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, Hélène Fenet, Serge Chiron, Claude Casellas, Elena Gomez

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

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 Martínez Bueno, C. Boillot, H. Fenet, S. Chiron, C. Casellas and E. Gómez*

Laboratory of Hydrosciences Montpellier UMR 5569. Dept. Environmental Sciences and Public Health. University of Montpellier I, 34000 Montpellier, France

*Corresponding author: Tel.: +33 (0) 4 11 75 94 18; Fax: +33 (0) 4 11 75 94 61. E-mail: mjbueno@ual.es

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;

1. Introduction

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

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

Most methods for the analysis of organic micropollotants 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 (Cbz 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 g ± 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 $Na_3Cit{:}2H_2O$ and 0.5 g $Na_2HCit{:}3H_2O$ 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 35ºC 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 g ± 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 H_2O. The extracts were passed through cartridges and then dried. Elution was carried out with 6 mL of MeOH (method 1) or 2mL 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).

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 μL/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 formiate 10 mM/formic acid 0.1% buffer) were assayed. Only Cbz showed a sensitivity improvement when ammonium formiate 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, 300ºC 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: 3x106 (high dynamic), 1x106 (balance scan) and 5x105 (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 5x105 (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 NH3 (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

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

3.2.2. Optimization of the QuEChERS procedure

Because the extraction quality depends on the sample homogenization step, mussel samples were freeze-dried with a lyophilizer. Moreover, as suggested in the EN 15662 standard, a minimum water percentage is necessary to get good recovery in the salting-out extractions [27]. For this reason, 10 mL of water was initially added to each sample prior to QuEChERS extraction and then the sample was vortexed for 30 sec. Alternatively, in this study, MgSO4 (anh) was substituted for Na_2SO4 (anh), and then the samples were allowed to stand for 5 min after centrifugation so as to efficiently absorb the water. Our first attempts were focused on d-SPE optimization for the development of a rapid and easy sample preparation protocol. Mussels are fat or lipid containing matrices (about 15%) and, although fats are not very soluble in AcN, a certain quantity of them will be co-extracted, so they have to be removed prior to the final determination step [20]. For that, different clean-up sorbent materials (Z-Sep-Plus, Z-Sep/C18 and PSA/C18) used to enhance matrix interference removal were evaluated. Z-Sep material contain zirconium atoms, which act as a Lewis acid, while the phosphate groups in phospholipids act as a strong Lewis base, strongly binding with zirconium atoms. On the other hand, until now, PSA and C18 phases have mainly been used for the clean-up step in the QuEChERS method. As seen in Table 2, comparable recoveries from spiked mussel samples were observed for all target compounds when a percentage of 2% formic acid was added. The addition of 2% formic acid was a critical step. Many sample preparation techniques for biological matrices use acid to disrupt compound-protein binding, which directly affects recovery and matrix effect. No significant differences were observed between Z-Sep+ and Z-Sep/C18. However, the mixture PSA/C18/Na2SO4 provided the best results in comparison to these new sorbent materials for TRANS and 10OH transformation products, possibly due to the fact that they allow the elimination of a greater amount of matrix co-extractive interference (see

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

3.3. Analytical performance

A rigorous validation procedure according to SANCO/10684/2009 and ISO/17025 Guidelines was used to ensure high quality analytical measurements [25,27]. The mean recovery data and deviations obtained, which highlights the precision of the extraction method, are given in Table 1. Satisfactory recoveries were achieved combined QuEChERS extraction with the mixture PSA/C18/Na_2SO4 for the clean-up step. The average recovery for both spike levels (10 and 50 ng/g dw) were higher than 75%, for all target compounds included in the study, with the exception of Cbz (67%). The linearity of the analytical response for all the studied compounds within the studied range of three orders of magnitude was very good, with correlation coefficients higher than 0.997 in all cases. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in matrix. When the percentage of the difference between these slopes is positive, then there is signal enhancement, whereas a negative value indicates signal suppression. Table 1 shows the percentage of enhancement or signal suppression found in the evaluated matrix. According to our results, no compound generated a relevant matrix effect higher than 50%, being AO the analyte that showed the greatest signal suppression effects of -40%. Thus, 4 compounds showed no matrix effect (<20%, because this variation is close to the repeatability values), and 3 analytes presented a medium effect (50-20%). To minimize matrix interference, matrix-matched calibration curves were used to compensate for the matrix effect and avoid any under/over estimation during quantification. R.S.D values for the intra-day analysis (repeatability) ranged between 5% and 11%, while they were 7% to 18% for the inter-day analysis. This demonstrates the repeatability of the method and therefore its effectiveness for quantitative purposes. The reporting levels of the studied compounds

