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RESEARCH PAPER

Analysis of lipophilic marine biotoxins by liquid chromatography coupled with high-resolution mass spectrometry in seawater from the Catalan Coast

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Abstract Marine biotoxins regularly occur along the coast, with several consequences for the environment as well as the food industry. Monitoring of these compounds in seawater is required to assure the safety of marine resources for human consumption, providing a means for forecasting shellfish contamination events. In this study, an analytical method was developed for the detection of ten lipophilic marine biotoxins in seawater: azaspiracids 1, 2, 3, 4 and 5, classified as azaspiracid shellfish poisoning toxins, and pectenotoxin 2, okadaic acid and the related dinophysistoxin 1, yessotoxin and homoyessotoxin, classified as diarrheic shellfish poisoning toxins. The method is based on the application of solid–liquid ultrasound-assisted extraction and solid-phase extraction, followed by high-performance liquid chromatography coupled with high-resolution mass spectrometry. The limits of detection of this method are in the range of nanograms per litre and picograms per litre for most of the compounds, and recoveries range from 20.5% to 97.2%. To validate the effectiveness of this method, 36 samples of surface water from open coastal areas and marinas located along the Catalan coast on the Mediterranean Sea were collected and analysed. Eighty-eight per cent of these samples exhibited okadaic acid in particulate and aqueous phases in concentrations ranging from 0.11 to 560 μg/g and from 2.1 to 1780 ng/L respectively. Samples from open coastal areas exhibited higher concentrations of okadaic acid in particulate material, whereas in samples collected in sportive ports, the particulate material exhibited lower levels than the aqueous phase.

Keywords Lipophilic marine biotoxins · Okadaic acid · Diarrhetic shellfish poisoning · Seawater · Solid phase extraction · High-performance liquid chromatography–high-resolution mass spectrometry

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Introduction

Marine biotoxins are secondary metabolites that are produced by marine photosynthetic organisms such as dinoflagellates, diatoms and cyanobacteria. Depending on the water temperature and quantity of organic matter, rapid growth of these microorganisms can occur, with the production of high amounts of marine biotoxins. This phenomenon is known as harmful algal blooms and can cause damage in marine ecosystems, and, in addition, biotoxins can be accumulated in certain marine organisms, passing then to the human food web.

In recent decades, an increase in proliferation, frequency, and persistence of toxic algal blooms has occurred because of eutrophication from agricultural run-off and global climate change [1]. As a consequence, the risk of intoxication via recreational bathing in contaminated waters and consumption of contaminated food has increased.

Marine biotoxins can be classified by their toxic mode of action, such as paralytic toxins, amnesic toxins, diarrheic

toxins and neurotoxins, or by their physicochemical properties, as in lipophilic and hydrophilic biotoxins.

To protect public health, there is Europe-wide legislation to limit their levels in seafood for human consumption. The maximum permitted levels in molluscs for human consumption are compiled in Commission Regulation (EU) 786/2013 [2]. In addition, the methods used for determining the marine biotoxins in seafood are described in Commission Regulation (EU) 15/2011, amending Regulation (EC) 2074/2005 [3]. Mouse and rat bioassays were the regulatory approaches for the control of different marine biotoxins in previous regulations. However, for ethical reasons and the lack of specificity and sensitivity for the determination of some toxins, these methods have been replaced by others, based on liquid chromatography (LC) coupled with tandem mass spectrometry (LC–MS/MS).

For the particular case of lipophilic marine biotoxins, in 2001 Quilliam et al. [4] reported for the first time their chromatographic separation using acidic conditions. Since then, the method has been applied in different studies and, 10 years later, the European Union Reference Laboratory for Marine Biotoxins reported another method, also based on acidic conditions [5]. However, in 2009, Gerssen et al. [6] achieved the separation of 28 lipophilic marine biotoxins using water and acetonitrile at pH 11 with limits of detection (LODs) in mussels ranging from 1 to 22 ng/g. Since then, other authors have reported different analytical approaches using slightly alkaline conditions for the chromatographic separation [7]. The success of the methods based on LC–MS/MS has been proven in different interlaboratory exercises, and was reflected in the regulation. However, with use of selected reaction monitoring or single ion monitoring methods for target analysis, data on non-target analytes such as potential transformation products is lost. In this sense, application of full-scan acquisition schemes with high-resolution mass spectrometry (HRMS) instrumentation allows parallel non-target screening of the acquired data to be performed.

A limited number of studies have explored the advantages of HRMS to assess marine biotoxins [8–11]. The first method using LC–HRMS was developed for the determination of azaspiracids (AZAs) in shellfish with a limit of quantification (LOQ) of 0.010 μg/g [12]. More recently, Domènech et al. [8], presented a multitoxin method for quantification and confirmation of okadaic acid (OA), yessotoxin (YTX), AZA-1, gymnodimine, 13-desmethyl spirolide C, pectenotoxin 2 (PTX-2) and brevetoxin B in mussels. The LOQs ranged from 0.9 to 4.8 pg on column. More recently, the same group of researchers [9] presented a new method combining a quick, easy, inexpensive, efficient, rugged and safe (QuEChERS) extraction and LC–HRMS for the analysis of lipophilic marine biotoxins in fresh and canned bivalves. The method fulfilled all the requirements of current European legislation [9]. Some analytical methods based on immunoassays have also been reported, achieving LODs of 150 mg/kg in shellfish [13, 14]. However, early determination of marine biotoxins in seawater to prevent seafood contamination events has been almost unexplored. Zendong et al. [15] reported an efficient passive-sampling-based extraction method for the high-resolution profiling of marine biotoxins in water from the French coast.

