34. 10.1016/j.chroma.2013.06.071 . hal-02406817

HAL Id: hal-02406817

<https://hal.science/hal-02406817>

Submitted on 12 Dec 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin ee au d ep ot et  a la diffusion de documents scientifiques de niveau recherche, publi es ou non,  emanant des  tablissements d'enseignement et de recherche fran ais ou  trangers, des laboratoires publics ou priv es.

Accepted Manuscript

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

Author: M.J Martínez Bueno C. Boillot H. Fenet S. Chiron C. Casellas E. Gómez

PII: S0021-9673(13)00996-5
DOI: <http://dx.doi.org/doi:10.1016/j.chroma.2013.06.071>
Reference: CHROMA 354470

To appear in: *Journal of Chromatography A*

Received date: 10-4-2013
Revised date: 6-6-2013
Accepted date: 27-6-2013

Please cite this article as: M.J.M. Bueno, C. Boillot, H. Fenet, S. Chiron, C. Casellas, E. Gómez, Fast and Easy extraction combined with High Resolution-Mass Spectrometry for Residue Analysis of two Anticonvulsants and their transformation products in Marine Mussels, *Journal of Chromatography A* (2013), <http://dx.doi.org/10.1016/j.chroma.2013.06.071>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



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

4
5
6 *M.J Martínez Bueno**, *C. Boillot*, *H. Fenet*, *S. Chiron*, *C. Casellas* and *E. Gómez*

7
8 Laboratory of Hydrosociences Montpellier UMR 5569. Dept. Environmental Sciences and Public
9 Health. University of Montpellier I, 34000 Montpellier, France

10
11
12
13
14
15
16
17 *Corresponding author: Tel.: +33 (0) 4 11 75 94 18; Fax: +33 (0) 4 11 75 94 61. E-mail:
18 mjbueno@ual.es

19

19 **ABSTRACT**

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

40

41

42

43 **Keywords:** pharmaceuticals; marine environment; aquatic organisms; biota;
44 QuEChERS; metabolites;

45

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 $\mu\text{g/L}$) [2,3,6], river water
60 (up to 1.1 $\mu\text{g/L}$) [3,7], drinking water (30 ng/L) [8] and even seawater (up to 1.1 $\mu\text{g/L}$)
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-dihydroxycarbamazepine (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 $\mu\text{g/L}$ 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).

77 Most methods for the analysis of organic micropollutants from aquatic
78 organisms are based on lipid isolation, which often involves complicated extraction and
79 clean up procedures to generate extracts ready for analytical determination. SPME
80 (solid-phase micro-extraction) [11], MASE (microwave-assisted solvent extraction)
81 [12], MSPD (matrix solid phase dispersion) [13], SFE (supercritical-fluid extraction)
82 [14], and especially PLE (pressurized liquid extraction) [15-18], have been reported.
83 Nevertheless, most of these methods are long, tedious, time-consuming and require
84 large volumes of organic solvents. In 2003, Anastassiades *et al.* [19] developed the
85 approach called QuEChES (quick, easy, cheap, effective, rugged and safe). This
86 procedure has frequently been used for the extraction of pesticides in food matrices
87 (milk, olive oil, several fruits and vegetables) [20,21]. However, there are few reported
88 studies related to pharmaceutical and personal care products (PPCPs) determination in
89 fish [22] and it has not been applied for the extraction of target anticonvulsants from
90 mussel samples.

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

98 Accordingly, our aim was to develop and validate a simple, rapid and sensitive
99 analytical strategy for detection, characterization and quantification of two
100 anticonvulsants (Cbz and Ox) and six of their main transformation products in mussels
101 by accurate mass measurements in MS and MS/MS modes. For that, an easy
102 QuEChERS extraction method followed by analysis with a liquid chromatography
103 coupled to full scan high resolution-mass spectrometry (LC-Orbitrap-MS) system was
104 developed and the procedure is described in this study. The effects of several parameters
105 were investigated and reported. The more demanding requirements regarding mass
106 spectrometric confirmation currently set by EU regulations (Commission Decision
107 2002/657/EC and SANCO/10684/2009 Guideline) were taken into account when
108 confirming and quantifying the target compounds [24,25]. We reported results obtained
109 during the optimization of the QuEChERS extraction method, evaluating the influence

