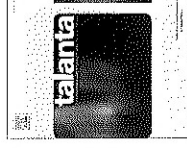




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Fast analysis of relevant contaminants mixture in commercial shellfish

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ABSTRACT

One of the major challenges currently faced is to develop systematic ways of addressing chemical mixtures in environmental assessment. With this purpose, a simple, rapid, and sensitive method for the detection and quantification of a mixture of relevant contaminants in molluscs has been developed. The method is based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and Ultra-High Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS). It includes a mixture of 23 compounds formed by pesticides, endocrine disruptors and pharmaceuticals (metolachlor, simazine, desethylatrazine, atrazine, triabenzazole, diazinon, malathion, bentazone, MCPA, propamit, acetamiprid, imidacloprid, caffeine, bisphenol A, tridocan, triclocarban, methylparaben, ethylparaben, propylparaben, 1H-benzotriazole, sulfamethoxazole, venlafaxine and carbamazepine). The method was developed and validated in 4 different types of shellfish of high commercial interest such as mussel (*Mytilus galloprovincialis*), oyster (*Crassostrea gigas*), cockle (*Cerastoderma edule*) and razor shell (*Solen marginatus*). The mean percentage of recoveries obtained for all the compounds in each mollusc type (intra-species) ranged from 96% to 107% showing the good performance of the method developed. The relative standard deviation was under 10% for the intra-day and 17% inter-day analyses. Method detection limits and method quantification limits were below 10 ng/g dry weight for all the species and compounds targeted. Finally, the method was applied to aquaculture samples, oysters and cockles, from Ebro Delta (Spain), after some episodes of mortality occurred in 2017. A high level of bisphenol A was detected in *C. edule* which may explain the mortality suffered by this organism. *C. gigas* presented low levels of metolachlor, bentazone, acetamiprid, and methylparaben.

1. Introduction

Multitude of contaminants derived from daily human activities are present in the aquatic environment and can interact with organisms such as molluscs. They can be simultaneously accumulated making a “cocktail” of hazardous substances with potential negative effects for the organism and for humans through the food chain. One of the major challenges currently faced is to develop systematic ways of addressing chemical mixtures in environmental assessment [1] and to identify priority mixtures of potential concern.

The analysis of contaminants mixtures in environmental samples is a difficult task. Concretely in seafood, when different groups of contaminants were targeted, the approach traditionally used was to apply as many different analytical methods as needed to the sample [2]. This strategy although useful is time- and money-consuming which is not suitable for use in regular analysis derived from quality control monitoring. Therefore, alternative fast and cheaper methods are needed for

covering the analysis of relevant contaminants mixtures in seafood. Currently, very few methods are available that allow to extract and quantify a certain mixture of contaminants in molluscs. Most of them are focused on the analysis of persistent organic pollutants (POPs) mixtures, containing chemicals from different groups, mainly polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), but also some polychlorinated naphthalenes (PCNs) and organo-chlorinated pesticides (OCPs) [3–5]. The development of methods for the analysis of contaminants mixtures containing contaminants of emerging concern (CECs) in shellfish is scarce. Bayen et al., in 2015 [6] developed a method for the extraction and identification of a mixture composed by a long list of pharmaceuticals compounds (PhACs) and some endocrine disrupting chemicals (EDCs) in molluscs [6]. Recently, [Mijangos et al [7], has published a method for the determination of multiclass organic pollutants including artificial sweeteners, industrial products, hormones, PhACs, personal care products, pesticides and phytoestrogens in tissues and biofluids of mussel and fish. Robust multi-

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replace: [2-5]

Replace by: Mijangos et al. [6]

and organo-chlorinated pesticides (OCPs) [3-5] → [6-8]
[6] → [9]
[9]

residue analytical methods for accurate determination of contaminants mixtures are essential for effective biomonitoring of environmental quality. Target analysis offers good sensitivity and reliable identification of the compounds, but it has a significant disadvantage as it always misses all compounds not included in the method. The use of High-Resolution Mass Spectrometry (HRMS) permits to overcome this issue through non-target analysis of the sample. Therefore, the selection of a high-resolution mass analyser when developing a target analytical method gives the opportunity of digging in the complexity of the contaminants mixture accumulated in a certain organism. Consequently, the present work aimed to develop and validate a simple, rapid, and sensitive method for the detection and quantification of a mixture of relevant contaminants in molluscs by using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and High-Resolution Mass Spectrometry. The mixture of contaminants selected was formed by pesticides (organonitrogen and organophosphorus pesticides, herbicides and insecticides), EDCs (stimulant, plasticiser, antibacterials, preservatives and triazole) and PhACs (psychiatric drugs and antibiotic) based on their occurrence and levels in the marine environment [8–11]. Twenty-three compounds were included and it was developed and validated in 4 different types of shellfish of high commercial interest such as mussel, oyster, cockle and razor shell. The method was successfully applied to aquaculture samples, oysters and cockles, from Ebro Delta (Spain) after some episodes of mortality occurred in 2017 in order to find out if there was any relationship with chemical contamination.

[11-14]

2. Material and methods

2.1. Chemicals and reagents

See supporting information for details.