In this article, we present the development and application of a method for the analysis of ten lipophilic marine biotoxins from different groups in seawater. The main objectives were as follows:

1. To develop a multiresidue method based on solid–liquid ultrasound-assisted extraction (UAE) and solid-phase extraction (SPE) followed by LC–HRMS for the detection and quantification of ten marine biotoxins in seawater, including five AZAs, OA and the related dinophysistoxin 1 (DTX-1), YTX, homoyessotoxin (hYTX) and PTX-2.
2. To apply the approach developed to assess the distribution of the selected marine biotoxins in seawater along the Catalan coast.

To the best of our knowledge, this is the first method which explores the use of HRMS for the analysis of marine biotoxins directly in seawater, and the method has improved the LOQ previously reported for marine biotoxins in seawater and in other matrices [16, 17]. Monitoring methods for the determination of marine biotoxins in seawater at trace levels can provide a means of forecasting shellfish contamination events in support of food safety.

Materials and methods

Chemicals and reagents

The structures and characteristics of the selected lipophilic marine biotoxins in this work are shown in Fig. 1.

Analytical standards of marine biotoxins were purchased from Cifga Laboratory (Lugo, Spain). The certified reference material were AZA-1 (1.36 ± 0.09 μg/g, purity 98% or greater; reference CRM-02-AZA1), AZA-2 (1.33 ± 0.09 μg/g, purity 97% or greater; reference CRM-02-AZA2), AZA-3 (1.30 ± 0.11 μg/g, purity 96% or greater; reference CRM-02-AZA3), OA (20.2 ± 1.4 μg/g, purity 99% or greater; reference CRM-00-OA), DTX-1 (8.08 ± 0.47 μg/g, purity 98% or greater; reference CRM-00-DTX1), YTX (7.42 ± 0.66 μg/g, purity 96% or greater; reference CRM-00-YTX) and hYTX (7.68 ± 0.49 μg/g, purity 99% or greater; reference CRM-00-hYTX). The quality control standards were AZA-4 (1.19 ± 0.07 μg/mL, purity 96% or greater; reference 02-AZA4), AZA-5 (1.20 ± 0.07 μg/mL, purity 97% or greater; reference 02-AZA5) and PTX-2 (7.16 ± 0.36 μg/g, purity 96% or greater; reference 00-PTX2). The auxiliary reagent ammonium hydroxide (99.99% purity) was

