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Purification of five azaspiracids from mussel samples contaminated with DSP toxins and azaspiracids

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ABSTRACT

Human intoxications during toxic episodes in shellfish are a very important concern for public health, as well as for economic interests of producer regions. Although initially each toxin appeared in a determined geographical zone, nowadays many of them are found in multiple places worldwide. In addition, more toxic compounds (new toxins or new analogs of known toxins) are being isolated and identified, which bring about new risks for public health. An example of this situation is the group of azaspiracids (AZAs). Initially these toxins were concentrated in Irish coasts but today appear in many different geographic locations; in the first toxic episode only three analogs were isolated, but now it is known that the group is comprised of at least eleven identified compounds. A substantial problem associated with all these new toxins is the extreme difficulty associated with the study of their toxic effects and mechanisms of action due to the very small quantities of purified toxin available. Therefore, the study of procedures to isolate them from contaminated shellfish or to synthesize them is of tremendous importance. In this paper we design a complete procedure to obtain AZAs analogs from mussels contaminated with DSP toxins and azaspiracids by means of three consecutive steps: an extraction procedure to remove toxins from shellfish, a solid phase extraction (SPE) to clean the samples and separate DSP toxins and AZAs, and a preparative HPLC to isolate each analog. In all the steps LC/MS is used to detect and quantify the toxins. Large amounts of AZA1, AZA2, AZA3, AZA4 and AZA5 were obtained by use of this procedure, which can be utilized in future studies relating to the toxins such as the production of certified materials and standards.
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1. Introduction

Azaspiracids (AZAs) are a new group of toxins described during the last decade. Their first toxic episodes were reported in 1995 and 1997 with mussels collected on the coast of Ireland [1,2], and produced similar human symptoms to those associated with DSP toxins but characterized by a slow progressive paralysis in the mouse bioassay. A new molecule was isolated in extracts from these mussels and named as azaspiracid (AZA1), due to its unusual structure with an azaspiro ring system fused to a 2,9-dioxabicyclo[3.3.1]-nonane ring, a trioxadispiroketal fused to a tetrahydrofuran ring and a carboxylic acid [3]. Synthesis of AZA1 showed that the stereochemistry initially proposed was incorrect and made possible the discovery of the correct structure [4–6].

Two analogs of AZA1 (Scheme 1) were isolated in mussels from one of the first toxic episodes and named as azaspiracid-2 (AZA2, 8-methylazaspiracid) and azaspiracid-3 (AZA3, 22-demethylazaspiracid) [7]. These have recently been successfully synthesized [8]. Over the last few years more analogs have been identified in shellfish (from AZA4 to AZA11) and their structures have been described [9–11]: AZA4 and AZA5 are the 3-hydroxy and the 23-hydroxy analogs of AZA3, AZA6 is a positional isomer of AZA1, AZA7 and AZA8 are hydroxyl analogs of AZA1, AZA9 and AZA10 are hydroxyl analogs of AZA6 and AZA11 is a hydroxyl analog of AZA2. The quantities of AZA7–AZA11 present in shellfish are extremely low, and usually account for less than 5% of the total AZAs and often even smaller ratios of these toxins are present [11].

Abbreviations: AZA, azaspiracid; DSP, diarrhoetic shellfish poisoning; SPE, solid phase extraction; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography coupled to mass spectrometry; YTX, yessotoxin; DTX, dinophysistoxin; OA, okadaic acid; HP, hepatopancreas; MeOH, methanol; AcH, acetic acid.

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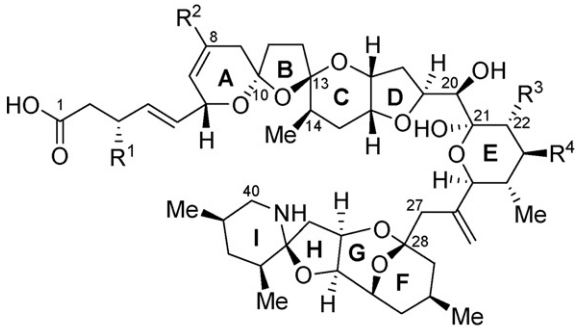


	R^1	R^2	R^3	R^4
1: azaspiracid-1	H	H	Me	H
2: azaspiracid-2	H	Me	Me	H
3: azaspiracid-3	H	H	H	H
4: azaspiracid-4	OH	H	H	H
5: azaspiracid-5	H	H	H	OH

Scheme 1. Structures of various AZAs analogs.