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

116 **2. Experimental**

117 **2.1. Chemicals and reagents**

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

120 **2.2. Sample preparation**

121 *2.2.1. QuEChERS extraction and d-SPE clean-up*

122 Freeze-dried mussels ($2 \text{ g} \pm 0.01$) were weighed in a 50 mL polypropylene
123 centrifuge tube and then 100 μL of a 2 mg/L methanolic surrogate standard solution was
124 added (Cbz-d8 and Epoxy-d10). Next, the mussels were rehydrated by adding 10 mL of
125 ultrapure water, and the mixture was vortexed for 30 sec. The tubes were then manually
126 and vigorously shaken for 1 min, after the addition 10 mL of AcN. Then 4 g Na_2SO_4
127 (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
128 each tube and the mixture was immediately vigorously shaken (manually) to avoid salt
129 agglomeration for 1 min more. A centrifugation (3500 rpm, 5 min) was performed, and
130 the mixture was then allowed to stand for 5 min, 2.5 mL of the upper AcN layer was
131 transferred into a 15 mL polypropylene tube, containing clean-up sorbent (750 mg
132 Na_2SO_4 , 125 mg PSA, 125 mg C18), and 50 μL of formic acid was added and shaken
133 for 1 min. After a second centrifugation step (5000 rpm, 5 min), 1 mL of mixture was
134 evaporated to dryness at 35°C under a nitrogen stream. The residue was reconstituted in
135 1 mL of AcN/water (1:9, v/v). Finally, the sample was centrifuged at 10.000 rpm for 10
136 min to separate the residual lipids and the extract was filtered directly into an analysis
137 vial using a 0.45 μm PTEF syringe filter

138 *2.2.2. PLE extraction and clean-up*

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

142 100 mesh, Sigma Aldrich), another GlassFiber filter and 50 g of glass beads (1 mm
143 diameter, Assistent) were placed in the stainless-steel extraction cells (66 mL). 3 g \pm 0.1
144 of homogenized mussel enriched with standard was next placed in the cells, mixed with
145 glass beads and finally covered with 15 g of glass beads. Extraction was carried out
146 with water/acetone (3/2, v/v). The extraction conditions were as follows: cell heating
147 time (5 min), static time (10 min), pressure (1500 psi), temperature (80°C), purging time
148 (200 s), flushing volume (60%) and cycles (2). After extraction, the acetone content was
149 evaporated using a rotary evaporator at 35°C. The remaining aqueous matrix was
150 diluted with 100 mL of water and filtered with a throw Glass Fiber filter (Whatman).
151 This matrix was cleaned by SPE using Oasis HLB extraction cartridges preconditioned
152 with 2 mL of MeOH and 2 ml of H₂O. The extracts were passed through cartridges and
153 then dried. Elution was carried out with 6 mL of MeOH (method 1) or 2mL MeOH + 4
154 ml EtAc (method 2). The eluate was then evaporated to dryness at 40°C, under a
155 nitrogen stream (Turbovap). The residue was dissolved in 1 mL of AcN/water (1:9).
156 After homogenization, centrifugation was carried out at 12.000 rpm for 8 min. The final
157 extract was transferred into vial after filtration using a 0.45 μ m PTEF filter. (Note: a
158 dilution 1:1 was necessary to apply the extracts obtained using method 1 before
159 injection into the LC-MS).

160 **2.3. LC-MS analysis**

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

174 For LC separation, a ZORBAX-XDB-C18 analytical column (100 mm length x
175 2.1 mm I.D and 1.8 μm particle size) from Agilent Technologies was used. Different
176 chromatographic conditions were evaluated in order to achieve the best analytical
177 results (flow rate, gradient time, mobile phase additives and two analytical columns).
178 The best separation was achieved using a 200 $\mu\text{L}/\text{min}$ flow rate and AcN and water with
179 0.1% formic acid in both mobile phases. The linear gradient was set at 10% to 100%
180 AcN for 10 min, and then maintained for 5 min. The re-equilibration time was 10 min.