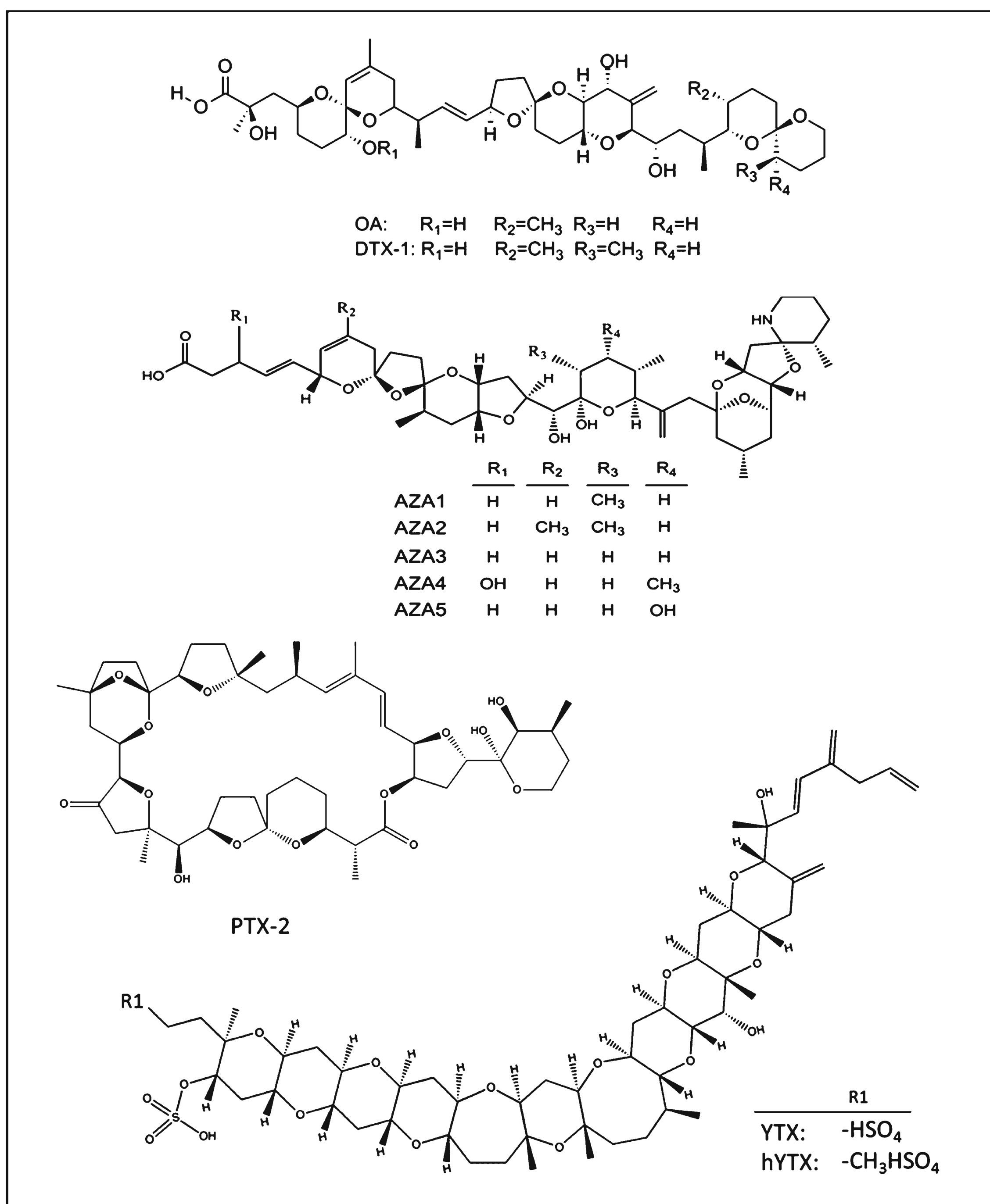

Fig. 1 Chemical structures of the selected lipophilic marine biotoxins in this study. *AZA* azaspiracid, *DTX-1* dinophysistoxin 1, *hYTX* homoyessotoxin, *OA* okadaic acid, *PTX-2* pectenotoxin 2, *YTX* yessotoxin

purchased from Sigma-Aldrich (Steinheim, Germany). High-performance LC grade methanol, acetonitrile, ultrapure water and formic acid were supplied by Merck (Darmstadt, Germany).

Sampling

In February and March 2015, seawater samples were collected from 18 different sampling sites along the Catalan coast (northeast Spain, Mediterranean Sea). Details of each location are summarised in Fig. S1. All of the selected locations have a marina and a public-access beach. Thirty-six samples of surface water (25-cm depth) from the shore of the marina and the beaches were collected in amber glass pots of 2.5 L, and they were immediately transported to the laboratory under cool conditions, and then frozen at −20 °C until their analysis.

Sample pretreatment

Each sample was analysed in triplicate. For each replicate, 500 mL of surface seawater was filtered through a nylon fibre filter of 0.45-μm mesh size (Whatman, Maidstone, UK). The seawater particulate and the dissolved phase were extracted separately. The particulate was extracted by UAE with 20 mL of methanol for 30 min. The filtrate phase was extracted and purified by SPE with 100-mg hydrophilic–lipophilic balance (HLB) cartridges (Waters, Cerdanyola del Vallès, Spain). Extraction consisted of preconditioning with 3 mL of methanol followed by 3 mL of water. Then, 500 mL of filtrate was loaded at 1 mL/min. Cartridges were washed with 3 mL of water to remove salts and interferences. Finally, the elution was completed with 6 mL of methanol. Extracts were concentrated to approximately 10 μL under a gentle N_2 stream and then reconstituted to 500 μL with the initial chromatographic mobile phase, 9:1 acetonitrile–water acidified with 0.1% formic acid.

Liquid chromatography coupled with high-resolution mass spectrometry

Chromatographic separation was performed with an Acquity ultra-high-performance LC system (Waters, Massachusetts, USA) using a reversed-phase column with C_{18} as the stationary phase (Synergy, 50 mm × 2 mm, 5 μm, 80 Å; from Phenomenex, Torrance, USA). The mobile phase was composed of acetonitrile (solvent A) and water (solvent B), both being acidified with 0.1% formic acid. The elution gradient was programmed as follows: 0 min (10% solvent A)—6 min (95% solvent A)—10 min (95% solvent A)—15 min (10% solvent A). The flow rate was maintained at 0.3 mL/min for the 15 min of the total chromatographic run. Samples were kept at 10 °C in the autosampler. The injection volume was 20 μL.

Mass spectrometry was performed with a Thermo Scientific Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionisation (HESI) probe operating in positive and negative ion modes.

The optimal source parameters were as follows: spray voltage of 3.5 kV for positive mode and 2.5 kV for negative mode, sheath flow gas of 60 a.u., auxiliary gas of 20 a.u. and sweep gas of 2 a.u. The heater temperature was set at 350 °C, the capillary temperature was set at 300 °C, and the S-lens RF level was 60%.

Acquisition was performed in full-scan and data-dependant MS/MS modes, simultaneously obtaining the full-scan mass spectrum with high resolution of 70,000 full width at half maximum (measured at *m/z* 200) and the tandem mass spectrum at medium resolution of 35000 full width at half maximum for every compound. The normalised collision energy (NCE) was set at different percentages of intensity for each compound (see Table 1).

Table 1 Fragmentation pattern of the marine biotoxins studied at optimal normalised collision energy (*NCE*)