AZA4–AZA11 have only been isolated in shellfish extracts, whereas AZA1–AZA3 have also been identified in extracts of cells of *Protoperidinium crassipes* [12], suggesting that the remainder of the analogs are products of bioconversion in shellfish. The predator characteristics of the identified microorganism make it impossible to determine if it produces the toxins or transforms a precursor molecule produced by another dinoflagellate or simply acquires them by feeding [12].

Although initially AZAs only appeared in mussels from Ireland, later they were detected in other bivalve molluscs, such as oysters, scallops, clams and cockles [13], and in other regions, such as north-east England and south-west Norway [14], north-west France and north-west Spain [15] and north-west Morocco [16]. Distribution of AZAs in mussel tissues has been studied with variable results. In one experiment AZA1–AZA3 were distributed throughout tissues, with AZA1 being predominant in the digestive glands and AZA3 in the remaining tissues [17]; whereas in another study AZAs were accumulated in the digestive glands only [18].

EU legislation established that the maximum level of AZAs (AZA1–AZA3) in bivalve molluscs, echinoderms, tunicates and marine gastropods is 160 μg of azaspiracid equivalents per kilogram (measured in the whole body or any part edible separately) [19].

Lack of large quantities of AZAs, isolated from naturally contaminated samples or synthesized, has made the study of their effects and mechanisms of action extremely difficult. An intraperitoneal lethal dose for AZA1 in mice has been determined to be 0.2 mg/kg, for AZA2 0.11 mg/kg and for AZA3 0.14 mg/kg [7]; whereas AZA4 and AZA5 showed less toxicity, as expected being products of bioconversion in shellfish [9]. Even several papers have been published about the cellular effects of AZAs, unfortunately the intracellular target of these toxins has not yet been determined.

Detection and quantification of marine toxins can be performed by liquid chromatography coupled to mass spectrometry [20,21]. This versatile technique has a high degree of sensitivity and selectivity and allows the simultaneous quantification of multiple compounds, obtaining information about their molecular weights and structural conformations.

The aim of the present study was to isolate AZA1–AZA5 analogs from contaminated mussels that contain DSP toxins and AZAs. These shellfish were extracted and partitioned with various solvents. Subsequently the two groups of toxins (DSP and AZAs) were separated by a solid phase extraction (SPE) procedure and finally AZAs analogs were isolated by HPLC. Quantification of toxins during the purification process was achieved by LC/MS/MS using atmospheric pressure ionization techniques.

2. Experimental

2.1. Chemicals

Solvents used in this paper were of HPLC or analytical grade quality. Acetone, methanol, *n*-hexane, ethyl acetate, acetic acid glacial, trifluoroacetic acid and acetonitrile were from Panreac (Spain). Formic acid was from Merck (Spain). Ammonium formate was from Fluka (Switzerland). Water with a high degree of purity was obtained by a prepurification filter system followed by the water purification system Arium 611 from Sartorius (Germany).

Standard solution of OA was provided by NRC Certified Reference Materials Program (Institute for Marine Biosciences, Halifax, NS, Canada). DTX-1, DTX-2, and YTX standards were kindly provided by Dr. T. Yasumoto; AZA1, AZA2, AZA3, AZA4 and AZA5 standards were kindly provided by Dr. M. Satake (Japan).

2.2. Technology

- Evaporators: Büchi® Syncore Systems for Parallel Evaporation (from Büchi, Switzerland), rotary evaporator R-200 (from Büchi) and VV2000 (from Heidolph, Germany) with vacuum controller (from Büchi) and centrifugal evaporator RC 10 09 (from Jouan, France).
- HPLC system: preparative and analytical HPLC were done with the same equipment from Waters (USA), which consists of pump (Delta 600), sample manager (2767), fraction collector (III) and controller (600), coupled to a column oven (CTO-10ACvp) from Shimadzu (Japan).
- LC/MS system: HPLC system Shimadzu, which consists of two pumps (LC-10ADvp), autoinjector (SIL-10ADvp) with refrigerated rack, degasser (DGU-14A), column oven (CTO-10ACvp) and system controller (SCL-10Avp), coupled to a QTRAP LC/MS/MS system (from Applied Biosystems, USA), which consists of a hybrid quadrupole-linear ion trap mass spectrometer equipped with an API fitted with an ESI source. Nitrogen generator NM20ZA (from Peak Scientific, USA).