181 **3. Results and discussion**

182 **3.1. LC-MS analysis**

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

197 The ionization source working parameters for the target compounds were
198 sequentially optimized by analysing a standard mixture at 10 ng/g. An increase in the
199 sensitivity and narrow peak resolution was achieved using a 65 V capillary voltage,
200 300°C temperature and 4 kV spray voltage. The tube lens voltage depends directly on
201 the molecular structure, and therefore different values were fully evaluated (90, 120 and
202 150 eV), on the basis of previous experience and published literature⁹. The best results
203 were obtained when a tube lens of 90 eV was applied. Continuing with the optimization,
204 AGC is a crucial parameter, because substantial data quality variations may occur when
205 the ion population is not accurately maintained. Previous studies have reported that the
206 ion density in the trap must be kept as low as possible to ensure the best resolution and

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

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

224 3.1.1. High resolution/high mass accuracy MS/MS experiments.

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

241 Da) from $[M+H]^+$. For Ox, a HCD of 10 eV yielded the ions of m/z 236 and 208,
242 associated with the loss of NH_3 (17 Da) and CONH (43 Da), respectively. Two major
243 ions at m/z 237 and 194, which correspond to losses of H_2O (18 Da) and CONH + H_2O
244 (43 + 18 Da), respectively, were observed for a mass spectrum of 10OH. The product
245 ions found in the 2OH spectrum were at m/z 210 and 180, corresponding to loss of
246 CONH (43 Da) and CONH + H_2CO (43 + 30 Da). More complex product ion mass
247 spectra were obtained for the protonated molecules of TRANS and Epoxy. Similar
248 product ions were found for both transformation products (180, 210 and 236 m/z),
249 except for the ion m/z 254 for 10OH. A fragmentation pathway for TRANS has
250 previously been proposed by Miao and Metcalfe [10]. In this pathway, the TRANS
251 molecule generated ion m/z 253 by loss of H_2O . Then two different fragmentation
252 pathways were planned, which originated ions at m/z 210 and 236 by loss of HNCO (43
253 Da) and NH_3 (17 Da), respectively. Finally, as a last step, these intermediate molecules
254 could yield the product ion of m/z 180 by rearrangement of the ring and loss of H_2CO
255 (30 Da) and 2CO (56 Da), respectively. However, based on the information obtained
256 through the use of HRMS technology, our study indicated a small difference in the
257 fragmentation route for TRANS transformation products (see Figure 2). The molecule
258 first generated the ion m/z 254, instead of m/z 253, by loss of NH_3 (17 Da). This gave
259 rise to ions m/z 236 and 210 by loss of H_2O (18 Da) and CO (28 Da), consecutively.
260 Finally, the product ion of m/z 180 was obtained by rearrangement of the ring and loss
261 of H_2CO (30 Da), as previously suggested [10]. The ring-double-bond equivalent
262 (RDBE) parameter is related to the degree of π -electron conjugation and was used as a
263 tool to support the structure of the intermediates or product ions obtained in the MS/MS
264 spectrum.

265 **3.2. Extraction method**

266 *3.2.1. QuEChERS versus PLE technique*

267 Table 2 summarizes the best results obtained with the two evaluated extraction
268 techniques. The total solvent consumption was: 70 mL H_2O :acetone (3:3, v/v) + 6 mL
269 MeOH (method 1) or 70 mL H_2O :acetone (3:3, v/v) + 2 mL MeOH + 4 mL EtAc
270 (method 2) with PLE, while it was only 10 mL AcN with the QuEChERS procedure.
271 The optimal sample amounts were 3 g in PLE and 2 g in QuEChERS. In a typical
272 preparation with QuEChERS extraction, a single analyst can manually prepare 8
273 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

374 found. The results obtained are in agreement with other studies in fish. For the first
375 time, Ramirez *et al.* detected the antiepileptic drug Cbz at a mean concentration of 1.1
376 ng/g in wet weight from fish muscle samples collected from two streams located in
377 Texas [28]. Finally, the results supports the hypothesis of low or no effective removal of
378 this pharmaceutical in conventional treatment plants, in line with previous studies [1-3].
379 However, none of the transformation products were detected in these tests. In any case,
380 recovery of the surrogate standards was above 70%, for both Cbz-d8 and Epoxy-d10.
381 These standards allowed us to verify that extraction method performance and analysis
382 were satisfactory. An example of identification of the targeted compound Cbz in a
383 marine mussel sample can see in supplementary data “Figure S5”. In full MS spectrum,
384 the measured mass for Cbz is shown at m/z 237.10221 which matches the theoretical
385 mass 237.10220 with an error of -0.1 ppm. The additional acquisition in full MS/MS
386 mode provided a more comprehensive identification of this anticonvulsant drug as well
387 as its structural characterization found for the characteristic fragment ion at m/z
388 194.09647 with a mass deviation of 0.2 ppm. Good mass accuracies were obtained in
389 the MS and MS/MS scans, which ensured correct identification in line with the other
390 evaluated parameters: retention time, empirical formula and RDBE.

