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Grau de Química

Treball Final de Grau

Identification of organic contaminants accumulated in mollusc by means of High Resolution Mass Spectrometry

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Science is the great antidote to the poison of enthusiasm and superstition.

Adam Smith

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REPORT

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1. SUMMARY

Chemical contamination of estuarine and coastal areas has negative implications for the environment and may also pose a risk for human health through the ingestion of contaminated fish and shellfish. Therefore, as part of the XENOMETABOLOMIC project, the present work aims to identify priority mixture of contaminants accumulated in wild mussels from Ebro Delta (*Mytilus galloprovincialis)* that may be of relevance for future monitoring and regulation. Moreover, it intends to elucidate potential differences in contamination patterns between the two Ebro Delta bays, Alfacs and Fangar, and provides data of the occurrence and distribution of the contaminants in mussels from each bay.

For this purpose, a previously developed analytical method was used, based on ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS). Sample extraction and purification were performed by using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology.

The analysis reveals the presence of 11 out of 23 compounds included in the analytical method, comprising organonitrogen pesticides, herbicides, endocrine disruptor compounds (EDCs), and pharmaceutically active compounds (PhACs). Their concentrations in mussels ranged from 0.19 ng/g dry weight (dw) of desethylatrazine to a maximum concentration of 19.25 ng/g dw of methylparaben. The total concentrations of contaminants (expressed as the sum of their single concentrations) at every sampling point did not exceed 25 ng/g dw.

The levels found in the present work are far below the maximum residues limits (MRL) established by the European Union, so that a potential risk to human health through mussel ingestion is unlikely to happen.

Keywords: priority contaminant mixture, mussel, bioaccumulation, risk, Ebro Delta, UHPLC-HRMS

2. RESUM

La contaminació química de les zones costeres i els estuaris té implicacions negatives pel medi ambient i podria suposar, a través de la ingesta de peix i marisc, un risc per la salut humana. Per això, com a part del projecte XENOMETABOLOMIC, aquest treball té l'objectiu d'identificar mescles prioritàries de contaminants acumulades en musclos silvestres *Mytilus galloprovincialis* del Delta del Ebre, que podrien ser rellevants per a una futura monitorització i regulació. A més a més, pretén dilucidar les possibles diferències en els patrons de contaminació entre les dues badies que formen el Delta del Ebre, Alfacs i Fangar, i proporciona dades de la presència i distribució dels contaminants en musclos dins d'una mateixa badia.

Amb aquest objectiu, s'ha utilitzat un mètode prèviament desenvolupat, basat en cromatografia de líquids d'ultra alta resolució (UHPLC) acoblada a espectrometria de masses d'alta resolució (HRMS). L'extracció i purificació de la mostra s'ha dut a terme basant-se en una metodologia QuEChERS (ràpid, fàcil, econòmic, eficaç, sòlid i segur).

L' anàlisi revela la presència d' 11 dels 23 compostos continguts en el mètode analític, incloent pesticides organonitrogenats, herbicides, disruptors endocrins (EDCs) i compostos farmacològicament actius (PhACs). Les seves concentracions oscil·len entre 0.19 ng/g pes sec de desetilatrazina i concentracions màximes de 19.25 ng/g pes sec de metilparabè. La concentració total de contaminants (expressada com la suma de les seves concentracions particulars) a cadascun dels punts de mostreig no sobrepassa els 25 ng/g pes sec.

Els nivells trobats en aquest treball es troben molt per sota del límit màxim de residus establerts per la Unió Europea. Per tant, és improbable que existeixi cap risc potencial per a la salut humana a través de la ingesta de musclos.

Paraules clau: mescles prioritàries de contaminants, musclos, bioacumulació, risc, Delta del Ebre, UHPLC-HRMS

3. INTRODUCTION

Large amounts of organic and inorganic pollutants are continuously introduced by human activities into the environment. This chemical contamination reaches coastal and estuarine areas from land based and diffuse sources [1], being municipal and industrial wastewater discharges, recreational activities, agricultural runoff and aquaculture the most important sources of contamination [2].

Chemical contamination of estuarine and coastal areas has negative implications for the environment and may also pose a risk for human health through the ingestion of contaminated fish and shellfish. Certain chemicals can exhibit a bioaccumulative potential and remain in marine organism tissues [2], with the consequent potential risk of exposure for consumers.

Moreover, most of these compounds can act at very low concentrations which raises the concern about their potential to cause adverse effects such as development of bacterial resistant or allergies [3] in wild organisms and also in humans.

3.1. AREA OF STUDY

The Ebro delta is a 320 km^2 wetland area of international importance for waterbird conservation [4] placed in the western Mediterranean (Spain).

The main economic activity of the area is agriculture, which is mostly dominated by rice (about 80% of the land is dedicated to its production) [4]. The rice fields are covered with running water during the growing season and the excess water is removed through drainage channels discharging into the two bays that form the delta: Alfacs (southern) and Fangar (northern).

Shellfish culture has been well developed and has become the second economic activity of the area after agriculture. Around 3.000 tonnes of bivalve molluscs are produced every year, being mussels the 95% of total production, which are distributed in 166 fixed mussel culture rafts that are spread between the two Ebro delta bays [4].

It is also important to notice that there is a nearby well developed industrial area located in the Ebro basin [5] besides many urban settlements close to the estuarine environment. Due to

the importance of tourism in the area, recreational activities have also increased, being considered another source of contamination. Therefore, a complex mixture of contaminants may potentially reach the coast generating a “cocktail” of contaminants of potential concern that can be accumulated in marine organisms such as mollusc. To identify the most relevant compounds included in this “cocktail” has become a hot topic and the European Union is aware of this necessity [6]. Hence, to develop systematic ways of addressing chemical mixtures in environmental assessment [7] and to identify priority mixtures of potential concern is one of the major challenges nowadays.

3.2. MUSSELS, SENTINEL ORGANISMS

Bivalves like mussels are mobility-limited filter feeders which draw in water and particulates from their surrounding environment and subsequently bioaccumulate contaminants in their tissues [8] . They are successfully used as indicator organisms for marine pollution monitoring [9] due to its global distribution of large and accessible population, its large size and sedentary adulthood, the ventilation of large volumes of water for nutrition and its ability to accumulate numerous contaminants [10], which enables them to concentrate chemicals in their tissues in proportional amounts to the levels present in water [11].