2.3. Mussel samples

Mussels were collected in Ireland during a harmful algal bloom (with OA, DTXs and AZAs) in 2006. The whole flesh tissue was removed from the shell and frozen.

Samples were defrosted, interstitial liquid was collected and digestive glands were carefully separated from the rest of the meat using clamps and scissors.

After this process 2.6 kg of digestive glands, 12.85 kg of meat and 5.6 kg of liquid were obtained. Meat and liquid were mixed and divided in six homogeneous portions of approximately 3 kg each one.

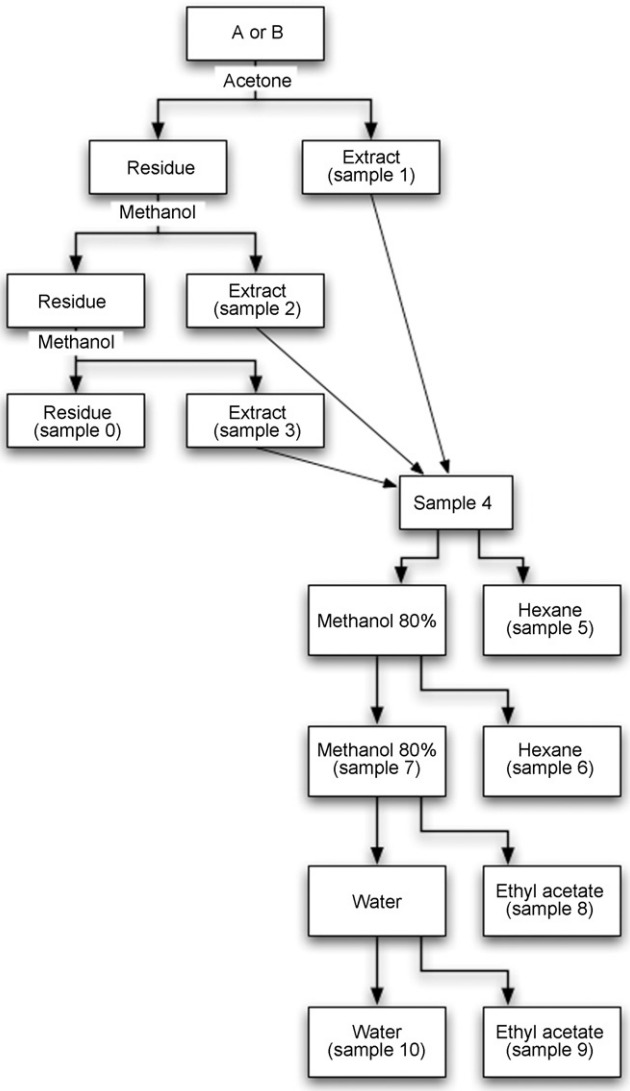
Two samples were processed:

- (A) Digestive glands (2.6 kg).
- (B) One portion of the mixture of meat and liquid (3 kg).

2.4. Extraction procedure (see Scheme 2)

The extraction procedure was the same for A and B (Scheme 2).

Sample was homogenized, mixed with acetone (using a volume of three times the weight, 3:1) and filtered. The extract (sample 1) was collected and vacuum dried. The residue was mixed with methanol (3:1) twice and filtered. These two extracts (samples 2



Scheme 2. Extraction procedure.

and 3) were mixed and vacuum dried. Finally samples 1–3 were mixed and named as sample 4.

Sample 4 was partitioned with 1:1 hexane and methanol (80%). The hexane layer (sample 5) was collected and the methanol layer was partitioned again with new hexane. The hexane layer (sample 6) was collected and the methanol layer, which contained the toxins, was vacuum dried (sample 7).