391 3.4.2. Identification of non-target compounds

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

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

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

430

431 **Conclusions**

432 The analytical method developed based on HRMS permitted us to obtain different
433 fragmentation pathways, which proved very useful for suitable identification of
434 isomeric compounds (2OH, Epoxy and Ox), which is a common problem when several
435 transformation products are analyzed. The performance of the two extraction techniques
436 (PLE and QuEChERS) was compared. An improved in the results in relation to
437 accuracy and sensitivity was obtained using QuEChERS extraction. The major
438 advantages of the protocol proposed in this study were the short extraction and clean-up
439 times, combined with a highly selective and sensitive final detection system. The
440 methodology proposed enabled the detection of target pharmaceuticals and their
441 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

456 **Acknowledgements**

457 The authors are very grateful for the financial support given by the Sanofi-Aventis and
458 the French Agence Nationale de la Recherche (Project PEPSEA) this work was
459 conducted within the Chair Emerging Pollutants (Veolia-Hydrosciences). The authors
460 also gratefully acknowledge IFREMER for assistance with mussel collection and to
461 Pesticide Residue Research Group of the University of Almeria by providing standard
462 solution. M.J. Martínez Bueno wishes to thank University of Montpellier for the
463 postdoc grant.

464

464 **References**

- 465
466 [1] M.J. Martínez Bueno, M.J. Gómez, S. Herrera, M.D. Hernando, A. Agüera and A.R.
467 Fernández-Alba. Occurrence and persistence of organic emerging contaminants and
468 priority pollutants in five sewage treatment plants of Spain: Two years pilot survey
469 monitoring. *Environ. Pollut.* 164 (2012) 267-273.
- 470 [2] B. Kasprzyk-Hordern, R.M. Dinsdale and A.J. Guwy. The removal of
471 pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during
472 wastewater treatment and its impact on the quality of receiving waters. *Water Res.* 43
473 (2009) 363-380.
- 474 [3] T.A. Ternes. Occurrence of drugs in German sewage treatment plants and rivers.
475 *Water Res.* 32 (1998) 3245–3260.
- 476 [4] Recommendation of the European parliament and of the council of 30 may 2002
477 concerning the implementation of integrated coastal zone management in Europe
478 (2002/413/EC) official journal of the European Communities 6.6.2002.
- 479 [5] T. Kosjek, H.R. Andersen, B. Kompare, A. Ledin and E. Heath. Fate of
480 carbamazepine during water treatment. *Environ. Sci. Technol.* 43 (2009) 6256-6261.
- 481 [6] M. Leclercq, O. Mathieu, E. Gomez, C. Casellas, H. Fenet and D. Hillaire-Buys.
482 Presence and fate of carbamazepine, oxcarbazepine, and seven of their metabolites at
483 wastewater treatment plants. *Arch. Environ. Contam. Toxicol.* 56 (2009) 408-415.
- 484 [7] C.D. Metcalfe, X.S. Miao, B.G. Koenig, and J. Struger, Distribution of acidic and
485 neutral drugs in surface waters near sewage treatment plants in the lower great lakes,
486 Canada. *Environ. Toxicol. Chem.* 22 (2003) 2881–2889.
- 487 [8] T.A. Ternes, In *Pharmaceuticals and Metabolites as Contaminants of the Aquatic*
488 *Environment*. In *Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues*; Daughton, C.G., Jones-Lepp, T.L., Ed.; ACS Symposium Series 791; American Chemical Society: Washington, DC, 2001.
- 491 [9] K. Wille, M. Claessens, K. Rappé, E. Monteyne, C.R. Janssen, H.F. de Brabander,
492 and L. Vanhaeck. Rapid quantification of pharmaceuticals and pesticides in passive