For instance, they are included in the Mussel Watch Program of the United States which measures the concentrations of coastal contaminants in bivalves and sediments to provide information for assessing the potential risk to marine wildlife and humans through the use of coastal resources [12].

Besides, in the 1990s the Spanish Institute of Oceanography (IEO) started a monitoring program along the Mediterranean coast of Spain, using *Mytilus galloprovincialis* as bioindicator species [13].

Moreover, mussels are very popular in human diet. The European market for mussels is estimated to be slightly below 600.000 tonnes in equivalent live animal weight [14] and Spain has one of the higher index of seafood consumption. Concretely, it has a per capita consumption over 3 Kg of mussel per year [14].

Mussels provide essential nutrients for humans but there might be as well transference of environmental contaminants, what underscores the necessity of identifying priority mixtures of contaminants accumulated in this organisms.

3.3. PREVIOUS STUDIES IN EBRO DELTA

The contamination status of the Ebro Delta has been previously researched, particularly for certain persistent organic pollutants (POPs) such as polychlorinated biphenils (PCBs), polycyclic aromatic hidrocarbons (PAHs) and pesticides. For instance, along the eighties and nineties, organochlorinated pesticides and PCBs were determined and monitored in mussels [15] and fish [16] from Ebro Delta. The levels found were of several dozens of ng/g wet weight (ww) and achieved maximum concentrations of hundreds ng/g ww [17]. A general decline in concentrations of all organochlorinated pesticides and PCBs was observed [15] and confirmed in subsequent reports [18], as a reflect of the regulations adopted in the early eighties.

Since the beginning of the century, the study was extended to organophosphorous pesticides (OPs) and PAHs in bivalves. Fenitrothion exhibited the highest concentration for a pesticide and was detected at concentrations around 5 ng/g wet weight in mussels [19]. PCBs presented similar levels to the ones found in last decades and PAHs were the family of compounds reaching the highest levels up to 100 ng/g wet weight in *Mytilus galloprovincialis* [19].

Moreover, in this last decade many studies began to question the effect of this pollutants in bivalve metabolome [20][21][22] while others noticed the relationship between high concentrations of pollutants and bivalves mortality episodes [4].

Recently, few novel studies have investigated the presence of emerging contaminants such as endocrine disruptor compounds (EDCs) and pharmaceutically active compounds (PhACs) in bivalves, fish and microalgae samples [3][23] from Ebro Delta. The most ubiquitous compounds detected were the psychiatric drug venlanfaxine and the antibiotic azithromycin, with the highest concentrations found in mussel (2.7 ng/g dw) and oyster (3.0 ng/g dw), respectively [3].

It has also been widely studied the contamination of bivalves with certain inorganic pollutants such as heavy metals (Pb, As, Cd…) coming from industrial activities and hunting practices [6]. Levels found were at around a few μg/g dw for most of them, being particularly high (hundreds of μg/g dw) for Zn and Fe [23][24][25].

So far, scientific reports have focused their study on the development of analytical methods to assess the presence of compounds from a single family of pollutants. To the best of our knowledge, there is not any work performed yet that aims to identify priority mixtures of contaminants of potential concern present in wild bivalves.

4. OBJECTIVES

The main objective of this proposal was to perform a novel study in wild mussels in order to identify a mixture of contaminants accumulated in this organism that may be of relevance for future monitoring and regulation. Some other specific objectives were:

- To quantify the contaminants positively identified with levels above method quantification limits.
- To study their occurrence and distribution inside each bay.
- To distinguish potential differences in contamination pattern between Alfacs and Fangar bays

5. EXPERIMENTAL SECTION

5.1. FIELD EXPERIMENT AND SAMPLING

A total of eight sampling points were allocated in Ebro Delta nearby contamination sources previously identified. Concretely, 5 sampling sites were located in Alfacs bay, named BAP1, BAP2, BAP3, BAP4 and BAP5 and 3 sampling sites in Fangar bay, named BFP1, BFP2, and BFP3. Their exact location are shown in figures 1 and 2. BAP1 was selected as external sampling site and initially considered as the "clean site". For mussels farming, a rope of around 3 m length was located in BAP1 hanging from rafts and fixed to the bottom. Specimens of *Mytilus galloprovincialis* native from Ebro Delta were cultivated and maintained there until the beginning of the field experiment. The experiment started in May 2017 when several nets containing 100 specimens each were deployed at each sampling site. The exposure to the natural waters at each site lasted for about 1 month (from May 25th to June 28th) in all sampling points except BAP5. This sampling site is located in a shallow area very close to an urban untreated waste water discharge. In previous experiments high mortalities were observed there after one week of exposure mainly due to the high load of faecal bacteria coming from the sewer. Therefore, in this sampling point the deployment and exposure of mussels was carried out only during the first week. Once the exposure period finished the samples were taken. The total number of individual organisms collected at each sampling site was 40. They were of similar size (5-7 cm of shell length) and satisfied the legal requirements of harvestable size or weight for human consumption. A pool was prepared with the edible content of the mussels, the shell was discarded and all edible tissue together with intervalvar liquid was added to the pool. Then, each pool was grinded, homogenized, freeze-dried and kept at -20ºC until its analysis.

Fig. 1: Sampling points in Alfacs bay (10/05/18 via Google Earth)

Fig. 2: Sampling points in Fangar bay (10/05/18 via Google Earth)

5.2. MATERIALS AND METHODS

5.2.1. Standards and reagents

All standards were of high purity grade (>90%) and they were purchased from Sigma-Aldrich. Isotopically labelled compounds used as internal standards were also purchased from Sigma Aldrich except metoalachlor-d6, thiabendazole-c13, malathion-d7 and triclosan-d3 that were purchased from Dr. Ehrenstorfer, propanil-d5 and sulfamethoxazole-d4 from Toronto Research Chemicals, and caffeine-d3 and bisphenol A d-4 from CDN Isotopes. Individual stock standards and isotopically labelled standards were prepared in methanol at a concentration of 10 ppm. Working standards solutions of 1 ppm, containing either standards or isotopically labelled internals standards were prepared in 100% acetonitrile (ACN) before each analytical run.