Sample 7 was partitioned with ethyl acetate and water (1:1). The ethyl acetate layer (sample 8) was collected and the water layer was partitioned again with new ethyl acetate. The two final layers (named as sample 9: ethyl acetate and sample 10: water) were collected separately.

The extracts obtained in all the steps of this procedure were analyzed by LC/MS.

2.5. Solid phase extraction

A SPE with silica was utilized to separate OA, DTXs and AZAs, using two types of cartridges: 2 ml empty polypropylene (from SUPELCO, Spain) filled with silica gel 60 (230–400 mesh ASTM, from Merck) and LC silica packing 20 ml (from SUPELCO). All cartridges were wetted with acetone before charging the sample, which was also dissolved in acetone. After adding the sample, cartridges were washed with acetone and finally they were eluted with methanol. The SPE was done using a VAC ELUT SPS 24 (from Varian, USA). The acetone and methanol extracts obtained during the SPE were collected separately and analyzed by LC/MS.

2.6. HPLC

Samples injected in the HPLC system were previously homogenized and filtered through 0.45 μm filters (Ultrafree-MC centrifugal filter devices from Millipore, Spain). Different AZAs analogs were separated using the following columns: Luna 5 μm C18(2) (150 mm × 2 mm) with 4 mm × 2 mm security guard cartridge for analytical assays and Luna 5 μm C18(2) (150 mm × 10 mm) with 10 mm × 10 mm security guard cartridge for preparative assays; all these materials were provided by Phenomenex (USA). Temperature of the column oven, sample volume injection, flow and mobile phase composition were modified to optimize the recovery and the separation of AZAs analogs in the column.

The final optimal conditions for preparative HPLC were: 35 °C in the column oven, 500 μl sample volume injection and 2.5 ml/min isocratic flow, using a mobile phase of MeOH:H_2O:AcH (700:300:1).

In all the experiments fractions were collected by time: one separated fraction was collected per minute. Each one of these fractions was vacuum dried, dissolved in methanol and analyzed by LC/MS.

2.7. LC/MS analysis

All the extracts collected during the extraction procedure and the SPE were analyzed by LC/MS to determine their toxins concentration. Before being injected in the LC/MS system, they were evaporated to dryness, resuspended in methanol and filtered through 0.45 μm filters (Ultrafree-MC centrifugal filter devices from Millipore, Spain). The column fractions from preparative HPLC were evaporated to dryness and resuspended in methanol.

Separation between toxins was achieved in a BDS-Hypersil C8 3 μm 120 A (50 mm × 2 mm) column (from Phenomenex, USA) with a 10 mm × 2.1 mm guard cartridge (from Thermo, USA), inside the column oven at 25 °C. Injection volume was 5 μl. Mobile phase consisted of 100% water with 2 mM ammonium formate and 50 mM formic acid in pump A and acetonitrile:water (95:5) with 2 mM ammonium formate and 50 mM formic acid in pump B. Analysis were carried out using a linear gradient elution with a constant total flow of 0.2 ml/min and a run time of 14 min. In each run % B started at 30%, achieving 90% in minute 8; these conditions held during 3 min (90% B and 10% A); % B decreased to 30% in minute 11.5 and it maintained this value until next run. Analyst software was used to control the instrument, process the data and analyze them.

Extracts were analyzed with the ESI interface operating simultaneously in both positive and negative modes using the following parameters: curtain gas, 15; CAD gas, 6; IonSpray voltage, 4000 or −4000; temperature, 450; gas 1, 50; gas 2, 50; these parameters had been previously optimized using toxin standards. Fractions from preparative HPLC were analyzed with the ESI interface operating in positive mode, with the parameters listed above (IonSpray voltage: 4000). The mass spectrometer was operated in multiple reaction-monitoring (MRM), analyzing two product ions per compound: one for quantification and the other for confirmation. For ESI negative the transitions selected were: OA and DTX-2, 803.6 > 255.2/209.2; DTX-1, 817.6 > 255.2/209.2; YTX, 1141.4 > 1061.5/855.4. For ESI positive the transitions selected were: AZA1, 842.6 > 824.2/806.1; AZA2, 856.7 > 838.6/820.6; AZA3, 828.7 > 810.7/792.7; AZA4 and AZA5, 844.5 > 826.6/808.7. Quantification was done with the most abundant ion in the fragment spectra: 255.2 (OA and DTX-2, DTX-1), 1061.5 (YTX), 824.2 (AZA1), 838.6 (AZA2), 810.7 (AZA3) and 826.6