Compound	Chemical formula	Molecular ion	*m/z* calculated	Product ion 1	*m/z* calculated	Relative abundance	Fragment ion ratio[a]	Product ion 2	*m/z* calculated	Relative abundance	Fragment ion ratio[a]	NCE (%)
Okadaic acid	$C_{44}H_{68}O_{13}$	$[M+Na]^+$	827.4547	$[C_{40}H_{60}O_{10}Na]^+$	723.4086	82	1.11 (0.06)	$[C_{44}H_{66}O_{12}Na]^+$	809.4454	58	1.62 (0.12)	40
Dinophysistoxin 1	$C_{45}H_{70}O_{13}$	$[M+Na]^+$	841.4710	$[C_{41}H_{62}O_{10}Na]^+$	737.4234	68	0.99 (0.05)	$[C_{45}H_{68}O_{12}Na]^+$	823.4606	49	1.71 (0.14)	30
Pectenotoxin 2	$C_{47}H_{70}O_{14}$	$[M+Na]^+$	881.4655	$[C_{46}H_{70}O_{12}Na]^+$	837.4758	30	20.97 (0.89)	$[C_{47}H_{68}O_{13}Na]^+$	863.4526	20	5.45 (0.62)	30
Azaspiracid 1	$C_{47}H_{71}NO_{12}$	$[M+H]^+$	842.5049	$[C_{47}H_{70}O_{11}N]^+$	824.4941	100	0.07 (0.00)	$[C_{47}H_{68}O_{10}N]^+$	806.4834	49	2.09 (0.12)	30
Azaspiracid 2	$C_{48}H_{73}NO_{12}$	$[M+H]^+$	856.5218	$[C_{48}H_{72}O_{11}N]^+$	838.5102	100	0.06 (0.01)	$[C_{48}H_{70}O_{10}N]^+$	820.4998	50	1.86 (0.02)	35
Azaspiracid 3	$C_{46}H_{69}NO_{12}$	$[M+H]^+$	828.4894	$[C_{46}H_{68}O_{11}N]^+$	810.4778	100	0.94 (0.07)	$[C_{46}H_{66}O_{10}N]^+$	792.4672	45	2.21 (0.14)	35
Azaspiracid 4	$C_{46}H_{71}NO_{13}$	$[M+H]^+$	844.4856	$[C_{46}H_{68}O_{12}N]^+$	826.4733	100	0.03 (0.00)	$[C_{46}H_{66}O_{11}N]^+$	808.4631	43	2.20 (0.18)	35
Azaspiracid 5	$C_{46}H_{71}NO_{13}$	$[M+H]^+$	844.4856	$[C_{46}H_{68}O_{12}N]^+$	826.4733	100	0.06 (0.00)	$[C_{46}H_{66}O_{11}N]^+$	808.4631	100	1.00 (0.03)	35
Yessotoxin	$C_{55}H_{82}O_{21}S_2$	$[M-H]^-$	1141.4739	$[C_{55}H_{81}O_{18}S]^-$	1061.5157	100	0.43 (0.04)	$[C_{42}H_{63}O_{16}S]^-$	855.3845	61	1.62 (0.04)	35
Homoyessotoxin	$C_{56}H_{84}O_{21}S_2$	$[M-H]^-$	1155.4892	$[C_{56}H_{83}O_{18}S]^-$	1075.5300	100	0.40 (0.01)	$[C_{43}H_{65}O_{16}S]^-$	869.4004	58	1.71 (0.04)	35

[a] The relative standard deviation is given in *parentheses*

Method validation and quality assurance/quality control

The proposed method was validated by our looking at the linearity range, intraday and interday precisions, method LODs and LOQs, and recovery rates. The validation experiments were performed by fortification of blank seawater samples with the selected marine biotoxins at three concentrations and by analysis of three replicates at each different spiking level following the method described in “Sample pretreatment” and “Liquid chromatography coupled with high-resolution mass spectrometry”.

Instrumental blanks, extraction blanks and procedural blanks were analysed at the beginning and in between fortified extracts. The instrument blanks were marine-biotoxin-free solvent (methanol) blanks that were analysed at the beginning of the run. The extraction blanks were marine-biotoxin-free ultrapure water that was extracted and analysed together with the samples. The procedural blanks were blanks that had been subjected to the sampling, the extraction and the instrumental analysis. No interference, contamination or carryover was detected in the blanks.

Selectivity

Identification of the target compounds was accomplished by comparison of the relevant retention time, exact mass and full-scan signals of the analytes in the matrix with those obtained for standard solutions, being analysed under the same experimental conditions.

Limits of detection and quantification

Instrumental LODs, defined as the lowest concentration at which each compound could be detected (with a Gaussian peak shape, less than 2 ppm of exact mass error and molecular isotopic pattern accomplishing the standard ratio) were determined by progressive dilution. A standard solution containing the ten selected biotoxins was prepared at an initial concentration of 50 μg/L, and serial dilutions of 1:5 and 1:10 were injected from 50 μg/L to 1 ng/L. The instrumental LOQ was estimated as 10/3 times the instrumental LOD.

In the same way, the method LODs of each analyte were defined as the lowest concentration for which the peak area was detected in matrix-matched calibration points. Progressive dilutions of the ten compounds in extracts from the particulate (seawater-suspended material) and in filtered seawater were used. The method LOQ were established as 10/3 times the method LOD.

Linearity

Instrumental linearity and sensitivity were estimated as the Pearson correlation coefficient (R^2) and the slopes of the calibration curves respectively. Average retention times and relative abundances for each analyte are shown in Table 2. An acceptable chromatographic separation was achieved for most of the target analytes.

Intraday and interday precision

Instrumental reproducibility (interday precision) was determined with six replicates of standard solutions on three consecutive days. Method reproducibility was calculated on three different days, and these values are shown in Table 2.

Recoveries and matrix effects

Repeatability and recoveries were obtained in fortified blank samples of seawater particulate and filtrate fractions. Three replicates for each matrix were used at two concentrations. Also, the matrix effect, in terms of signal suppression/enhancement, was estimated for particulate and filtrate extracts. These parameters are shown in Table 3.

Safety conditions

Because of the toxic properties of these compounds, extreme caution was used during the manipulation of the standards and samples. All solutions were maintained and all extractions were prepared under a fume hood. Moreover, the instrumental analysis was performed with a covering curtain surrounding the equipment. Microsyringes and glass material in contact with standards and samples were carefully rinsed after their use and heated at 400 °C overnight.