2.2. Sampling

Organisms corresponding to different species of shellfish were collected from Ebro Delta (Catalonia, Spain) between April and Nov 2017. Spring and summer are the seasons when the highest load of contaminants are released to the Delta mainly due to agriculture (discharge of drainage channels from rice crops) and urban activities (industry and tourism).

Mussels (*Mytilus galloprovincialis*) and razor shell (*Solen marginatus*) were sampled from Alfacs bay (located at the south of the delta), oyster (*Crassostrea gigas*) and cockle (*Cerastoderma edule*) were taken from Fangar Bay (located at the north of the delta). For the method development the samples were collected from a shellfish farm allocated outside of Alfacs Bay and considered as clean site. The target bivalve species were selected due to their high consumption by the population. Thirty organisms were collected per species, their shells were discarded, and a pool was made by homogenising their tissues. The composite sample was freeze-dried, grounded in a mortar, and kept at -20°C until its analysis.

2.3. Extraction and purification

One-gram dry weight (dw) of the sample was extracted by using QuEChERS Bekolult Citrat-Kit-01. Prior to the extraction, the internal standards mixture was added at a concentration of 50 ng/g dw (Table 1), vortexed and left to equilibrate overnight in refrigerated conditions. Procedural blank extractions were also simultaneously carried out. The next day, 10 mL of ACN and 5 mL of HPLC water were added together with a mixture of salts containing 4 g of MgSO_4 , 1 g of NaCl , 1 g of sodium citrate and 0.5 g of disodium citrate sesquihydrate. Then the sample was vortexed (1 min) and centrifuged (5 min at 3220 RCF and 15°C). Immediately after, the supernatant liquid (6 mL) was transferred to a centrifuge tube to perform the dispersive solid phase extraction (dSPE) by adding QuEChERS Bekolult PSA-Kit-04A that

consists of 4 mg of primary secondary amine (PSA), 400 mg of octadecylsilane (C18e) and 1200 mg of MgSO_4 . It was vortexed (1 min) and centrifuged again (5 min at 3220 RCF and 15°C). The supernatant liquid was transferred to a glass tube to evaporate under a gentle stream of nitrogen until complete dryness. Then it was re-dissolved in 1 mL of ACN:water (50:50) and filtered through Ostro™, a phospholipids removal plate. The filtered sample was then transferred to an appropriate vial for its injection in UHPLC-HRMS.

2.4. Analysis by UHPLC-HRMS

For the identification and quantification of the target compounds, the shellfish extracts were injected in an UHPLC-HRMS instrument. Chromatographic separations were carried out with an Acquity Ultra-Performance™ Water liquid chromatograph system from Waters (Milford, MA, USA), equipped with two binary pumps systems using a Purospher STAR RP-18 end-capped column (150 mm \times 2.1 mm, 2 μm particle size) (Merk, Darmstadt, Germany) for both positive and negative electrospray ionisation (Table 1). The mobile phase consisted of acetonitrile (A) and water (B) at a flow rate of 0.2 mL/min. A gradient elution was applied for chromatographic separation: 0 min: 10%A; 2.5 min: 50%A; 12.5 min: 80%A, 13.5 min: 100%A; 16.5 min: 10%A; 25 min: 10%A. The column temperature was set at 25°C ; the injection volume was 20 μL . Detection was performed using an Orbitrap Q-Exactive™ mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). Full scan data in both positive and negative mode were acquired at a resolving power of 70,000 FWHM. Ion source parameters in positive electrospray mode (ESI+) were: spray voltage 3.5 kV, sheath gas ($\text{N}_2 > 95\%$) 35, auxiliary gas ($\text{N}_2 > 95\%$) 10, capillary temperature 350°C , S-lens RF level 60, auxiliary gas heater temperature 250°C . Ion source parameters in negative mode (ESI-) were: spray voltage -3.5 kV, sheath gas ($\text{N}_2 > 95\%$) 25, auxiliary gas ($\text{N}_2 > 95\%$) 10, capillary temperature 350°C , S-lens RF level 60, auxiliary gas heater temperature 250°C . For the compounds of interest, a scan range of m/z 70–1000 was selected; the automatic gain control (AGC) was set at 1×10^6 and the maximum injection time was set to 50 ms. Data analysis and processing were done using Thermo Xcalibur Software v. 3.1.

The selectivity and specificity of the method were verified by analysing samples and standard solutions. The peaks of the target compounds in the samples were confirmed by comparing their retention times with those in the standard solutions and also by identifying the precursor ion with a mass error below 5 ppm. Blank samples (100% ACN) were run every 3 samples on the sample queue both between standards, spiked and non-spiked samples in order to detect any possible carryover effect.