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

3.4. Analysis of real samples

3.4.1. Identification and quantitation of target compound

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

With regard to marine waters, Togola and Budzinski reported the presence of Cbz in the Mediterranean Sea at concentrations of approximately 10-40 ng/L [29]. More recently, higher concentrations were noted by Wille *et al*. in 2010 in a Belgian coastal region (up to 732 ng/L) [9]. Concerning the results obtained for target compounds in marine mussels collected from the Mediterranean Sea (n=10), the psychiatric drug Cbz was only found in two samples at concentrations above its MQL (1.5 ng/g). In the sample collected in the vicinity of an emissary area, Cbz was detected at levels of up to 3.5 ng/g dw, while in the lagoon sample, the concentration was below the MDL (0.5 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* 237.10221which 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

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

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

Acknowledgements

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

References

[1] M.J. Martínez Bueno, M.J. Gómez, S. Herrera, M.D. Hernando, A. Agüera and A.R. Fernández-Alba. Occurrence and persistence of organic emerging contaminants and priority pollutants in five sewage treatment plants of Spain: Two years pilot survey monitoring. Environ. Pollut. 164 (2012) 267-273.

[2] B. Kaspryk-Hordern, R.M. Dinsdale and A.J. Guwy. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res. 43 (2009) 363-380.

[3] T.A. Ternes. Occurence of drugs in German sewage treatment plants and rivers. Water Res. 32 (1998) 3245–3260.

[4] Recommendation of the European parliament and of the council of 30 may 2002 concerning the implementation of integrated coastal zone management in Europe (2002/413/EC) official journal of the European Communities 6.6.2002.

[5] T. Kosjek, H.R. Andersen, B. Kompare, A. Ledin and E. Heath. Fate of carbamazepine during water treatment. Environ. Sci. Technol. 43 (2009) 6256-6261.

[6] M. Leclercq, O. Mathieu, E. Gomez,C. Casellas, H. Fenet and D. Hillaire-Buys. Presence and fate of carbamazepine, oxcarbazepine, and seven of their metabolites at wastewater treatment plants. Arch. Environ. Contam. Toxicol. 56 (2009) 408-415.

[7] C.D. Metcalfe, X.S. Miao, B.G. Koenig, and J. Struger, Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower great lakes, Canada. Environ. Toxicol. Chem. 22 (2003) 2881–2889.

[8] T.A. Ternes, In Pharmaceuticals and Metabolites as Contaminants of the Aquatic 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: Washingon, DC, 2001.

[9] K. Wille, M. Claessens, K. Rappé, E. Monteyne, C.R. Janssen, H.F. de Brabander, 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.

[17] G. Bianco, G. Novario, G. Anzilotta, A. Palma, A. Mangone and T.R. Cataldi, Polybrominated diphenyl ethers (PBDEs) in Mediterranean mussels (*Mytilus galloprovincialis*) from selected Apulia coastal sites evaluated by GC–HRMS. J. Mass. Spectrom. 45 (2010) 1046–1055.

[18] L. Liguori, K. Heggstad, H.T. Hove and K. Julshamn. An automated extraction approach for isolation of 24 polyaromatic hydrocarbons (PAHs) from various marine matrixes. Anal. Chim. Acta. 573–574 (2006) 181–188.