Results and discussion

Optimisation of the analytical methods

Sample preparation

Because of interferences that are commonly present in complex matrices, such as seawater, and the low concentrations expected in seawater, a purification step is needed to increase the sensitivity and to reduce matrix interferences and the concentration of salts in the extract. Therefore, 500 mL of each seawater sample was filtered through a 0.45-μm nylon filter and submitted to SPE. Considering the versatility and the results that were previously reported with use of OASIS HLB cartridges [7], this type of stationary phase was preselected. After the preconditioning of the cartridges with 3 mL of methanol and 3 mL of ultrapure water, the optimal loading volume of the seawater sample was evaluated: 100-, 250-, 500- and 1000-mL samples were tested. The highest intensities were obtained for 500 and 1000 mL, but to avoid clogging of the

Table 2 Analytical parameters for the ten lipophilic marine biotoxins: average retention time, linearity and sensitivity expressed as Pearson correlation coefficients (R^2) of the calibration curves for the instrument and the analytical methods of particulate and the seawater, and intraday and interday instrumental and method precision represented as the relative standard deviation (*RSD*) of the standard solution

Compound	Instrumental parameters							Particulate				Filtrate seawater			
	Retention time (min)	Linearity (μg/L)	R^2	RSD (%) Intraday ($n = 6$) (10 μg/L)	RSD (%) Interday ($n = 3$) (10 μg/L)	ILOD (fg on column)	ILOQ (fg on column)	Linearity (ng/L)	R^2	MLOD (ng/L)	MLOQ (ng/L)	Linearity (ng/L)	R^2	MLOD (ng/L)	MLOQ (ng/L)
Okadaic acid	4.97	0.25–50	0.99	3.39	7.92	5	15	0.5–50	0.99	0.4	1	0.5–50	1.00	0.3	1
Dinophysistoxin 1	5.86	0.25–50	0.99	7.89	6.14	5	15	0.8–50	0.98	0.3	1	0.2–20	0.99	0.3	1
Pectenotoxin 2	5.47	0.5–50	0.99	10.2	27.5	2	6	0.8–50	0.98	0.5	2	0.5–50	1.00	0.5	2
Azaspiracid 1	5.3	0.5–50	0.99	7.06	10.8	10	30	0.8–50	0.97	0.003	0.01	0.5–50	0.99	0.002	0.007
Azaspiracid 2	5.5	0.1–50	0.99	5.56	14.3	10	30	0.5–50	0.99	0.003	0.01	0.5–50	1.00	0.002	0.007
Azaspiracid 3	4.96	0.025–50	1.00	4.38	7.67	2	6	0.5–50	0.99	0.003	0.01	0.5–50	0.99	0.002	0.007
Azaspiracid 4	4.21	0.025–50	1.00	8.25	7	1	3	0.5–50	0.98	0.003	0.01	0.5–50	0.99	0.003	0.01
Azaspiracid 5	4.49	0.8–50	0.96	8.33	12.1	1	3	1–50	0.98	0.003	0.01	1–20	1.00	0.003	0.01
Yessotoxin	5.59	0.5–50	0.99	10.8	10.7	16	48	1–50	0.98	0.3	1	0.5–50	0.99	0.3	1
Homoyessotoxin	5.61	0.5–50	0.99	9.56	7.26	10	30	1–40	0.99	0.5	2	1–40	0.99	0.5	2

Limits of detection and quantification obtained for each compound in the instrumental approach and in both analytical methods are shown

ILOD instrumental limit of detection, *ILOQ* instrumental limit of quantitation, *MLOD* method limit of detection, *MLOQ* method limit of quantitation

Table 3 Recoveries of the analytical method for the ten lipophilic marine biotoxins at two concentrations for the particulate and the filtrate, and the corresponding matrix effect

Compound	Particulate									Filtrate				
	Recovery (%)								Matrix effect	Recovery (%)				Matrix effect
	30-min ultrasonication				3-min ultrasonication									
	10 ng/L[a]	RSD (n= 3)	1 ng/L[b]	RSD (n = 3)	10 ng/L[a]	RSD (n = 3)	1 ng/L[b]	RSD (n = 3)		25 ng/L	RSD (n = 3)	10 ng/L	RSD (n = 3)	
Okadaic acid	83.4	10.1	69.9	0.2	91.6	2.2	80.68	3.07	1.0	84.8	11.4	97.2	6.03	1.4
Dinophysistoxin 1	89.1	5.8	-	-	-	-	-	-	0.3	75.0	8.6	61.2	3.7	0.3
Pectenotoxin 2	75.3	3.2	-	-	64.2	21.5	55.1	2.0	1.3	84.7	19.7	81.5	9.6	1.5
Azaspiracid 1	33.9	5.1	69.5	39.6	106.8	4.0	64.6	19.9	0.8	40.1	11.9	33.8	11.1	1.8
Azaspiracid 2	20.5	0.3	61.3	51.2	66.2	8.8	48.0	13.4	1.8	41.0	13.5	24.0	0.8	2.0
Azaspiracid 3	57.1	8.0	29.4	3.4	104.4	21.5	49.2	8.9	1.0	83.3	7.8	44.4	11.2	1.3
Azaspiracid 4	74.4	13.8	72.4	4.9	121.2	34.0	63.8	14.0	0.9	66.4	2.7	47.4	2.4	1.1
Azaspiracid 5	76.2	22.2	51.2	1.6	101.8	38.7	58.4	21.2	1.0	50.9	9.5	38.8	7.8	2.0
Yessotoxin	43.0	2.1	76.1	4.2	47.6	8.4	29.5	0.5	0.6	53.2	14.9	51.5	20.1	1.5
Homoyessotoxin	46.1	1.6	79.2	5.8	35.6	8.1	45.6	4.3	0.6	47.6	12.5	48.8	24.0	1.5

RSD relative standard deviation

[a] Considering 17 mg of particulate per litre of seawater (diameter greater than 0.45 μm), this concentration is equivalent to 588 μg of okadaic acid per kilogram of particulate.