2.5. Recovery study

Mussel, oyster, cockle and razor shell (1 g of dw sample per triplicate ($n = 3$), for each matrix) were spiked with the contaminants mixture (Table 1) in order to study the extraction efficiency of the proposed methodology. The spike concentration was 50 ng/g dw. The internal standards mixture was also added at the same concentration prior to the extraction. The samples were immediately vortexed after addition of every mixture, and they were kept at 4°C overnight before the extraction process was carried out. Triplicate control samples of each matrix were also analysed to determine possible background levels of the target compounds. Total recoveries were calculated by comparing the concentrations measured in the sample after the analytical procedure with the initial spiked concentration. The concentrations measured in the sample were determined by using internal calibration. For this purpose, an 8 points calibration curve (between 0.05 and 50 ng/mL) of the target compounds was made up in acetonitrile.

Replace by:
ACN: water (50:50)

Table 1

Target compounds organized by chemical family, ESI mode, m/z and isotopically labelled internal standard assigned for their quantification.

Chemical family	Compound	ESI	m/z	RT (min)	Internal Standard	m/z	RT (min)
Organonitrogen pesticides	Atrazine (ATRA)	POS	216.1010	7.13	Atrazine-d5	221.1324	7.09
	Desethylatrazine (DEA)	POS	188.0697	4.65	Atrazine-d5	221.1324	7.07
	Metolachlor (METO)	POS	284.1411	10.77	Metolachlor-d6	290.1789	10.66
	Simazine (SIMA)	POS	202.0853	5.97	Simazine-d10	212.1481	5.87
	Diazinon (DIAZ)	POS	305.1083	12.98	Diazinon-d10	315.1710	12.82
Organophosphorus pesticides	Malathion (MALA)	POS	331.0433	10.42	Malathion-d7	338.0875	10.31
	Thiabendazole (THIA)	POS	202.0433	5.10	Thiabendazole-C13	208.0636	5.10
	Bentazone (BEN)	NEG	239.0487	3.49	Bentazon-d7	246.0926	2.48
	MCPA	NEG	199.0155	4.06	MCPA-d5	202.0342	4.04
	Propanil (PROP)	NEG	217.9948	8.44	Propanil-d5	223.0261	8.39
Insecticides	Acetamiprid (ACET)	POS	223.0748	4.70	Acetamiprid-d3	226.0935	4.69
	Imidacloprid (IMIDA)	POS	256.0599	4.62	Imidacloprid-d4	260.0850	4.60
EDCs	1H-benzotriazole (BENZOT)	POS	120.0556	4.18	Benzotriazole-d4	124.0814	4.15
	Bisphenol A (BPA)	NEG	227.1067	6.49	Bisphenol A d-4	231.1325	6.45
	Caffeine (CAF)	POS	195.0876	3.60	Caffeine-d3	198.1064	3.59
	Ethylparaben (EP)	NEG	165.0543	5.90	Ethylparaben-C13	171.0743	5.90
	Methylparaben (MP)	NEG	151.0385	5.12	Ethylparaben-C13	171.0743	5.90
	Propylparaben (PP)	NEG	179.0713	6.95	Ethylparaben-C13	171.0743	5.90
	Triclocarban (TCC)	NEG	312.9707	12.87	Triclosan-d3	289.9622	12.92
	Triclosan (TCS)	NEG	286.9438	12.99	Triclosan-d3	289.9622	12.92
	Carbamazepine (CBZ)	POS	237.1022	5.53	Carbamazepine-d10	247.1653	5.46
	Sulfamethoxazole (SMX)	POS	254.0593	4.84	Sulfamethoxazole-d4	258.0844	4.83
PhACs	Venlafaxine (VEN)	POS	278.2114	9.98	Venlafaxine-d6	284.2491	9.97

2.6. Statistical analysis

The mean percentage of recovery and its relative standard deviation (RSD) were calculated for each compound and matrix. Besides, statistical analysis of the percentages of recoveries obtained for all compounds in every matrix was carried out with "R" software v 3.5.1. The median, percentile 25th and 75th are shown by the box plot (see Fig. S1). Whiskers above and below the box indicate the 10th and 90th percentiles. Points above and below the whiskers indicate outliers.

3. Results and discussion

3.1. Extraction and purification

QuEChERS was proposed as extraction and purification technique because it is simple, fast, it offers high sample throughput and the possibility of obtaining high recoveries for a wide variety of organic chemicals, from polar to non-polar compounds in biological matrices [12]. It was compared with Pressurised Liquid Extraction (PLE) using different solvents mixtures (acetonitrile, methanol:water (1:2) and dichloromethane:acetone (1:1)). For the purification stage Solid Phase Extraction (SPE), on different cartridges such as HLB and ENV + and dispersive SPE (dSPE) were evaluated. The results indicated that PLE (performing the extraction at 50 °C, 1500 psi and 3 static cycles) followed by SPE was not appropriate because most of the target chemicals were either non-detected or there was co-extraction of other matrix compounds that interfered with the analysis (data not shown).

The total recoveries obtained for the four-target species *M. galloprovincialis*, *C. gigas*, *C. edule* and *S. marginatus* at the spiking level chosen are shown in Fig. 1. The numeric values and their respective RSD are also presented in the supporting information (Table S1). The spike concentration used was 50 ng/g dw, which was selected considering that levels of pesticides, EDCs and pharmaceuticals in marine biota usually ranged in the low ppb levels [8]. Even so, sometimes depending on the specific compound (mainly pesticides and EDCs), type of sample and origin, the concentrations can be much higher reaching the ppm levels [9,13]. The extracted ion chromatograms of the 23 compounds included in the method in a standard and in the 4 different matrices studied are presented in Fig. 2, Fig. S2, S3 and S4. They show the elution order in both positive and negative ionisation modes.