5.2.2. Samples analysis

Each lyophilized sample was grounded in a mortar and three replicates, each of 1g, were weighted and analysed by using QuEChERS (Bekolut Citrat-Kit-01). Prior to the extraction, the internal standards mixture was added (see appendix 2), vortexed and left to equilibrate overnight in refrigerated conditions. The next day 10 mL of ACN and 5 mL of HPLC water were added to each replicate together with a mixture of salts containing 4g of $MgSO_4$, 1g of NaCl, 1g of NaCitrate and 0.5g of disodium citrate sesquihydrate. Then the sample was vortexed (1 min at 2500 rpm) and centrifuged (5 min at 4000 rpm at 15ºC). Immediately after, 6 mL of the supernatant liquid was transferred to a centrifuge tube of 15 mL to perform the dispersive solid phase extraction (dSPE) by adding Quechers Bekolut PSA-Kit-04A consisting in 4mg of primary secondary amine (PSA), 400mg of octadecylsilane (C18e) and 1200mg of $MgSO_4$. The mixture was vortexed and centrifuged again (1 min at 2500 rpm and 5 min at 4000 rpm at 15ºC respectively). The supernatant liquid was transferred to a glass tube to evaporate under nitrogen until complete dryness, redissolved in 1 mL of ACN and filtered through a phospholipids removal plate for purification. The filtered liquid was transferred to appropriate vials for their injection in Ultra-High Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS).

5.2.3. Analysis by UHPLC-HRMS

The mussel extracts were analysed by ultra-high performance liquid chromatography coupled to orbitrap Q-exactive high resolution mass spectrometry. Chromatographic separations were

carried out with an Acquity Ultra-PerformanceTM Water liquid chromatograph system (Milford, MA, USA), equipped with two binary pumps systems using a Purospher STAR RP-18 end-capped column (150 mm x 2.1 mm, 2 µm particle size).

The optimized separation conditions were a regular flow rate of 0.2 mL/min and an elution gradient varying the concentration of ACN from 10% to 100% after 13.5 minutes, with a total runtime of 25 minutes (see table 1). Volume of injection was 20 µL.

The UHPLC instrument was coupled to a Q-exactive orbitrap mass spectrometer (Q exactive™ Thermofischer Scientific, San Jose, CA, USA) equipped with an electrospray ionization source. The samples were run both in positive and negative ionisation modes.

Table 1: Elution gradient. A=ACN B=H_2O

Time (min)	% A	%B
0	10	90
2.5	50	50
12.5	80	20
13.5	100	-
15.5	100	-
16.5	10	90
25	10	90

The concentrations measured in the sample were determined by using internal calibration. For this purpose a calibration curve ranged between 1 and 50 ng/mL of the target compounds was prepared containing as well 50 ng/mL of the internal standards used (for details see appendix 2). The quantification was done by using Thermo Xcalibur Software v. 3.1.

5.2.4. Statistical analysis

For comparison of the contaminant concentration between the sample points at each bay, statistical analysis of two independent groups was performed according to the Mann-Whitney U-test for non-parametric data, identified as such by the Kolmogorov-Smirnov test. The significance level was set at $p \leq 0.5$. Kolmogorov-Smirnov test and Mann-Whitney U-test were performed with IBM SPSS Statistics Software v.24.

Principal Component Analysis (PCA) was conducted using SOLO Ink Software v.8.6.1 to study differences between bays and contamination patterns and levels in sampling points.

6. RESULTS AND DISCUSSION

6.1. OCCURRENCE AND LEVELS OF CONTAMINANTS

The analysis of the mussel samples revealed the presence of 11 out of 23 compounds included in the analytical method, considering both bays and all sampling points. Only three of them (desethylatrazine, atrazine and propylparaben) were detected below the limit of quantification (LOQ) established in the analytical method (appendix 1, table 3). The remaining 10 compounds were quantifiable (levels above their respective LOQ) in at least one sampling point. Their concentrations ranged from 0.19 ng/g dry weight (dw) of desethylatrazine in mussels from Alfacs bay, sampling point BAP4, to a maximum concentration of 19.25 ng/g dw of methylparaben in the same bay, sampling point BAP1 (table 2, page 21). The contaminants quantified in samples from Fangar bay ranged in a smaller interval of concentration, from 0.38 ng/g dw of carbamazepine in sampling point BFP3 up to 9.36 ng/g dw of bentazone in the same sampling point.

Four out of the six families of contaminants included in the analytical method were identified in mussel samples from at least one location (table 2). Therefore, the mixture of contaminants predominant in the area of study was formed by organonitrogen pesticides, herbicides, endocrine disrupting compounds (EDCs) and pharmaceuticals compounds (PhACs). Concretely, by the following compounds: desethylatrazine, atrazine, bentazone, MCPA, triclosan, methylparaben, ethylparaben, propylparaben, 1H-benzotriazole, venlafaxine and carbamazepine. The group of contaminants with more positive identifications (5 contaminants) was EDCs. It deserves to be mentioned that this group is also the one with more representative compounds, since a wide range of chemicals can cause endocrine disruption, so this group encompasses a heterogeneous class of molecules. In contrast to EDCs, organophosphorus pesticides and insecticides were not detected in any sampling site (levels below their respective limit of detection (LOD), appendix 1, table 3).

The levels of contaminants mixture found at each sampling site are presented in figure 3 as the sum of the compounds' concentrations measured. They ranged between 6.02 ng/g dw in BAP3 and 24.02 ng/g dw in BFP3. However, most of the sampling points showed a total pollutants concentration between 15 and 20 ng/g dw. The levels detected in the present study (in the low nanograms per grams) are in the same range that the ones reported previously in the literature by other authors for EDCs and PhACs [3][23][8], and similar pesticides [24] in marine organisms.