Table 1
Results obtained during the extraction procedure of sample A

Sample	AZA1 (μg)	OA (μg)	DTX-2 (μg)
0	121	21	4
4	28,200	2910	548
5	0	0	0
6	61	0	0
7	43,750	2045	515
8	41,125	2088	366
9	239	5	3
10	272	0	0

Digestive glands from mussels (A) were collected, homogenized and extracted with acetone and methanol. Later on the extracts followed various partitions. Different samples, from 0 to 10, were obtained along the processes (Scheme 2) and their toxin concentrations were determined by LC/MS.

(AZA4 and AZA5), using the calibration curves obtained for each one of the standards.

3. Results

The objective of this study is to obtain purified AZAs analogs from contaminated mussels. These toxins must be extracted from the shellfish and separated from OA and DTXs; later on each analog must be isolated. To perform this process we use three steps: an extraction procedure from shellfish, a SPE with silica and an HPLC separation. LC/MS is used to quantify toxins concentration along the process.

3.1. Extraction procedure

The extraction procedure is the same for A and B (Scheme 2). They are extracted with acetone and later on twice with methanol. The three extracts are vacuum dried and mixed, obtaining a dense final material (sample 4). To guarantee that all the toxins have been extracted, the final extraction residue (sample 0) is mixed with methanol and the toxin profile of this methanolic extract is measured, obtaining only trace amounts of OA, DTX-2 and AZA1.

Sample 4 contains OA, DTXs and AZAs, together with contaminants extracted from initial samples (A or B). Various partitions are used to eliminate undesirable compounds. The sample is solved in methanol (80%) and partitioned twice with hexane, obtaining two hexane layers (samples 5 and 6) with trace amounts of AZAs and one methanolic final layer (sample 7) with the rest of the toxins. In this step various fat layers are separated in the interface methanol/hexane, eliminating lipophilic contaminants from the final sample.

Sample 7 is vacuum dried, solved in water and partitioned twice with ethyl acetate. In this case hydrophilic contaminants are eliminated in the final watery layer (sample 10) and the toxins are concentrated in the two ethyl acetate layers (samples 8 and 9).

Results obtained during the extraction of A are shown in Table 1. AZA1 concentration in sample 4 is low with respect to samples 7 or 8, the high density of the first one produced a lack of homogeneity in the measured aliquot which can be the explication of this value. Sample 8 contains almost the overall of the initial toxins, without lipophilic and hydrophilic contaminants eliminated with the partitions.

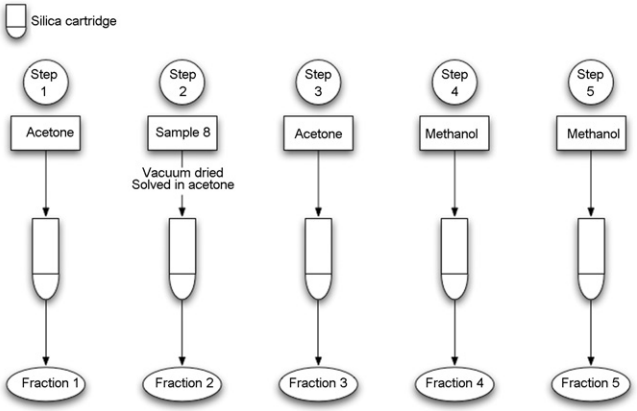
A summary of the results obtained for B is shown in Table 2. Concentrations of sample 4 are also low, due to the same difficulties than for sample A. Sample B does not contain DTX-2, but the distribution of AZA1 and OA in the different steps of the extraction corresponds with the obtained for sample A.

After analyze these results we decided to use sample 8 (from A or B) to perform the solid phase extraction and separate different groups of toxins.