- 493 samplers using ultrahigh performance liquid chromatography coupled to high resolution
494 mass spectrometry. *J. Chromatogr. A* 1218 (2011) 9162– 9173.
- 495 [10] X. Miao and C.D. Metcalfe. Determination of Carbamazepine and Its Metabolites
496 in Aqueous Samples Using Liquid Chromatography-Electrospray Tandem Mass
497 Spectrometry. *Anal. Chem.* 75 (2003) 3731-3738.
- 498 [11] O.P. Togunde, K.D. Oakes, M.R. Servos and J. Pawliszyn, Determination of
499 Pharmaceutical Residues in Fish Bile by Solid-Phase Microextraction Couple with
500 Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS). *J. Environ. Sci.*
501 *Technol.* 46 (2012) 5302–5309.
- 502 [12] R. Cueva-Mestanza, M.E. Torres-Padrón, Z. Sosa-Ferrera and J. Santana-
503 Rodríguez. Microwave-assisted micellar extraction coupled with solidphase extraction
504 for preconcentration of pharmaceuticals in molluscs prior to determination by HPLC. *J.*
505 *Biomed. Chromatogr.* 22 (2008) 1115–1122.
- 506 [13] A.M. Carro, D. García-Rodríguez, P. Gonzalez-Siso and R.A. Lorenzo.
507 Determination of chemotherapeutic agents in fish and shellfish by matrix solid-phase
508 dispersion and liquid chromatography-tandem mass spectrometry. *J. Sep. Sci.* 35 (2012)
509 2866–2874.
- 510 [14] E.D. Ramseya, A.T. Reesb, G. Weia, J.Y. Liua and X.H. Wua. Direct aqueous
511 supercritical fluid extraction coupled on-line with liquid chromatography–tandem mass
512 spectrometry for the analysis of polyether ionophore antibiotics in water. *J. Chromatogr.*
513 *A* 1217 (2010) 3348–3356.
- 514 [15] B. Subedi, M.A. Mottaleb, C.K. Chamblissa and S. Usenko. Simultaneous analysis
515 of select pharmaceuticals and personal care products in fish tissue using pressurized
516 liquid extraction combined with silica gel cleanup. *J. Chromatogr. A* 1218 (2011) 6278–
517 6284.
- 518 [16] I. García, M. Ignacio, A. Mouteira, J. Cobas and N. Carro. Assisted solvent
519 extraction and ion-trap tandem mass spectrometry for the determination of
520 polychlorinated biphenyls in mussels. Comparison with other extraction techniques.
521 *Anal. Bioanal. Chem.* 390 (2008) 729–737.

- 522 [17] G. Bianco, G. Novario, G. Anzilotta, A. Palma, A. Mangone and T.R. Cataldi,
523 Polybrominated diphenyl ethers (PBDEs) in Mediterranean mussels (*Mytilus*
524 *galloprovincialis*) from selected Apulia coastal sites evaluated by GC–HRMS. *J. Mass.*
525 *Spectrom.* 45 (2010) 1046–1055.
- 526 [18] L. Liguori, K. Heggstad, H.T. Hove and K. Julshamn. An automated extraction
527 approach for isolation of 24 polyaromatic hydrocarbons (PAHs) from various marine
528 matrixes. *Anal. Chim. Acta.* 573–574 (2006) 181–188.
- 529 [19] M. Anastassiades, S.J. Lehotay, D. Stajnbaher and F.J. Schenck. Fast and Easy
530 Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive
531 Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce. *J.*
532 *AOAC International.* 86 (2003) 412-431.
- 533 [20] A. Wilkowska, and M. Biziuk. Determination of pesticide residues in food matrices
534 using the QuEChERS Methodology. *Food Chem.* 125 (2011) 803–812.
- 535 [21] B. Kmellar, P. Fodor, L. Pareja, C. Ferrer, M.A. Martinez-Uroz, A. Valverde and
536 A.R. Fernandez-Alba. Validation and uncertainty study of a comprehensive list of 160
537 pesticide residues in multi-class vegetables by liquid chromatography–tandem mass
538 spectrometry. *J. Chromatogr. A* 1215 (2008) 37–50.
- 539 [22] S.C. Cunha, C. Cunha, A.R. Ferreira and J.O. Fernandes, Determination of
540 bisphenol A and bisphenol B in canned seafood combining QuEChERS extraction with
541 dispersive liquid–liquid microextraction followed by gas chromatography–mass
542 spectrometry. *Anal. Bioanal. Chem.* 404 (2012) 2453–2463.
- 543 [23] E. Gomez, M. Bachelot, C. Boillot, D. Munaron, S. Chiron, C. Casellas and H.
544 Fenet, H. Bioconcentration of two pharmaceuticals (benzodiazepines) and two personal
545 care products (UV filters) in marine mussels (*Mytilus galloprovincialis*) under
546 controlled laboratory conditions. *Environ. Sci. Pollut. Res.* 19 (2012) 2561–2569.
- 547 [24] Commission Decision 2002/657/EC, *Off. J. Eur. Commun.* L221 (2002) 8.
- 548 [25] SANCO/10684/2009. Method validation and Quality Control procedure for
549 pesticides residues analysis in food and feed, 2010. Document No. SANCO/10684/2009