Regarding mussel's safety for human consumption, the levels found here are far below the maximum residues limits (MRL) established by the European Union. Usually, they are around 200 ng/g for pesticides in food from animal origin (comparison done with terrestrial animals because for aquatic organisms the levels haven't been set yet [25]) and for pharmaceuticals in fish [26]. Therefore, a potential risk to human health through mussel ingestion is unlikely to happen.

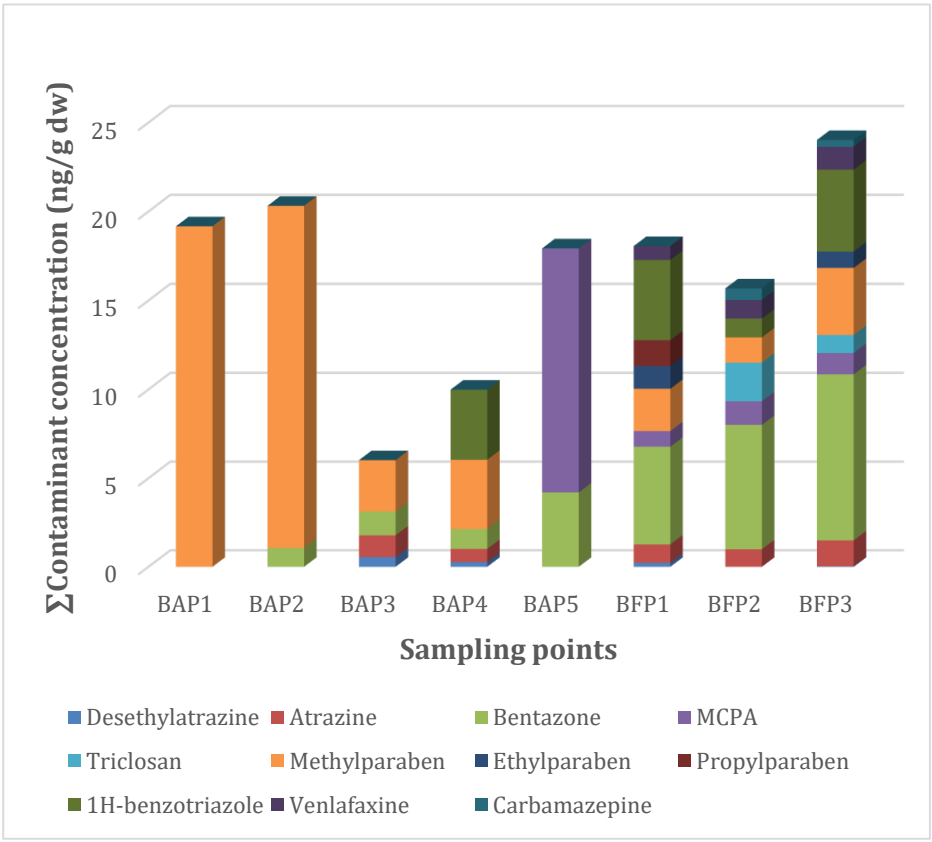

Fig. 3: Total contaminants concentration (ng/g dw) in mussel samples from Alfacs and Fangar bays

6.2. CONTAMINATION DIFFERENCES BETWEEN BAYS

Clear differences in contamination pattern have been found between the two bays after a PCA multivariate analysis (figure 4). Total variability explained by this PCA accounted for 68%. The first axis (PC1) explains the highest variability (almost 45%) and clearly depicts two different clusters depending on the bay origin. The second axis (PC2) explains 23% of the variability and is mainly associated with the differences between sampling points in the same bay. These results highlight the existence of different patterns of pollution considering the presence and levels of the contaminants in each bay.

Regarding the presence, between 1 and 5 different contaminants were found in mussels from Alfacs bay, while Fangar's samples presented 8 or 9 different compounds in the mixture. Moreover, the chemical group of the compounds present in each bay was also a differentiating factor among them. Most of the quantified PhACs and EDCs (with the exception of methylparaben and 1H-benzotriazole) were only present in Fangar bay, while pesticides and herbicides were detected and quantified in both bays.

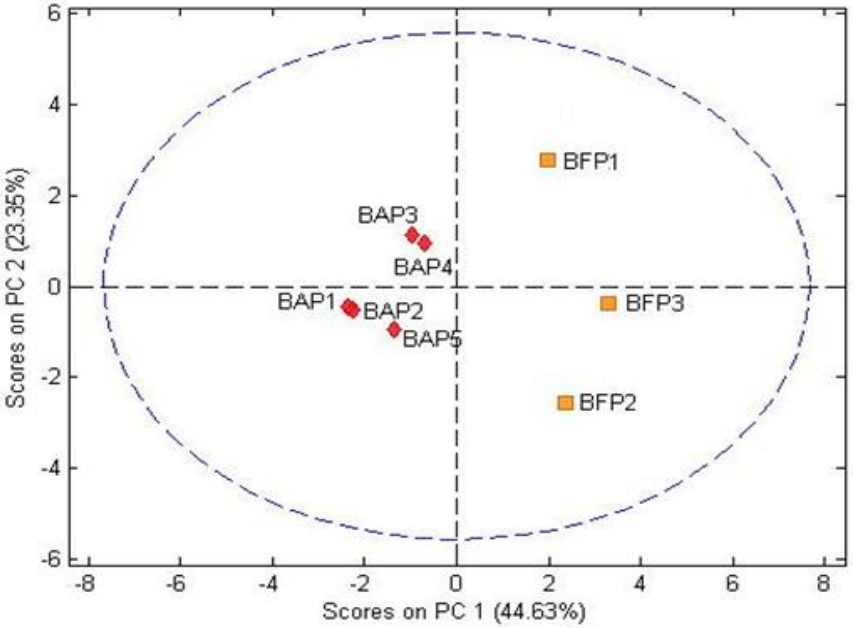

Fig. 4: PCA score plots of the sampling points in Alfacs and Fangar bays described by the mixture of contaminants and their average levels in each sample. The percentage of explained variation of the first two components is displayed on the relative axes.