[19] M. Anastassiades, S.J. Lehotay, D. Stajnbaher and F.J. Schenck. Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce. J. AOAC International. 86 (2003) 412-431.

[20] A. Wilkowska, and M. Biziuk. Determination of pesticide residues in food matrices using the QuEChERS Methodology. Food Chem. 125 (2011) 803–812.

[21] B. Kmellar, P. Fodor, L. Pareja, C. Ferrer, M.A. Martinez-Uroz, A. Valverde and A.R. Fernandez-Alba. Validation and uncertainty study of a comprehensive list of 160 pesticide residues in multi-class vegetables by liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1215 (2008) 37–50.

[22] S.C. Cunha, C. Cunha, A.R. Ferreira and J.O. Fernandes, Determination of bisphenol A and bisphenol B in canned seafood combining QuEChERS extraction with dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry. Anal. Bioanal. Chem. 404 (2012) 2453–2463.

[23] E. Gomez, M. Bachelot, C. Boillot, D. Munaron, S. Chiron, C. Casellas and H. Fenet, H. Bioconcentration of two pharmaceuticals (benzodiazepines) and two personal care products (UV filters) in marine mussels (*Mytilus galloprovincialis*) under controlled laboratory conditions. Environ. Sci. Pollut. Res. 19 (2012) 2561–2569.

[24] Commission Decision 2002/657/EC, Off. J. Eur. Commun. L221 (2002) 8.

[25] SANCO/10684/2009. Method validation and Quality Control procedure for 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/Na_2SO4 (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.