Recoveries ranged between 62% and 148%. However, when the

variation of the dataset was studied, the box plot diagram (Fig. S1) identified both percentages as extremes outliers. The mean percentage of recoveries obtained for all the compounds in each mollusc type (intra-specie) ranged from 96% to 107%, being very close to the medians, which ranged between 96 and 105% (Fig. S1). Their RSD reached a maximum of 17%, although in the majority of the cases it was below 10% which indicates a high degree of repeatability (Table S1). The recoveries obtained for the four mollusc types (inter-species) ranged in a very narrow interval, which shows the robustness of the proposed methodology. This interval was particularly close for *M. galloprovincialis*, *C. gigas* and *C. edule*. In the case of *S. marginatus* slightly lower recoveries were achieved for the majority of the compounds (Fig. 1).

As a limitation of the method, it was found that the extractions of BPA from *C. gigas* and VEN from *S. marginatus* were not possible due to the co-extraction of others matrix compounds that interfered with the analysis. Changes in the chromatography were tested in order to improve their separation (using different solvents and elution gradient), but this was a detriment to the others compounds analysed. Anyway, considering the good performance of the original method for BPA and VEN in the other target organisms, and the importance of these contaminants from an environmental point of view, it was decided to keep them.

3.2. Matrix effects

The effects caused by the 4 different matrices studied (mussel, oyster, cockle and razor shell) on the analysis of the target compounds were checked. For this purpose, the peak areas of the shellfish's extracts (first subtracted by the peak areas corresponding to the native analytes present) spiked at 1, 5, 10, 25 and 50 ng/g were compared to those of the analytes in solvent (ACN:water 50:50) spiked at the same levels. The percentages of signal reduction or enhancement are shown in Table 2 and Fig. S5. Ion suppression was the effect mostly observed with few exceptions (parabens in *M. galloprovincialis*, VEN in *C. gigas* and BPA and TCS in *C. edule*). It ranged in a wide interval from practically not suppressed (i.e. METO in *M. galloprovincialis*) to strongly suppressed (i.e. BEN in *S. marginatus*). It was noted that *M. galloprovincialis* was the shellfish presenting less ion suppression (mean percentage of ion suppression 16%) followed by *C. gigas* (42%), *C. edule* (51%) and finally *S. marginatus* (70%). Matrix effects, typically matrix suppression, are a well-known problem in LC-MS analysis and need to