Although total concentration ranges (the sum of all concentrations of contaminants) were similar in both bays (figure 3), the levels of some particular compounds were different between them. On the one hand, methylparaben concentration was found to be the most discriminant factor in the separation of Alfacs sampling points as a group. On the other hand, the levels of bentazone were an important differentiation factor for Fangar samples (figure 5).

The prior statements may indicate different sources of contamination in each bay, since the nature (or chemical group), concentration and number of compounds detected were significantly different in Alfacs and Fangar. Contamination input from agricultural runoff was common in both bays as it was expected (pesticides like desethylatrazine and MCPA appeared between the two clusters) but Fangar bay received a higher pollutant's input from urban sources (such as parabens, venlafaxine, carbamazepine and triclosan) (figure 5).

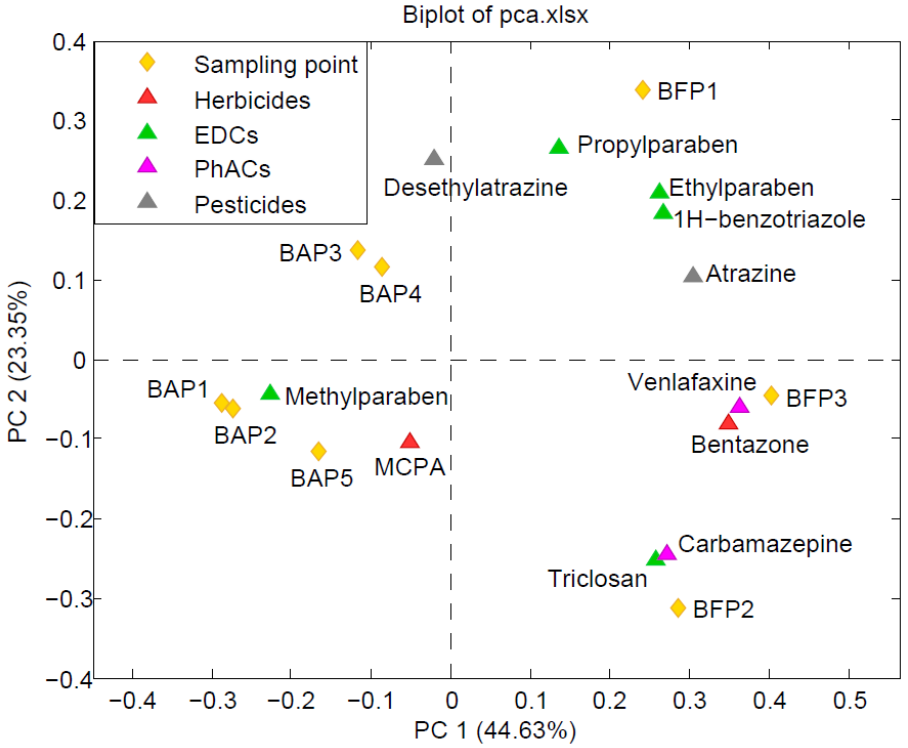

Fig.5: PCA biplot of the loadings (contaminants, in triangles) and scores (sampling points, in diamonds) of the first two Principal Components. The percentage of explained variation of the first two components is displayed on the relative axes

6.3. CONTAMINATION DIFFERENCES BETWEEN SAMPLING POINTS IN THE SAME BAY

6.3.1. ALFACS BAY

Differences among sampling points located in the same bay were also found. Concretely, in Alfacs bay three clusters were observed in the PCA analysis (figures 4 and 5). The first cluster includes BAP1 and BAP2, the second BAP3 and BAP4 and the third BAP5.

The first group (BAP1 and BAP2) is clearly separated by methylparaben's concentration (figure 5) with levels significantly higher ($p\ value \leq 0.05$ according to Mann-Whitney test) than in the other sampling points (figure 6 A). The highest concentrations of this compound (near 20 ng/g dw) were detected in BAP1 and BAP2. BAP1 was initially considered as the "clean" site because it is located outside of Alfacs bay in an area without any direct contaminant input such as effluents from waste water discharges or agricultural waste. Therefore, a potential source of methylparaben contamination could be anti-fouling paintings for boats, since BAP1 and BAP2 are located close to maritime ports and methylparaben can be used as additive in the formulation of biocides [27][28]. Besides, methylparaben is the most polar compound among the parabens studied here, therefore it could be more likely solubilized in sea water. This is an initially hypothesis and further investigation are required.

The second cluster formed by BAP3 and BAP4 is characterised by holding the lowest total contaminants concentration in the whole study (BAP3 less contaminated site, figure 3) and for the presence of organonitrogen pesticides such as desethylatrazine and atrazine. These are the only sampling points in this bay where this family of pollutants was found. Besides, differences within these two sampling points lie in the significantly higher concentrations ($p \leq 0.05$) of these two pesticides in BAP3 (figure 6A). This results evidence a higher load of contamination coming from rice fields' runoff in this zone of the Alfacs bay.

Finally, BAP5 presented significantly higher concentrations of herbicides (bentazone and MCPA) while no other contaminant was found above the limit of detection.

6.3.2. FANGAR BAY

Opposite to Alfacs bay, every single Fangar sampling point presented different characteristics and, therefore, no possible cluster could be done. In this bay the mixture of contaminants presents indicated a higher complexity in the contamination features.

BFP1 presented significantly higher concentrations of ethylparaben, propylparaben and 1H-benzotriazole ($p \leq 0.05$), all them EDCs, showing a high importance of urban sources of contamination in this sampling point (figure 6 B). Herbicides such as bentazone and MCPA, which could be related to agricultural sources of contamination, were also quantified but significant differences were not found (according to Mann-Whitney test) with the other sampling sites.

BFP2 presented significantly higher concentrations of the EDC triclosan respect the other sampling points ($p \leq 0.05$) (figure 6 B) with a maximum concentration of 2 ng/g dw. Triclosan was only present in BFP2 and BFP3 and a clear trend could not be established although it comes mainly from urban discharges of waste water.

BFP3 presented significantly higher concentrations ($p \leq 0.05$) of atrazine, bentazone, methylparaben and 1-H benzotriazole compared with the other sampling sites in this bay. These compounds indicate an important input of both agricultural and urban discharges into this bay. Moreover, this is the sampling point with the highest total level of contaminants measured in the present work (see figure 3, BFP3 most contaminated site).