Table 2
Results obtained during the extraction procedure of sample B

Sample	AZA1 (μg)	OA (μg)	DTX-2 (μg)
0	79	5	0
4	14,325	264	0
5+6	4	0	0
7	29,475	699	0
8+9	20,966	649	0
10	385	0	0

Meat and liquid from mussels were collected, weighted and divided in six aliquots. One of them (B) was extracted with acetone and methanol. Later on the extracts followed various partitions. Different samples, from 0 to 10, were obtained along the processes (Scheme 2) and their toxin concentrations were determined by LC/MS.



Scheme 3. Solid phase extraction (SPE).

3.2. SPE

Sample 8 contains toxins from the original sample (A or B) solved in ethyl acetate. We do a solid phase extraction with silica to separate AZAs from DSP toxins, using 600 mg of silica for 5 g of initial sample (digestive glands or meat mixed with liquid). Sample 8 must be dried and solved in acetone previously to the SPE. The process is as follows: silica cartridge is wetted with acetone and sample is charged; then the cartridge is washed with acetone and finally it is eluted with methanol. For each SPE we obtain five liquid fractions (Scheme 3), but fraction 1 only contains acetone. Results using sample 8 from digestive glands appear in Table 3. High density of sample 8 makes difficult to obtain homogeneous aliquots and due to this results in Table 3 show high variability. However, the SPE achieves the separation between OA and DTX-2, mainly in fractions 2 and 3, and AZA1, mainly in fractions 4 and 5.

Finally we scale the described process and use 64 cartridges with 5 g of silica each one, with the corresponding solvent volumes, separating OA and DTX-2 in the acetone fractions (mixed and called as sample C) and AZA1 in the methanol fractions (mixed and called as sample D). An example of the results with these cartridges appears in Table 4. It shows the high variability of the process, again due

Table 3
SPE results obtained using sample 8 from digestive glands

	AZA1 (ng)	OA (ng)	DTX-2 (ng)
Fraction 2	53.5 ± 43.3	435.8 ± 9.5	79.5 ± 2.8
Fraction 3	736.1 ± 318.2	344.4 ± 65.1	68.7 ± 4.8
Fraction 4	33,757.5 ± 2,992.5	18.8 ± 3.5	5.1 ± 0.7
Fraction 5	12,799.5 ± 1,606.5	18.4 ± 1.1	4.4 ± 0.3

Three aliquots of 210 μl of sample 8 were collected, vacuum dried and solved in acetone. They were charged in 2 ml silica cartridges previously wetted with acetone. Later on they were washed with the same solvent and eluted with methanol. Different fractions, from 1 to 5, were obtained along the process (Scheme 3) and their toxin concentrations were determined by LC/MS. Mean ± S.E.M. of three experiments.

Table 4
SPE results obtained using sample 8 from digestive glands and 20 ml silica cartridges

	AZA1 (μg)	OA (ng)	DTX-2 (ng)
Fraction 2 + 3	2.129 ± 0.003	11,655 ± 2,575	759.3 ± 124.8
Fraction 4 + 5	651.31 ± 111.19	0	0

Three aliquots of 1750 μl of sample 8 were collected, vacuum dried and solved in acetone. They were charged in 20 ml silica cartridges previously wetted with acetone. Later on they were washed with the same solvent and eluted with methanol. Different fractions, from 1 to 5, were obtained along the process (Scheme 3) and their toxin concentrations were determined by LC/MS. Mean ± S.E.M. of three experiments.

to the high density of sample 8, and the suitability of this SPE to achieve an AZA1 extract (fractions 4 and 5) without OA and DTX-2.

Similar results (not shown) are obtained using sample 8 from meat and liquid.

3.3. HPLC separation

Separation between different groups of toxins was achieved in the previous step, obtaining a sample D without OA and DTX-2. HPLC is then used to isolate different AZAs analogs (1–5) in this sample. Bibliography describes various protocols to achieve this objective, using different columns, flows, temperatures and mobile phases [10–12,17,18,22–27]. In a first step we try to optimize all these parameters by analytical HPLC and later on scale the process to preparative HPLC.

Different protocols in analytical HPLC were tested to determine which one achieves greater recovery and better separation of AZAs analogs. Several mobile phases with different compositions, various heater conditions and sample volumes were tested. The best results were obtained using MeOH:H_2O:AcH (700:300:1) at 0.2 ml/min, 35 °C and 20 μl injection volume.