[b] Considering 17 mg of particulate per litre of seawater (diameter greater than 0.45 μm), this concentration is equivalent to 58.8 μg of okadaic acid per kilogram of particulate.

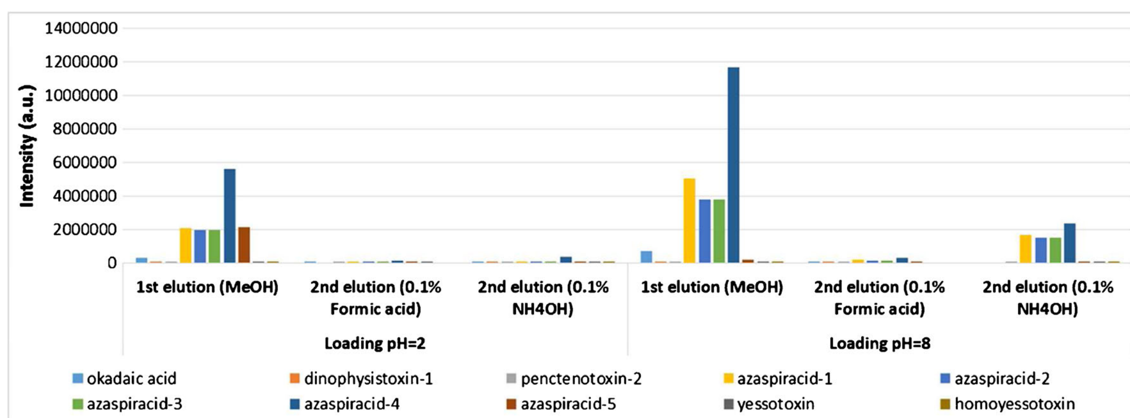

Fig. 2 Amount of marine biotoxin eluted from the cartridge under different loading and elution conditions

cartridges, the optimal volume was set at 500 mL. The effect of pH was also considered, and the sample pH was adjusted to 2 and 8. For most of the compounds, the highest intensity was obtained at pH 8 (Fig. 2). YTX and the related hYTX exhibited slightly higher recoveries when the sample was acidified, but pH 8 was set as the optimal value as a compromise. Also, different elution conditions were tested. Two cycles were tested: the first cycle, with 3 mL of methanol, and the second cycle, with either 3 mL of methanol with 0.1% ammonium hydroxide, or 3 mL of methanol with 0.1% formic acid. As shown in Fig. 2, second elutions with acidified methanol or with basified methanol did not improve significantly the performance of the method. Overall, the best performance was obtained when the seawater samples were loaded at pH 8 and the elution was performed with methanol in two cycles. As can be seen in Table 3, under these conditions, the recovery rates were above 40% for most of the compounds in seawater.

On the other hand, to analyse those biotoxins trapped in the particulate matter during the filtration step, a method based on UAE was optimised. In this case, the use of different solvents was studied, including methanol, methanol with 0.1% ammonium hydroxide, methanol with 0.1% formic acid, and acetonitrile. Each of the solvents tested was used in two extraction cycles of 3 and 30 min, and each with 10 mL of extractant. Two cycle times were tested because although some of the marine biotoxins are degraded by prolonged UAE, other toxins exhibited better recoveries with extraction for 30 min. The recoveries obtained for the extraction from seawater particulate with different solvents for 30 minutes are presented in Fig. 3. For OA and DTX-1, higher recoveries were obtained with use of methanol with 0.1% ammonium hydroxide for the extraction. However, for the rest of the biotoxins studied here, the best recoveries were obtained with methanol. Therefore, methanol was selected as the extraction solvent. As can be seen in Table 3, for most of the compounds, the recovery rates ranged from 43% to 89%, with the exception of AZA-1 and AZA-2, for which they were lower.

Optimisation of the analytical procedure

To optimise the chromatographic separation, different mobile phases composed of water and acetonitrile or water and methanol in different proportions were tested. In Table S1, the tailing factor of each compound is shown under different conditions.

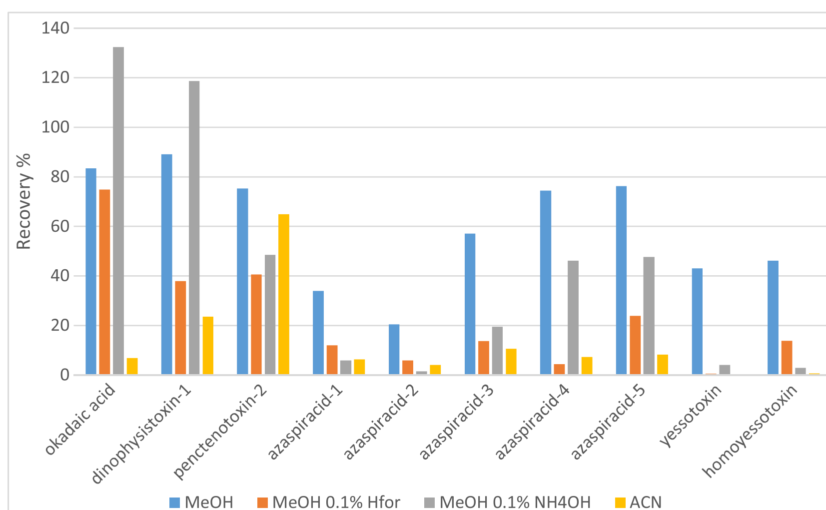

Fig. 3 Absolute recovery for the extraction of the particulate using different solvents: methanol (*MeOH*), MeOH with 0.1% formic acid (*Hfor*), MeOH with 0.1% NH_4OH, and acetonitrile (*ACN*)

The acidic conditions with acetonitrile–water with 0.1% formic acid were selected to give the best shape of the chromatographic peaks for most of the compounds, as in previous studies [18]. Good separation was achieved for the target analytes as presented in the extracted ion chromatograms in Fig. S2.