Table 2: Quantification results

		Concentrations (ppb=ng/g dry weight) ± RSD (3 replicates)							
Family	**Compound**	**BAP1**	**BAP2**	**BAP3**	**BAP4**	**BAP5**	**BFP1**	**BFP2**	**BFP3**
Organonitrogen pesticides	Metolachlor	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Simazine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Desethylatrazine	<LOD	<LOD	0.55±0.26	<LOQ	<LOD	0.23±0.05	<LOD	<LOD
	Atrazine	<LOD	<LOD	1.23±0.15	<LOQ	<LOD	<LOQ	<LOQ	1.46±0.05
Organophosphorus pesticides	Thiabendiazole	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Diazinon	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Malathion	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Herbicide	Bentazone	<LOD	1.07±0.13	1.34±0.28	1.13±0.11	4.20±0.26	5.52±0.32	7.02±0.35	9.36±0.04
	MCPA	<LOD	<LOD	<LOD	<LOD	13.73±1.32	0.88±0.33	1.33±0.80	1.20±0.28
	Propanil	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Insecticide	Acetamiprid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Imidacloprid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EDCs	Caffeine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Bisphenol A	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Triclosan	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.17±0.29	1.01±0.11
	Triclocarban	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Methylparaben	19.17±2.57	19.25±2.70	2.90±0.34	3.90±0.64	<LOD	2.38±0.46	1.42±0.20	3.77±0.22
	Ethylparaben	<LOD	<LOD	<LOD	<LOD	<LOD	1.28±0.17	<LOD	0.92±0.06
	Propylparaben	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD
	1H-benzotriazole	<LOD	<LOD	<LOD	3.95±0.10	<LOD	4.51±0.30	1.06±0.17	4.60±1.06
PhACs	Sulfamethozaxole	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Venlafaxine	<LOD	<LOD	<LOD	<LOD	<LOD	0.78±0.40	1.04±0.56	1.29±0.35
	Carbamazepine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.65±0.30	0.38±0.22

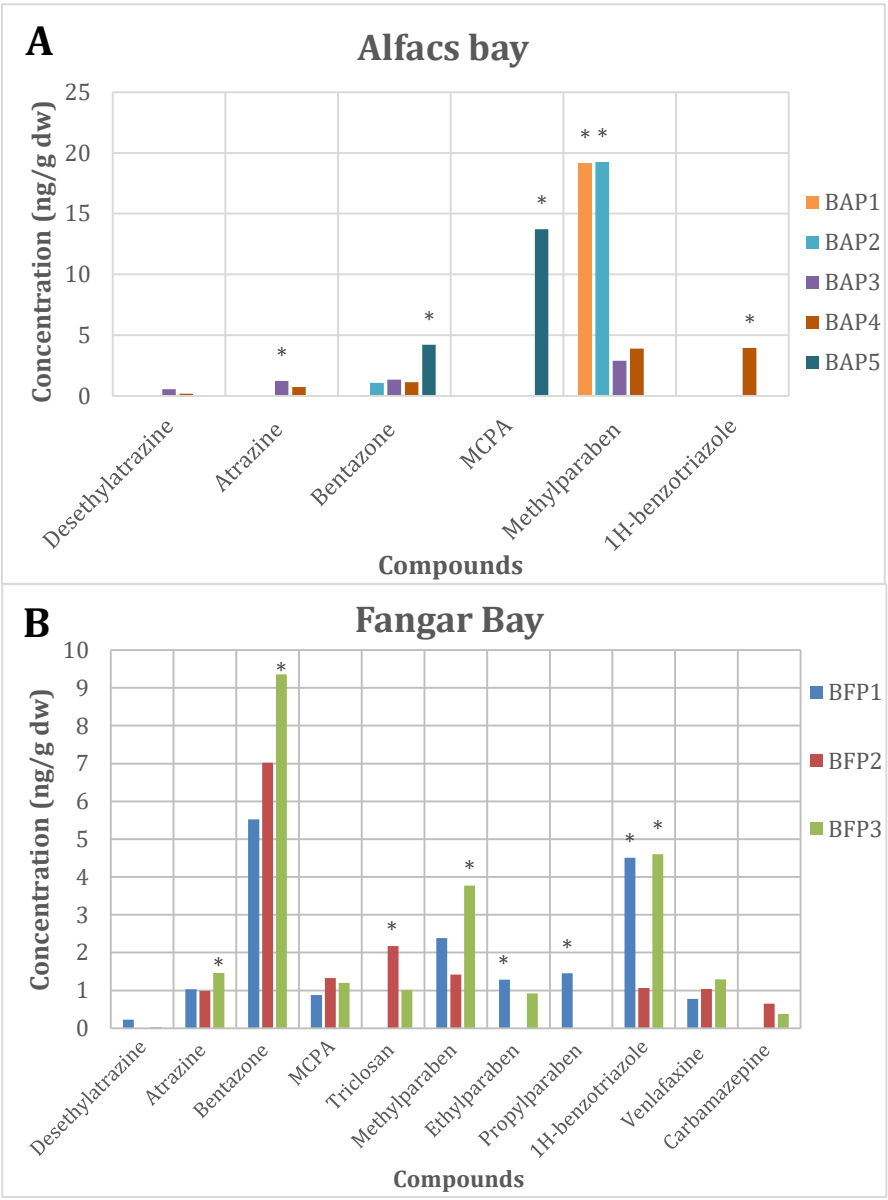

Fig. 6: Concentration of each contaminant in each sampling point. Significant differences have been established according to Mann-Whitney test (significant p≤0.05).
** The highest concentrated sampling points have a significantly different concentration respect to the smaller ones.*

7. CONCLUSIONS

The occurrence and levels of different families of contaminants in mussel samples from Ebro Delta have been studied in the present work. The research has led to the detection of 11 out of 23 of the target contaminants, including organonitrogen pesticides, herbicides, endocrine disruptor compounds and pharmaceutically active compounds.