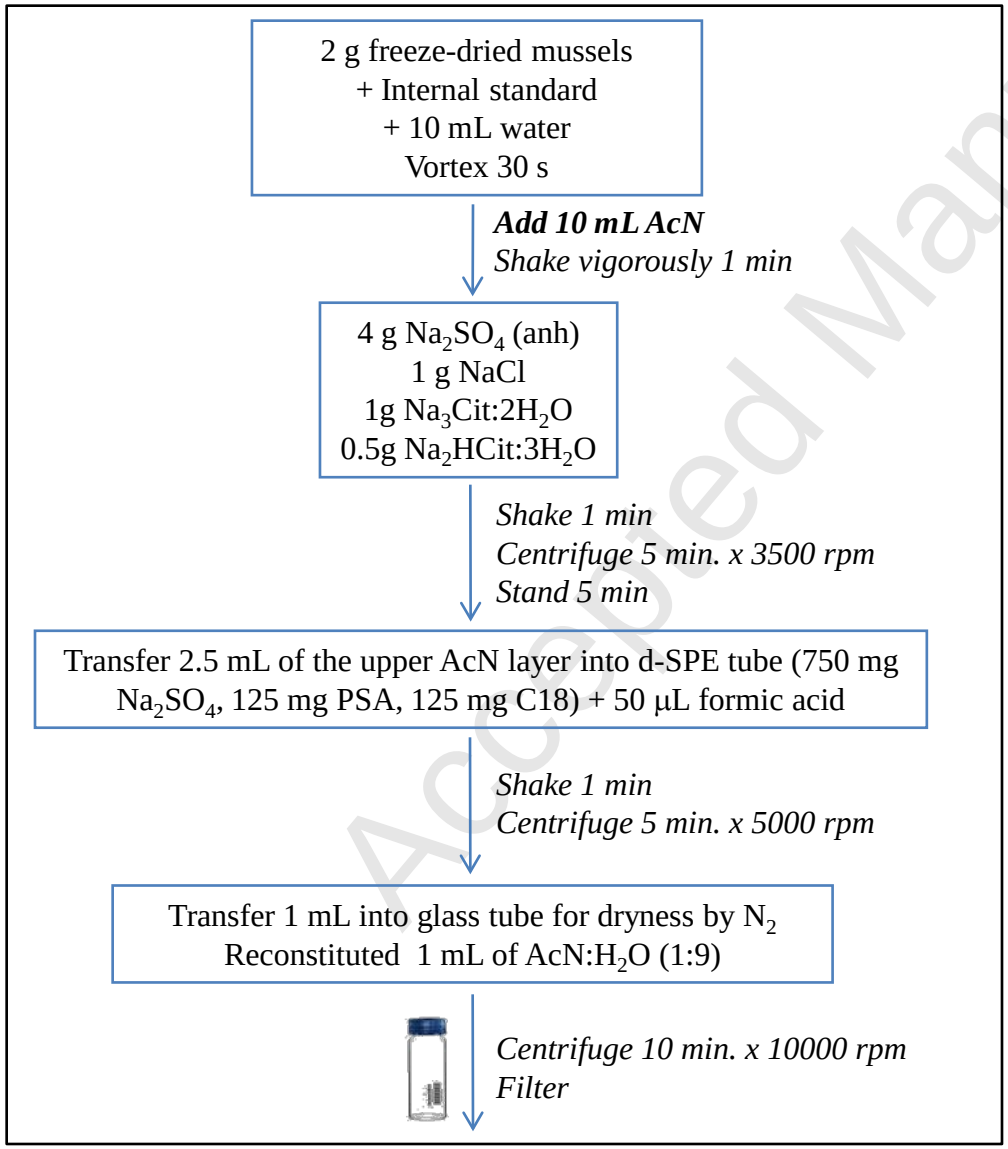


Figure 2. Product ion mass spectrum obtained for Epoxy and TRANS at 10 eV, and proposed fragmentation pathway for TRANS.

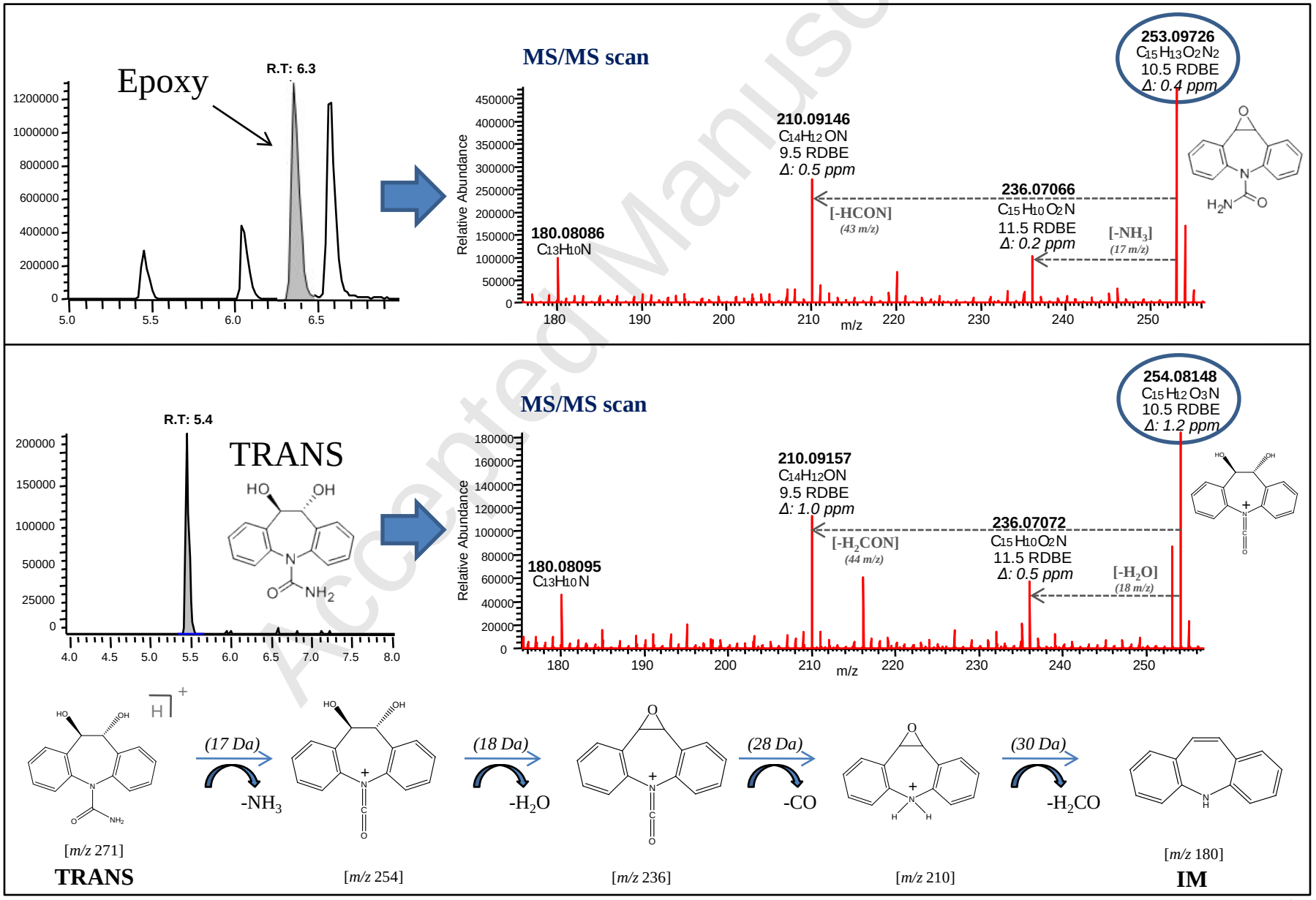
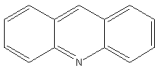
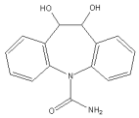
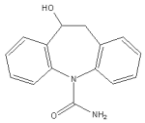
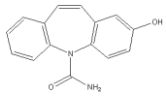
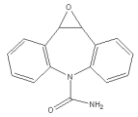
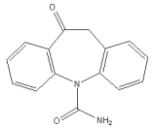
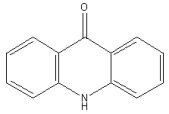
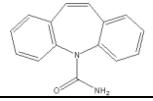