- 550 [26] A.C. Hogenboom, J.A. van Leerda and P. de Voogt. Accurate mass screening and
551 identification of emerging contaminants in environmental samples by liquid
552 chromatography-hybrid linear ion trap Orbitrap mass spectrometry. *J. Chromatogr. A.*
553 1216 (2009) 510–519.
- 554 [27] ISO/IEC/EN 17025. General Requirements for the Competence of Calibration and
555 Testing Laboratories.
- 556 [28] A.J. Ramirez, M.A. Mottaleb, B.W. Brooks and C.K. Chambliss. Analysis of
557 pharmaceuticals in fish tissue using liquid chromatography - tandem mass spectrometry.
558 *Anal. Chem.* 79 (2007) 3155-3163.
- 559 [29] A. Togola and H. Budzinski. Multi-residue analysis of pharmaceutical compounds
560 in aqueous samples. *J. Chromatogr. A* 1177 (2008) 150–158.
- 561 [30] C.L. Chitescu, E. Oosterink, J. Jong and A.A.M. Stolker. Accurate mass screening
562 of pharmaceuticals and fungicides in water by U-HPLC_Exactive Orbitrap MS. *Ana.*
563 *Bioanal. Chem.* 403 (2012) 2997-3011.
- 564 [31] M.J. Martínez Bueno, S. Uclés, M.D. Hernando, E. Dávoli and A.R. Fernández-
565 Alba. Evaluation of selected ubiquitous contaminants in the aquatic environment and
566 their transformation products. A pilot study of their removal from a sewage treatment
567 plant. *Water Research* 45 (2011) 2331-2341.

568

569

570

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.