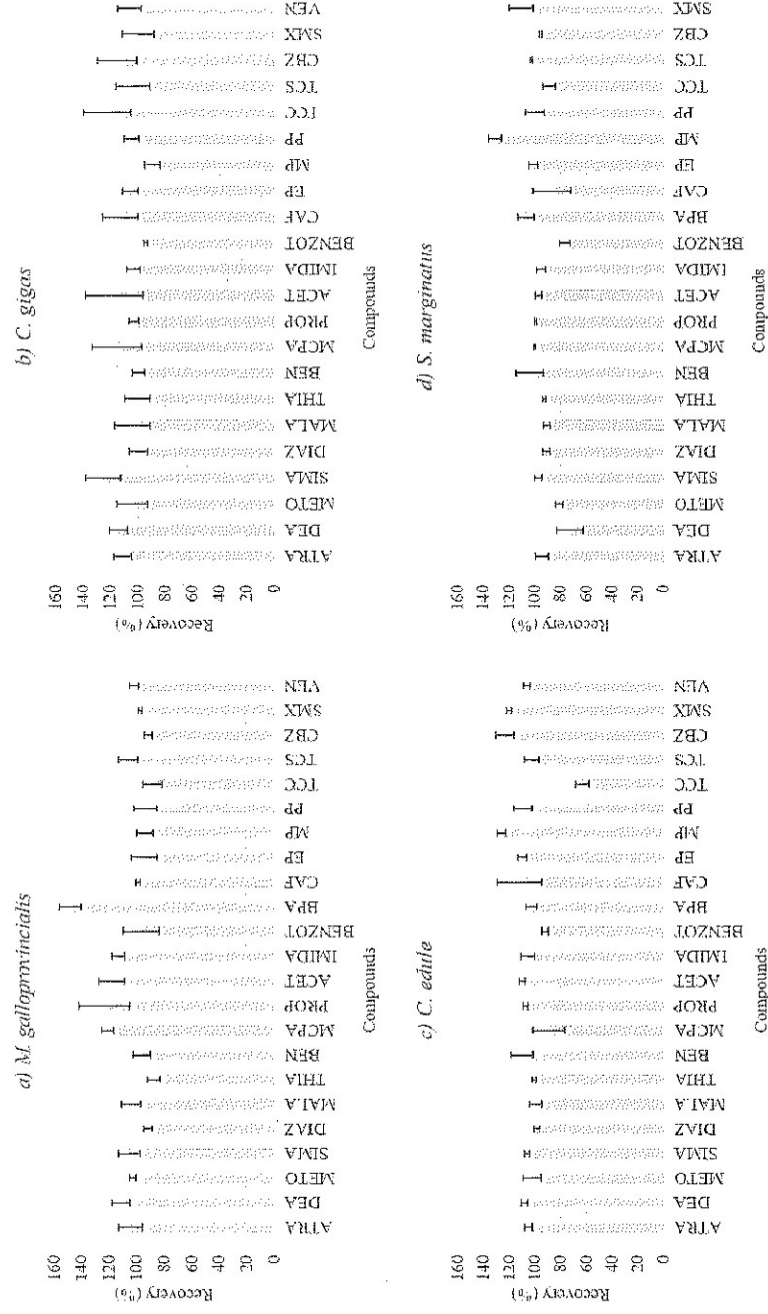


Fig. 1. Mean percentages recoveries ($n = 3$) of the target compounds in a) *M. galloprovincialis* (mussel), b) *C. gigas* (cockle) and d) *S. marginatus* (razor shell) spiked at 50 ng/g dry weight. [15]

be evaluated together with recoveries [12] in order to minimize matrix interferences and avoid any under or overestimation during quantification internal sample calibration can be performed [14]. In the present work, the use of internal sample calibration (also known as matrix-matched calibration) together with isotopically labelled internal

standards was initially considered, and 4 calibration curves (one per target species) were made up in the corresponding shellfish extracts. However, the recoveries obtained were not significantly different to the ones obtained with solvent calibration (t -test, $p > 0.05$), and this approach was discarded.

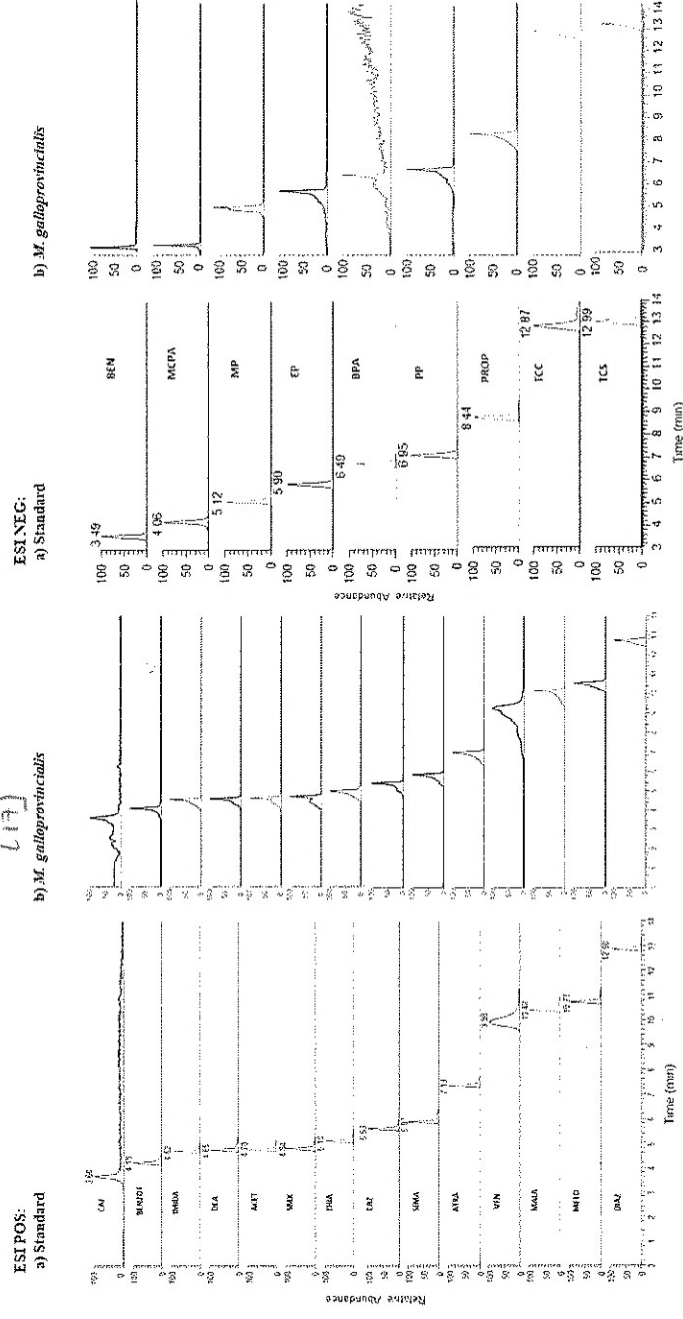


Fig. 2. Extracted ion chromatograms of the compounds analysed in a) a standard b) *M. galloprovincialis* spiked at 50 ng/g in positive and negative electrospray ionisation.

Table 2
Matrix effect (%) expressed as ion suppression (-) or enhancement (+) for the target compounds tested in *M. galloprovincialis*, *C. gigas*, *C. edule* and *S. marginatus*.