The identified mix of contaminants is formed by desethylatrazine, atrazine, bentazone, MCPA, triclosan, methylparaben, ethylparaben, propylparaben, 1H-benzotriazole, venlafaxine and carbamazepine.

Concentrations of these compounds in mussel ranged between 0.19 ng/g dw for desethylatrazine to 19.25 ng/g for methylparaben, these levels are similar to the ones recently reported for other matrices in Ebro Delta. Moreover, the total contaminants concentration (expressed as the sum of their single concentrations) of every sampling point did not exceed 25 ng/g dw.

Differences between bays have been shown by means of PCA, which clearly depicted samples in two clusters that matched with the bay of origin. The number of compounds, their chemical group and concentration have been detected as the main factors for this differentiation.

Intra-bay differences have been detected by means of statistical tests and PCA, allowing to hypothesize with the relationship between contamination patterns and contamination main sources. Agricultural runoff is a common source of contamination in both bays but also Fangar bay suffers a higher pollutant's input from urban source.

To conclude, it deserves to be mentioned that although the concentrations detected did not exceed the maximum residue levels established for these contaminants in foodstuff from animal origin and therefore there is no risk for human health, the occurrence and frequency of detection of these pollutants in shellfish points out the necessity of permanently monitoring contaminants' levels. Moreover, further research on the effect of cooking on these concentrations is needed in order to know the real levels to which consumers are exposed after cooking a meal.

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9. ACRONYMS

ACET	Acetamiprid
ACN	Acetonitrile
ATRA	Atrazine
BAPx	Alfacs bay samplint point
BEN	Bentazone
BFPx	Fangar bay sampling point
BPA	Bisphenol A
C18	Octadecylsilane
CAF	Caffeine
DEA	Desethylatrazine
DIAZ	Diazinon
dSPE	Dispersive solid phase extraction
DW	Dry weight
EDC	Endocrine disruptor compound
EP	Ethylparaben
ESI	Electrospray ionization
HRMS	High resolution mass spectrometry
IMIDA	Imidacloprid
IS	Internal standard
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MALA	Malathion
MCPA	(4-Chloro-2-methylphenoxy)acetic acid
METO	Metolachlor

MP	Methylparaben
MRL	Maximum residue limits
MS	Mass spectrometry
PCA	Principal component analysis
PhAC	Pharmaceutically active compound
PP	Propylparaben
PROP	Propanil
PSA	Primary secondary amine
RT	Retention time
SIMA	Simazine
TCC	Triclocarban
TCS	Triclosan
THIA	Thiabendiazole
UHPLC	Ultra-high performance liquid chromatography
WW	Wet weight

APPENDIX 1: METHOD VALIDATION

1.1. LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

Both LOD and LOQ were determined in spiked samples, being LOD the minimum amount of analyte with signal-to-noise ratio of 3 and LOQ the minimum amount of analyte with signal-to noise ratio of 10. LODs were in the range of 0.002-3,000 ng/g and LOQs ranged between 0.010 and 10,000 ng/g.

Table 3: Detection and quantification limits

	LOD	**LOQ**
Compound	s/n = 3 (ppb=ng/g)	s/n = 10 (ppb= ng/g)
Metolachlor (METO)	0.020	0.080
Simazine (SIMA)	0.020	0.080
Desethylatrazine(DEA)	0.070	0.230
Atrazine (ATRA)	0.330	1.090
Thiabendazole (THIA)	0.020	0.080
Diazinon (DIAZ)	0.100	0.330
Malathion (MALA)	0.250	0.500
Bentazone (BEN)	0.100	0.500
MCPA	0.050	0.250
Propanil (PROP)	0.250	0.500

Acetamiprid (ACET)	0.021	0.070
Imidacloprid (IMIDA)	0.500	1,000
Caffeine (CAF)	3,000	10,000
Bisphenol A (BPA)	3,000	10,000
Triclosan (TCS)	0.500	1,000
Triclocarban (TCC)	0.500	1,000
Methylparaben (MP)	0.002	0.010
Ethylparaben (EP)	0.004	0.010
Propylparaben (PP)	0.490	1.650
1H-benzotriazole	0.160	0.520
Sulfamethozaxole	0.050	0.170
Venlafaxine hydrochloride	0.070	0.240
Carbamazepine	0.090	0.300

1.1.2. ACCURACY AND PRECISION

Accuracy and precision were calculated from five repeated injections of a sample spiked at 50 ng/g for intra-day assay, and from three injections of the same sample ran on three different days for inter-day evaluation. Accuracy values ranged between 0.2 and 17.1% for intra-day and between 1.5 and 18.8 for inter-day analyses. RSD values for intra-day assays (repeatability) were in the range of 0.4-10% and RSD values for inter-day analyses (reproducibility) ranged from 0.1 to 14.9%.

It is important to point out that both repeatability and reproducibility were below 20%, consequently being effective for quantification purposes[2].

Table 4: Accuracy and precision

Compound	Intra-day accuracy (%)	RSD (%)	Inter-day accuracy (%)	RSD (%)
Metolachlor	-5.8	0.4	-1.5	1.6
Simazine	-3.3	1.6	-5.6	0.1
Desethylatrazine	2.4	1.3	-12.1	14.9
Atrazine	0.2	1.3	-3	1.5
Thiabendazole	13.6	1.8	15.8	0.6
Diazinon	1	0.6	-3.2	1.8
Malathion	-6.3	0.7	-3.3	0.2
Bentazone	1.2	0.5	2.6	0.6
MCPA	-15	1.5	-13.1	2
Propanil	-15.5	2.6	-12.2	1.1
Acetamiprid	-5.1	1	4.6	0.8
Imidacloprid	-6.1	0.3	4.6	2.2
Caffeine	13.3	10	16.1	10.2
Bisphenol A	-17.1	3.2	-14.1	8.9
Triclosan	-8	0.6	-7.9	1.4
Triclocarban	2.6	7.4	18.8	16.9
Methylparaben	5.3	3.5	10.6	1.1
Ethylparaben	18.7	1.1	15.1	1.7
Propylparaben	7	1.2	8	2.2
1H-benzotriazole	7.6	1.7	-1	1.2
Sulfamethozaxole	1.3	1.3	2.4	1.9
Venlafaxine	16.7	0.7	6.8	3.4
Carbamazepine	-5.2	0.9	17.9	4.8

1.1.3. RECOVERIES

One gram dry weight (dw) was spiked per triplicate with the mixture of compounds and internal standards mixture at 50 ng/g dw. Triplicate control samples were also analysed in order to determine the background levels of the target compounds.