Accepted Manuscript

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

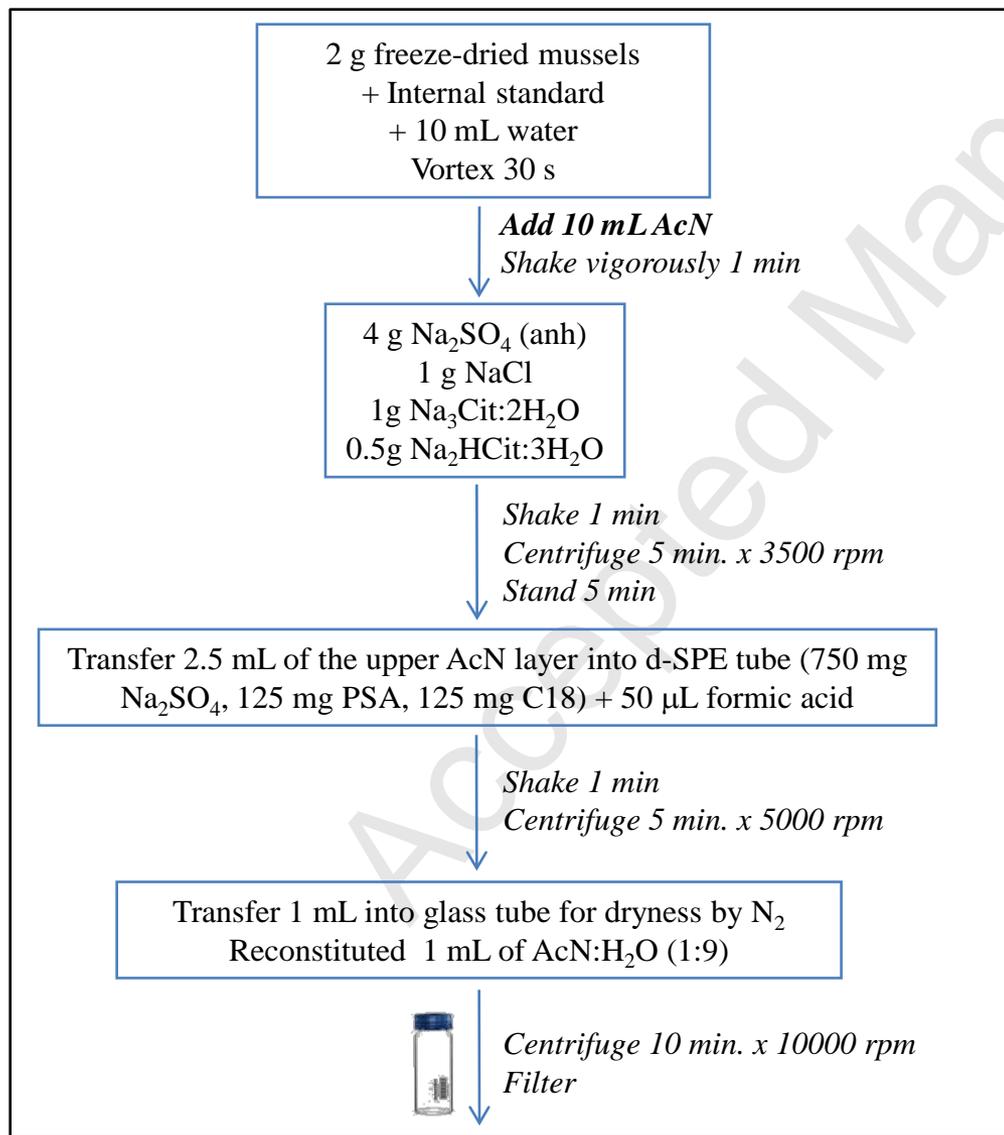


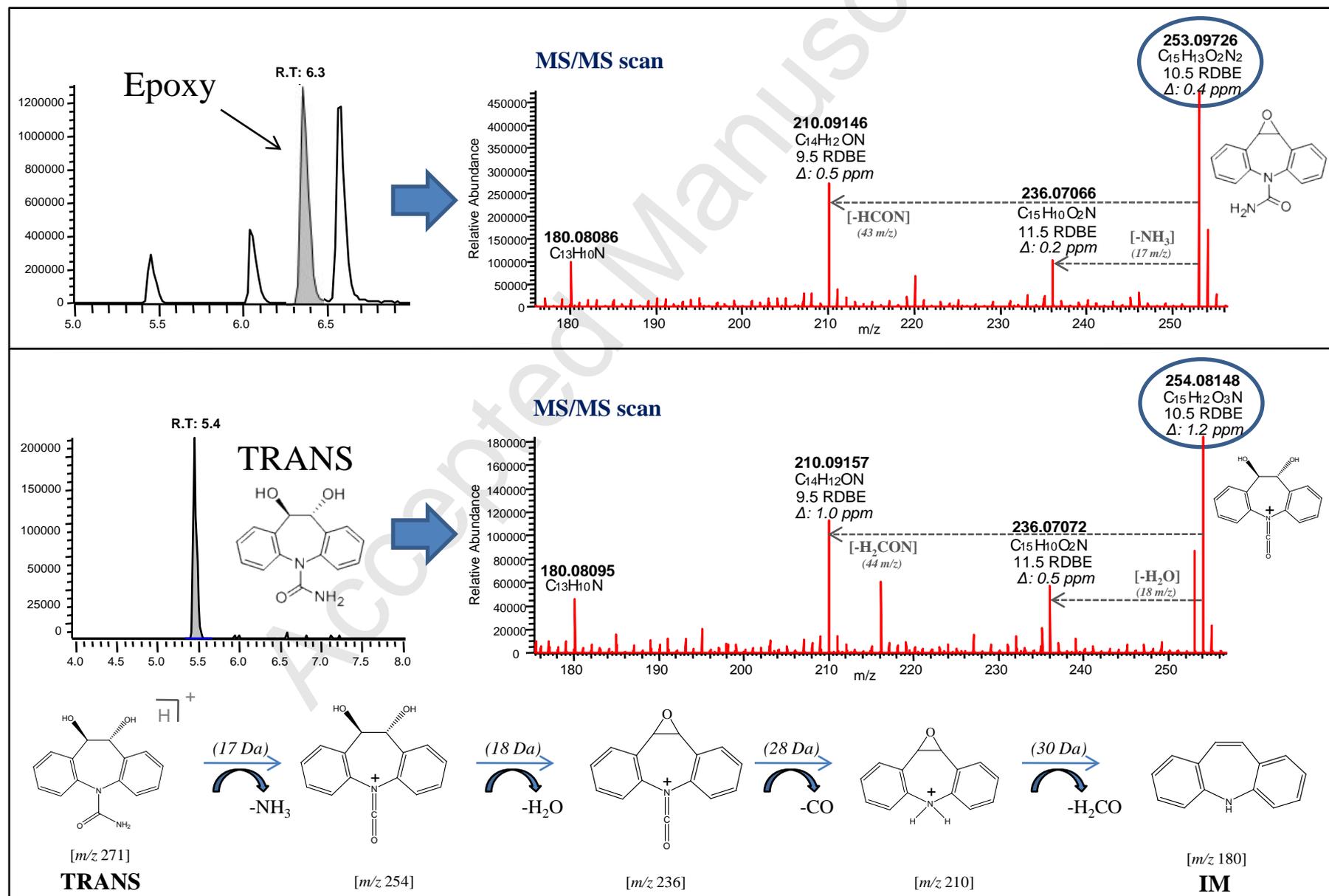
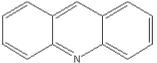
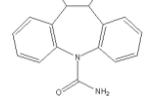
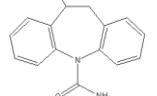
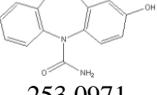
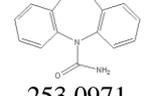
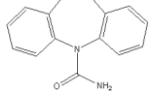
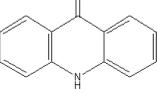
Figure 2. Product ion mass spectrum obtained for Epoxy and TRANS at 10 eV, and proposed fragmentation pathway for TRANS.