Compound	Matrix effect (%)			
	<i>M. galloprovincialis</i>	<i>C. gigas</i>	<i>C. edule</i>	<i>S. marginatus</i>
Atrazine	-6.62	9.74	-58.12	12.70
Desethylatrazine	-38.09	27.68	-85.33	6.70
Metolachlor	-0.41	9.22	-50.34	18.90
Simazine	-15.92	10.45	-71.68	7.48
Diazinon	-15.35	3.29	-63.18	12.71
Malathion	-6.31	16.81	-52.16	15.87
Thiabendazole	-4.19	8.11	-58.58	15.03
Benazone	-45.39	11.32	-94.80	1.63
MCPA	-14.57	17.58	-85.45	8.90
Propanil	-5.25	4.42	-9.42	8.98
Acetamiprid	-34.91	14.07	-76.01	5.67
Imidacloprid	-9.30	38.28	-83.56	7.04
1H-benzotriazole	-74.17	8.79	-89.62	7.48
Bisphenol A	-6.97	13.39	14.08	5.63
Caffeine	-73.53	10.37	-55.54	2.73
Ethylparaben	5.74	10.04	-29.73	4.21
Methylparaben	25.30	22.54	-29.40	8.47
Propylparaben	2.83	7.88	-23.70	7.68
Triclocarban	-8.52	4.49	-51.23	2.08
Triclosan	-2.91	7.02	32.88	5.76
Carbamazepine	-6.02	9.79	-53.41	15.11
Sulfamethoxazole	-32.09	25.30	-82.80	7.94
Venlafaxine	-7.40	2.53	-36.18	28.62

3.3. Method validation

Method detection limits (MDLs) and method quantification limits (MQLs) for *M. galloprovincialis*, *C. gigas*, *C. edule* and *S. marginatus* are presented in Table 3. They were determined in spiked samples ($n = 3$) of the four shellfish types. The calculation was based on signal-to-noise ratio (S/N) for those compounds and matrices which exhibited baseline noise. The acceptance criteria were $S/N = 3$ for MDL and $S/N = 10$ for MQL. For those compounds and matrices which baseline was completely flat (and therefore N was equal to 0), the MDL and MQL were established by visual inspection of the calibration curve made up in the corresponding matrix extract [15]. The concentration of the lowest standard that could be clearly distinguished was used as MDL and

double it as MQL. Calibration curve range was between 0.01 and 50 ng/g, they were generated using linear regression (regression-coefficients shown in Table 2). MDLs and MQLs were below 10 ng/g dw for all the species and compounds targeted. Similar MQLs were described by Baduel et al. [12] for the extraction of a mixture of contaminants from fish muscle using LC-QTOF-MS/MS. Bayen et al. [6] reported MDLs for a mix of contaminants in mussel generally below 1 ng/g but expressed as wet weight. According to our previous experience [16], the conversion factor from wet weight to dry weight concentration for mussels ranged from 4 to 7. Therefore, MDLs in the same range (expressed in dw) than the ones reported here were obtained for similar compounds.

Accuracy and precision for the developed method were determined for the same spiked sample (*M. galloprovincialis*) intra-day ($n = 5$

Table 3

Method detection and quantification limits (MDL, MQL) of the target compounds in *M. galloprovincialis*, *C. gigas*, *C. edule* and *S. marginatus*.

Compound	MDL (ng/g dw)				MQL (ng/g dw)			
	<i>M. galloprovincialis</i>	<i>C. gigas</i>	<i>C. edule</i>	<i>S. marginatus</i>	<i>M. galloprovincialis</i>	<i>C. gigas</i>	<i>C. edule</i>	<i>S. marginatus</i>
Atrazine	0.33	0.04	0.05	0.05	1.09	0.12	0.10	0.10
Desethylatrazine	0.07	0.22	0.50	0.10	0.23	0.75	1.00	0.50
Metolachlor	0.02	0.05	0.05	0.03	0.08	0.16	0.10	0.11
Simazine	0.02	0.28	0.02	0.05	0.08	0.95	0.05	0.10
Diazinon	0.10	0.28	0.05	0.11	0.33	0.93	0.10	0.37
Malathion	0.25	0.50	0.25	0.10	0.50	1.00	0.50	0.50
Thiabendazole	0.02	0.07	0.05	0.16	0.08	0.25	0.10	0.95
Benazone	0.10	0.05	0.01	0.02	0.50	0.16	0.02	0.05
MCPA	0.05	0.03	0.10	0.08	0.25	0.09	0.50	0.27
Propanil	0.25	0.50	0.10	0.22	1.00	1.00	0.50	0.73
Acetamiprid	0.02	0.07	0.50	0.05	0.07	0.24	1.00	0.10
Imidacloprid	0.50	0.50	0.50	0.50	1.00	1.00	1.00	1.00
1H-benzotriazole	0.16	0.10	0.50	0.10	0.52	0.25	1.00	0.50
Bisphenol A	3.00	10.00	0.50	10.00	10.00	10.00	1.00	10.00
Caffeine	3.00	2.00	2.50	5.00	10.00	4.00	5.00	5.00
Ethylparaben	0.01	0.15	0.01	0.50	0.01	0.50	0.02	1.00
Methylparaben	0.01	0.29	0.02	0.02	0.01	0.98	0.05	0.05
Propylparaben	0.49	0.50	0.05	0.35	1.66	1.66	0.10	1.18
Triclocarban	0.50	0.50	0.25	1.24	1.00	1.00	0.50	4.13
Triclosan	0.50	0.50	0.25	1.07	1.00	1.00	0.50	3.57
Carbamazepine	0.09	0.20	0.02	0.20	0.30	0.66	0.05	0.66
Sulfamethoxazole	0.05	0.25	1.00	0.10	0.17	0.50	1.50	0.50
Venlafaxine	0.07	0.20	0.50	10.00	0.24	0.65	1.00	10.00