Toxin standards were directly infused into the HESI source to determine the optimal mass spectrometry conditions. For each analyte, the mass of the corresponding ion obtained was compared with the theoretical mass calculated by Xcalibur 2.1. Mass deviations were found to be below 2 ppm.

The mass spectral characterisation of selected toxins is described in Table 1. Under the optimum working conditions, each compound was identified and several fragment ions were obtained, some of them being in agreement with previous studies [19], and some others being determined during the optimisation.

Identification of the ten lipophilic marine biotoxins was successfully achieved by comparison of the average retention time (Table 2) and the exact mass of the precursor and product ions for each compound (Table 1), both in the matrix and in standard solutions that were analysed under the same experimental conditions.

LODs and LOQs are presented in Table 2. As can be seen, the best instrumental LODs were obtained for AZA toxins, with the method LOQ in the picogram per litre range, showing a high sensitivity of the method, especially for the filtrate portion. The instrumental linearity and sensitivity were estimated for all compounds in the different matrices. For this, calibration curves were prepared in solvent- and matrix-matched extracts of the particulate and filtrate. The concentration range of the calibration curves and Pearson correlation coefficients (R^2) are shown in Table 2 for all analytes in each matrix.

Recoveries were estimated at two concentrations: 588.2 and 58.82 μg/kg for the particulate fraction and 25 and 10 ng/L for the seawater filtrate. Three replicates were used for each matrix and each concentration. Blank samples were treated with the same extraction procedure and then spiked at the same concentration to be used as references.

For the particulate fraction, two different treatments (3 and 30 min of UAE) were required because some of the marine biotoxins can be degraded by prolonged UAE, but prolonged extraction is recommended to be sure of the extraction of toxins from algal cells in suspension. Low recoveries were also

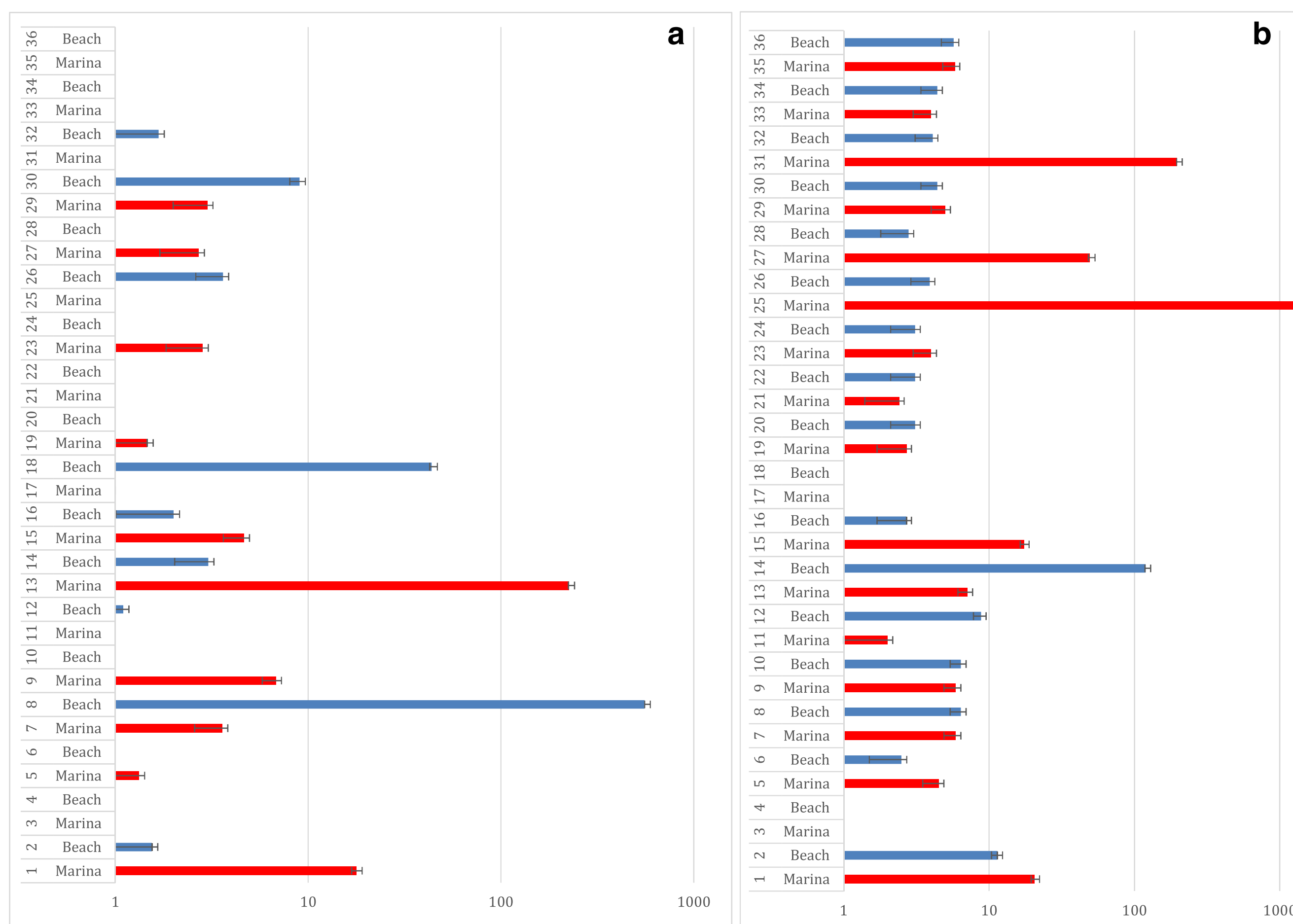

Fig. 4 Occurrence of okadaic acid on a logarithmic scale (**a**) in the particulate fraction expressed as micrograms per gram and (**b**) in seawater expressed as nanograms per litre for the different sampling sites

affected by the low concentrations considered, but in all cases these were corrected by the use of matrix-matched standards.