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. (%, ± σ)	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 ± 8	0.4	0.1	0.5
TRANS 5.4 min		C ₁₅ H ₁₅ N ₂ O ₃	9.5	271.1081	1.2	254.0812	C ₁₅ H ₁₂ NO ₃	254.0815	1.5	-17	5/9	93 ± 9	0.2	0.1	0.5
						210.0913	C ₁₄ H ₁₂ NO	210.0916	1.2						
						236.0706	C ₁₅ H ₁₀ NO ₂	236.0709	1.3						
10OH 5.8 min		C ₁₅ H ₁₅ N ₂ O ₂	9.5	255.1129	0.5	237.1022	C ₁₅ H ₁₃ N ₂ O	237.1025	1.0	-18	9/10	110 ± 2	0.8	0.3	1.5
						194.0966	C ₁₄ H ₁₂ N	194.0967	1.2						
2OH 6.1 min		C ₁₅ H ₁₃ N ₂ O ₂	10.5	253.0972	0.3	210.0913	C ₁₄ H ₁₂ NO	210.0914	0.6	-24	9/17	104 ± 10	1.0	0.3	1.5
						180.0807	C ₁₃ H ₁₀ N	180.0808	0.8						
EPOXY 6.4 min		C ₁₅ H ₁₃ N ₂ O ₂	10.5	253.0972	0.5	210.0913	C ₁₄ H ₁₂ NO	210.0915	1.1	1	11/14	75 ± 9	0.6	0.2	1.0
						180.0807	C ₁₃ H ₁₀ N	180.0808	0.7						
						236.0706	C ₁₅ H ₁₀ NO ₂	236.0717	2.0						
OX 6.6 min		C ₁₅ H ₁₃ N ₂ O ₂	10.5	253.0970	-0.6	236.0706	C ₁₅ H ₁₀ NO ₂	236.0706	0.2	-22	9/18	90 ± 6	0.6	0.2	1.0
						208.0756	C ₁₄ H ₁₀ NO	208.0758	0.8						
AO 6.8 min		C ₁₃ H ₁₀ NO	9.5	196.0758	0.6	-	-	-	-	-40	11/10	78 ± 2	0.2	0.1	0.5
CBZ 7.1 min		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 ± 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;