Table 4
Accuracy and precision in *M. galloprovincialis*.

Compound	Intra-day		Repeatability		Inter-day		Reproducibility	
	Error (%)	RSD (%)	Error (%)	RSD (%)	Error (%)	RSD (%)	Error (%)	RSD (%)
Atrazine	0.2	1.3	-3.0	1.5				
Desethylatrazine	2.4	1.3	-12.1	14.9				
Metolachlor	-5.8	0.4	-1.5	1.6				
Simazine	-9.3	1.6	-5.6	0.1				
Diazinon	1.0	0.6	-3.2	1.8				
Malathion	-6.3	0.7	-3.3	0.2				
Thiabendazole	13.6	1.8	15.8	0.6				
Bentazone	1.2	0.5	2.6	0.6				
MGPA	-15.0	1.5	-13.1	2.0				
Propanil	-15.5	2.6	-12.2	1.1				
Acetamiprid	-5.1	1.0	4.6	0.8				
Imidacloprid	-6.1	0.3	4.6	2.2				
1H-benzotriazole	7.6	1.7	-1.0	1.2				
Bisphenol A	-17.1	3.2	-14.1	8.9				
Caffeine	13.3	10.0	16.1	10.2				
Ethylparaben	18.7	1.1	15.1	1.7				
Methylparaben	5.3	3.5	10.6	1.1				
Propylparaben	7.0	1.2	8.0	2.2				
Triclocarban	2.6	7.4	18.8	16.9				
Triclosan	-8.0	0.6	-7.9	1.4				
Carbamazepine	-5.2	0.9	17.9	4.8				
Sulfamethoxazole	1.3	1.3	2.4	1.9				
Venlafaxine	16.7	0.7	6.8	3.4				

injections the same day) and inter-day ($n = 3$ injections performed in 3 different days, one per day). Accuracy was defined as the deviation of the measured mean concentration from the spiked concentration, expressed in percentage, as described by Bogialli et al. [17]. Accuracy values are expressed as percentage of error and they ranged between -17.1 and 18.8% (which correspond to an accuracy of 82.9% and 118.8%) (Table 4). Precision was expressed as the relative standard deviation of the measured concentration. RSD values for the intra-day analysis

3.4. Application to aquaculture samples

The method developed was applied to samples of *C. gigas* and *C. edule* collected from Ebro Delta during several mortality episodes occurred in 2017. In May, when temperatures were around 20 °C, *C. gigas* cultured at Fangar bay, experimented a massive first mortality event (up to 85% accumulative mortality) of commercial size oyster. This event repeated later on in June and October (12 and 30% of mortality, water temperature 28 °C and 22 °C respectively). In these events, the mortality was significantly lower, especially in June considered as normal mortality for that time of the year. This is noteworthy because June was the month that registered the highest water temperature. In September, a massive mortality event of *C. edule* was also registered (100% of mortality, water temperature 20 °C). The results obtained after the analysis of these samples are presented in Table 5. Ten out of the 23 contaminants included in the method were determined at concentrations above their respective MDLs. Concretely 4 pesticides (METO, ATRA, BEN and ACET) and 6 EDCs (CAF, BPA, MP, EP, PP and BENZOT). Their levels ranged from below MQL up to 4277.40 ng/g dw of BPA in *C. edule* dead in September. Unfortunately, water samples were not taken during this mortality event, and the concentration of BPA in water was not measured. Anyhow, considering the high level of BPA bioaccumulated in the shellfish a very high concentration in water would be expected (probably due to a direct spill). Based on reported EC50 and LC50 BPA is classified as "moderately toxic" and "toxic" to aquatic biota by the European Commission and the United States Environmental Protection Agency (US EPA), respectively [18]. Therefore, its occurrence and level in *C. edule* could explain the mortality suffered at that specific moment. However, pathogens associated with cockle massive mortality events such as *Marteilia cochlinalis* [19] have been previously reported in the same area

Table 5

Concentrations measured (ng/g dry weight, $n = 3$) in *C. gigas* and *C. edule* collected from Ebro Delta (Spain).