Table 1. Analytical performance and MS - MS/MS data for each target compound studied by LC-Orbitrap-MS method.

Compound tr (min)	MS					MS/MS									
	Theo. Mass	**Formula $[M+H]^+$**	**RDBE**	**Exp. Mass[1]**	**Δ^2** *(ppm)*	**Theo. Mass**	**Formula**	**Ex. Mass[1]**	**Δ^2** *(ppm)*	**Matrix Effect**	**Inter/intra -day** *(%)*	**Rec.** *(%, ± σ)*	**LOQ** *(ng/g)*	**LOD** *(ng/g)*	**MDL** *(ng/g)*
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	271.1077 	$C_{15}H_{15}N_2O_3$	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 ± 9	0.2	0.1	0.5
10OH 5.8 min	255.1128 	$C_{15}H_{15}N_2O_2$	9.5	255.1129	0.5	237.1022 194.0966	$C_{15}H_{13}N_2O$ $C_{14}H_{12}N$	237.1025 194.0967	1.0 1.2	-18	9/10	110 ± 2	0.8	0.3	1.5
2OH 6.1 min	253.0971 	$C_{15}H_{13}N_2O_2$	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 ± 10	1.0	0.3	1.5
EPOXY 6.4 min	253.0971 	$C_{15}H_{13}N_2O_2$	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 ± 9	0.6	0.2	1.0
OX 6.6 min	253.0971 	$C_{15}H_{13}N_2O_2$	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 ± 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 ± 2	0.2	0.1	0.5
CBZ 7.1 min	237.1022 	$C_{15}H_{13}N_2O$	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).

	QuEChERS				**PLE**	
Clean-up (QuEchERS) / Elution solvent (PLE)	Z-Sep+ 50 µL FA*	Z-Sep+	Z-Sep/C18 50 µL FA*	PSA/C18 50 µL FA*	MeOH	EtAc
Sample amount *(g)*	2				3	
Solvent total volume *(mL)*	10 mL AcN				70 mL H_2O:acetone + 6 mL MeOH	70 mL H_2O: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;