The matrix effect was calculated for each type of extract by division of the slope of the calibration curve of the particulate and filtrate by the slope of the calibration curve prepared in solvent. The values are summarised in Table 3. In general, matrix enhancement was observed for most of the compounds. In spite of the filtrate fraction being cleaned up by SPE, the effect was greater in this fraction because of the presence of coeluted compounds, as reported previously [20–24]. Previous studies of marine biotoxins also reported matrix enhancement [7, 23], which in our case was compensated for by the use of matrix-matched standards.

Occurrence of the biotoxins on the Catalan coast

Thirty-six surface seawater samples collected according to the description in "Sampling" were analysed. The main result of this study was the presence of a single lipophilic marine biotoxin, OA, which was present in 88% of the samples. The concentrations of OA at each sampling site are presented in Fig. 4. Taking into account the total concentration of OA in both the particulate and the filtrate, the mean value was 730 ng/L and the median value was 71 ng/L. Samples taken near highly urbanised, industrialised or agricultural areas recorded the highest concentrations of OA. The presence of this lipophilic biotoxin has been evidenced in a considerable number of studies related to biota and water from the Mediterranean Sea [26, 27].

The highest concentrations of OA were present in the particulate fraction with a mean partition ratio (K) of 0.298, calculated as the concentration of the filtrate divided by the concentration of the particulate, both in nanograms per litre. For OA, K ranged from 1.00×10^{-3} to 3.82 inside ports and from 1.00×10^{-3} to 0.832 in open coastal areas. Inside ports, the mean of K was 0.405, while in open coastal areas, the mean was 0.166.

The concentrations in suspended material are summarised in Fig. 4a. In the particulate, the concentrations of OA ranged from 0.09 to 560 μg/g. The mean and median concentrations were 24.9 and 1.21 μg/g respectively. Considering only the samples with positives, the mean and median concentrations were 34.5 and 2.36 μg/g respectively. The highest concentration of OA was 560 μg/g, corresponding to site 8—L'Ametlla de Mar beach. This high concentration could be related to high water temperatures accompanied by high amounts of organic matter, partially generated in the aquaculture facilities nearby, that could contribute to the eutrophication, promoting phytoplankton growth. The mean concentration of OA in positive open coastal samples was 52.03 ± 152.5 μg/g, whereas inside ports it was almost half that value, 19.41 ± 59.28 μg/g, although this difference was shown not to be statistically significant ($p > 0.050$) according to the t test and non-parametric tests (Wilcoxon signed rank test).

Related to the occurrence of OA in the filtrate seawater, the concentrations in nanograms per litre are shown in Fig. 4b. In seawater, the concentrations of OA ranged from 2.10 to 1780 ng/L. The mean concentration was 64.0 ng/L and the median concentration was 4.40 ng/L. Considering only the samples with positives, the mean and median concentrations were 72.0 and 4.75 ng/L respectively. The sample with the highest concentration corresponded to site 25—Masnou marina—with 1780 ng/L. Contrary to what happens with the particulate samples in water solution, the highest OA concentrations were found in samples from the interiors of ports.

In addition, no clear tendency was found between the concentration of OA in samples coming from inside marinas and those from open coastal areas. The highest concentration of OA in marinas was 9600 ng/L, whereas in open coastal areas it was 8600 ng/L. The mean concentration inside marinas was 770 ng/L, and the median concentration was 110 ng/L, whereas in open areas the concentrations were 700 and 57 ng/L respectively. No significant correlations were found between the concentrations of OA and measured pH and salinity.

OA is produced by different species of dinoflagellates of the genera *Dinophysis* and *Prorocentrum*, which are present in oceans and seas worldwide [25], including the Mediterranean Sea. The rest of the lipophilic marine biotoxins were not detected in any samples in this study, even those which are produced by species of the same class (Dinophyceae). However, according to our results, the presence of some species of this phylum in the Catalan coast, during the sampling weeks, can be suggested. Some reports have shown that OA is one of the biotoxins that accumulates in higher amounts in Mediterranean shellfish of some areas [27–29].

Conclusions

A multiresidue method has been developed and evaluated for the analysis of ten lipophilic marine toxins in seawater, by our considering two fractions of water and particulate material, showing a solid performance at the parts per trillion level.

The method was assessed with respect to accuracy, specificity, selectivity, repeatability, within-laboratory reproducibility, LOD, LOQ and linearity.

Good performance has been demonstrated, permitting quantitative analysis of selected analytes and fast screening of non-target biotoxins by retrospective screening using its full-scan capabilities.

The method was applied to characterise the occurrence of these contaminants in samples from the western Mediterranean coast. OA was the only biotoxin detected, and this compound was found in most of the samples (88%) in the range between 0.11 and 560 μg/g in the particulate and between 2.1 and 1780 ng/L in the filtrate in positive samples in non-algal bloom conditions. These results support the need for monitoring

programs in Europe, and highlight the importance of further studying the degradation patterns, distribution and chronic toxic effects to properly perform risk assessment studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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