Compound	Compounds concentrations (ng/g dry weight) \pm RSD									
	<i>C. gigas</i> control May	<i>C. gigas</i> dead May	<i>C. gigas</i> control June	<i>C. gigas</i> dead June	<i>C. gigas</i> control Nov	<i>C. gigas</i> dead Oct	<i>C. edule</i> control Dec	<i>C. edule</i> dead Sept		
Atrazine	nd ^d	nd	nd	nd	nd	nd	nd	2.64 \pm 0.03		
Desethylatrazine	nd	nd	nd	nd	nd	nd	nd	nd		
Metolachlor	nd	nd	nd	1.15 \pm 0.05	nd	nd	nd	nd		
Simazine	nd	nd	nd	nd	nd	nd	nd	nd		
Diazinon	nd	nd	nd	nd	nd	nd	nd	nd		
Malathion	nd	nd	nd	nd	nd	nd	nd	nd		
Thiabendazole	nd	nd	nd	nd	nd	nd	nd	nd		
Bentazone	nd	nd	1.56 \pm 0.13	0.14 \pm 0.09	0.61 \pm 0.02	1.24 \pm 0.14	0.35 \pm 0.02	5.11 \pm 0.33		
MGPA	nd	nd	nd	nd	nd	nd	nd	nd		
Propanil	nd	nd	nd	nd	nd	nd	nd	nd		
Acetamiprid	3.68 ^b	6.71 ^b	9.51 \pm 0.96	nd	nd	nd	nd	3.50 \pm 0.86		
Imidacloprid	nd	nd	nd	nd	nd	nd	nd	nd		
1H-benzotriazole	nd	nd	nd	nd	nd	nd	nd	2.43 \pm 0.89		
Bisphenol A	ml ^c	ml	ml	ml	ml	ml	nd	4277.40 \pm 13.84		
Caffeine	nd	nd	nd	nd	nd	nd	nd	99.85 \pm 23.84		
Ethylparaben	nd	nd	nd	nd	nd	nd	3.56 \pm 0.09	nd		
Methylparaben	nd	nd	nd	nd	1.10 \pm 0.39	0.29 \pm 0.07	6.69 \pm 0.25	nd		
Propylparaben	nd	nd	nd	nd	nd	nd	0.56 \pm 0.05	nd		
Triclocarban	nd	nd	nd	nd	nd	nd	nd	nd		
Triclosan	nd	nd	nd	nd	nd	nd	nd	nd		
Carbamazepine	nd	nd	nd	nd	nd	nd	nd	nd		
Sulfamethoxazole	nd	nd	nd	nd	nd	nd	nd	nd		
Venlafaxine	nd	nd	nd	nd	nd	nd	ml	nd		

^a nd = non detected (< MDL).

^b Low amount of sample, only one replicate analysed.

^c ml = method limitation.

^d ng = non quantified (< MQL).

and they might be also present in the samples. On the contrary, the mortality events suffered by *C. gigas* were not related to any particular compound included in the method since their levels ranged in the low ng/g dw. Eight years ago Köck et al. [19] found very high levels of malathion in shellfish from the same area (53 mg/kg) which could explain the mortalities registered by them. However, in the present research malathion was not detected because this pesticide was banned in 2007 by the European Commission [20]. Therefore, there must be other reasons that may explain such high mortalities percentages like the presence of potential pathogens, for example *Vibrio aestuvarius* and *Vibrio splendidus* that have been recently reported for the first time in Pacific oyster cultured in the Spanish Mediterranean [21].

4. Conclusions

A simple, rapid, and sensitive method has been developed and validated for the simultaneous detection and quantification of a mixture of relevant contaminants in molluscs by using QuEChERS and UHPLC-HRMS. The mixture of contaminants selected was formed by 23 compounds including pesticides (organonitrogen and organophosphorus pesticides, herbicides and insecticides), EDCs (stimulant, plasticizer, antibacterials, preservatives and triazole) and PhACs (psychiatric drugs and antibiotic). The mean percentage of recoveries obtained for all compounds in each mollusc type (*M. galloprovincialis*, *C. gigas*, *C. edule* and *S. marginatus*) ranged from 96% to 107%. As a limitation of the method, it was found that the extractions of BPA from *C. gigas* and VBN from *S. marginatus* were not possible due to the co-extraction of others matrix compounds that interfered with the analysis. Other than that, RSD values for the intra-day analysis were below 10%, and below 17% for the inter-day analysis. This demonstrates the repeatability and reproducibility of the method and therefore its effectiveness for quantification purposes. MDLs and MQLs were below 10 ng/g dw. Ion suppression was the effect mostly observed with few exceptions, and it was compensated by the use of internal calibration. The method developed was applied to samples of *C. gigas* and *C. edule* collected from Ebro Delta during several mortality episodes occurred in 2017. Ten out of the 23 contaminants included in the method were determine at concentrations above their respective MDLs. Concretely 4 pesticides (METO, ATRA, BEN and ACET) and 6 EDCs (CAF, BPA, MP, EP, PP and BENZOT). The occurrence and level of BPA in *C. edule* could explain the mortality events suffered at that specific moment. On the contrary, the mortality events suffered by *C. gigas* were not related to any particular compound included in the method since their levels ranged in the low ng/g dw. No unknown large peaks potentially behind the mortality were neither observed in the scan screening of chromatograms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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A attached word file and include

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Declarations of interest

None.

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Supplementary data to this article can be found online at <https://>

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