Total recoveries were calculated by comparing the concentrations measured in the sample after analytical procedure with the initial spiked concentration. The concentrations measured in samples were determined by using internal calibration as reported in section 5.2.3.

Table 5: Recoveries

Family	Compound	Mytilus galloprovincialis	
		Recovery %	RSD
Organonitrogen pesticides	Metolachlor	102.6	2.1
	Simazine	105.1	7.7
	Desethylatrazine	111.2	6.4
	Atrazine	104.3	8.2
Organophosphorus pesticides	Thiabendazole	87.4	4.6
	Diazinon	91.6	3.3
	Malathion	103.7	7.0
Herbicide	Bentazone	95.8	6.0
	MCPA	120.6	4.1
	Propanil	123.3	18.2
Insecticide	Acetamiprid	118.0	9.1
	Imidacloprid	113.0	4.4
EDCs	Caffeine	99.0	1.4
	Bisphenol A	148.2	8.0
	Triclosan	106.1	6.6
	Triclocarban	88.3	7.0
	Methylparaben	94.0	5.8
	Ethylparaben	94.5	9.1
	Propylparaben	93.4	8.1
	1H-benzotriazole	96.6	13.1
PhACs	Sulfamethozaxole	97.4	1.3
	Venlafaxine	101.8	2.9
	Carbamazepine	91.4	2.9

1.4. MATRIX EFFECTS

To evaluate matrix effects, peak areas of mussel extracts spiked at 5, 10, 25 and 50 ng/g were compared to those of the analytes in solvent (ACN 100%) spiked at the same concentrations.

Table 6: Matrix effects

		Compound	Effect	RSD(%)
Organonitrogen pesticides		Metolachlor	0.41	9.22
		Simazine	15.92	10.45
		Desethylatrazine	38.09	27.68
		Atrazine	6.62	9.74
Organophosphorus pesticides		Thiabendazole	4.19	8.11
		Diazinon	15.35	3.29
		Malathion	6.31	16.81
Herbicide		Bentazone	45.39	11.32
		MCPA	14.57	17.58
		Propanil	5.25	4.42
Insecticide		Acetamiprid	34.91	14.07
		Imidacloprid	9.30	38.28
EDCs	Stimulant	Caffeine	73.53	10.37
	Plasticizer	Bisphenol A	6.97	13.39
	Antibacterial	Triclosan	2.91	7.02
		Triclocarban	8.52	4.49
	Preservatives	Methylparaben	-25.30	22.54
		Ethylparaben	-5.74	10.04
		Propylparaben	-2.83	7.88
	Triazole	1H-benzotriazole	74.17	8.79
	Antibiotic	Sulfamethozaxole	32.09	25.30
PhACs	Psychiatric drug	Venlafaxine	7.40	2.53
		Carbamazepine	6.02	9.79

APPENDIX 2: UHPLC-HRMS INFORMATION

Table 7: Target compounds organized by their family, ionization mode, precursor ions, retention time and isotopically labelled standards.

Family	Compound	ESI	m/z	RT	IS	m/z	RT
Organonitrogen pesticides	Metolachlor (METO)	POS	284.1411	10.77	Metoalachlor-d6	290.1789	10.66
	Simazine (SIMA)	POS	202.0853	5.97	Simazine-d10	212.1481	5.87
	Desethylatrazine(DEA)	POS	188.0697	4.65	Atrazine-d5	221.1324	7.07
	Atrazine (ATRA)	POS	216.1010	7.13	Atrazine-d5	221.1324	7.09
Organophosphorus pesticides	Thiabendazole (THIA)	POS	202.0433	5.10	Thiabendazole-C13	208.0636	5.10
	Diazinon (DIAZ)	POS	305.1083	12.98	Diazinon-(diethyl-d10)	315.1710	12.82
	Malathion (MALA)	POS	331.0433	10.42	Malathion-d4	338.0875	10.31
Herbicide	Bentazone= bentazon (BEN)	NEG	239.0487	3.49	Bentazon-d4	246.0926	2.48
	MCPA	NEG	199.0155	4.06	MCPA-d3	202.0342	4.04
	Propanil (PROP)	NEG	217.9948	8.44	Propanil-d5	223.0261	8.39
Insecticide	Acetamiprid (ACET)	POS	223.0748	4.70	Acetamiprid-d3	226.0935	4.69
	Imidacloprid (IMIDA)	POS	256.0599	4.62	Imidaclo prid-d4	260.0850	4.60
EDCs	Caffeine (CAF)	POS	195.0876	3.60	Caffeine-d3	198.1064	3.59
	Bisphenol A (BPA)	NEG	227.1067	6.63	Bisphenol A-C13	239.1470	6.61
	Triclosan (TCS)	NEG	286.9438	12.99	triclosan-d3	289.9622	12.92

	Triclocarban (TCC)	NEG	312.9707	12.87	triclosan -d3	289.9622	12.92
	Methylparaben (MP)	NEG	151.0385	5.12	Ethylparaben-C13	171.0743	5.90
	Ethylparaben (EP)	NEG	165.0543	5.90	Ethylparaben-C13	171.0743	5.90
	Propylparaben (PP)	NEG	179.0713	6.95	Ethylparaben-C13	171.0743	5.90
	1H-benzotriazole	POS	120.0556	4.18	Benzotriazole-d4	124.0814	4.15
PhACs	Sulfamethoxazole	POS	254.0593	4.84	Sulfamethoxazole-d4	258.0844	4.83
	Venlafaxine hydrochloride	POS	278.2114	9.98	Venlafaxine-d6 hydrochloride	284.2491	9.97
	Carbamazepine	POS	237.1022	5.53	Carbamazepine-d10	247.1653	5.46