The characteristics used in preparative HPLC are similar to analytical, except volume of injected sample (500 μl instead of 20 μl) and mobile phase flow (5 ml/min instead of 0.2 ml/min). Fractions are collected each minute, dried, solved in methanol and analyzed by LC/MS. Results appear in Table 5 and Fig. 1. Percentages of recovery for AZA1, AZA3 and AZA4 are high, whereas for AZA2 and AZA5 are lower, always above 50%. Fig. 1 shows percentages of purity of the analyzed fractions. Separation between AZAs analogs is not good since all of them leave the column in the first 15 min and most of the fractions contain more than one analog. To avoid this problem the mobile phase flow is modified, using 2.5 ml/min instead of 5 ml/min. Results appear in Table 6 and Fig. 2. This modification maintains high percentages of recovery (except for AZA2, probably due to an experimental mistake) and achieves better separation between analogs. Therefore, purification of AZAs analogs can be done in preparative HPLC using MeOH:H_2O:AcH (700:300:1) at 2.5 ml/min, 35 °C and 500 μl injection volume.

Table 5
AZAs recovery in preparative HPLC

	m (ng)	% recovery
AZA1	147,727	81.8
AZA2	23,047	64.7
AZA3	4,122	88.6
AZA4	970	86
AZA5	982	61

An isocratic flow of 5 ml/min was used, with a mobile phase composed by MeOH:H_2O:AcH (700:300:1). The oven temperature was 35 °C and the injected sample volume was 500 μl. 60 fractions of 1 min each one were collected and their toxin concentrations were measured by LC/MS. % recovery was calculated comparing the total amounts in the 60 fractions collected with the injected quantities in the 500 μl initial sample.

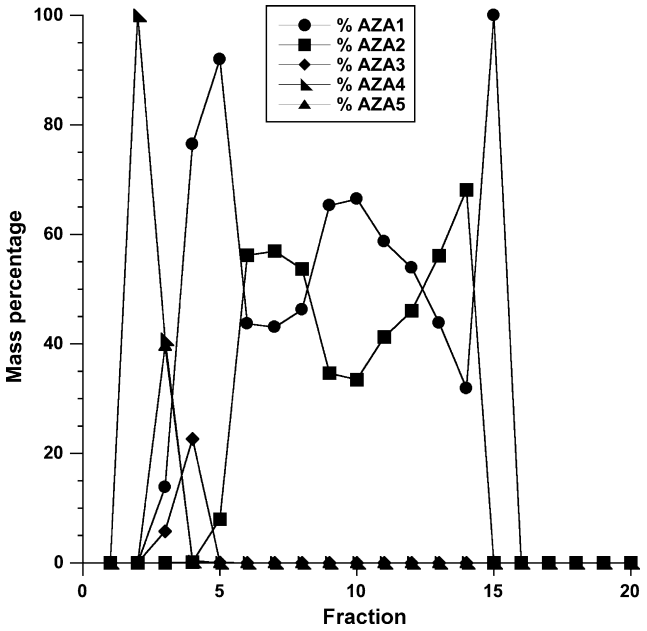


Fig. 1. Mass percentage of AZAs analogs in fractions collected in preparative HPLC. The mass percentage of each AZA analog was calculated in fractions obtained in the assay described in Table 5, although fractions from 21 to 60 are not represented because they do not contain any analog.

Table 6
AZAs recovery in preparative HPLC, with a mobile phase flow of 2.5 ml/min

	m (ng)	% recovery
AZA1	161,902	89.7
AZA2	13,506	37.9
AZA3	3,033	65.2
AZA4	1,000	88.7
AZA5	1,383	85.5

An isocratic flow of 2.5 ml/min was used, with a mobile phase composed by MeOH:H_2O:AcH (700:300:1). The oven temperature was 35 °C and the injected sample volume was 500 μl. Sixty fractions of 1 min each one were collected and their toxin concentrations were measured by LC/MS. % recovery was calculated comparing the total amounts in the 60 fractions collected with the injected quantities in the 500-μl initial sample.

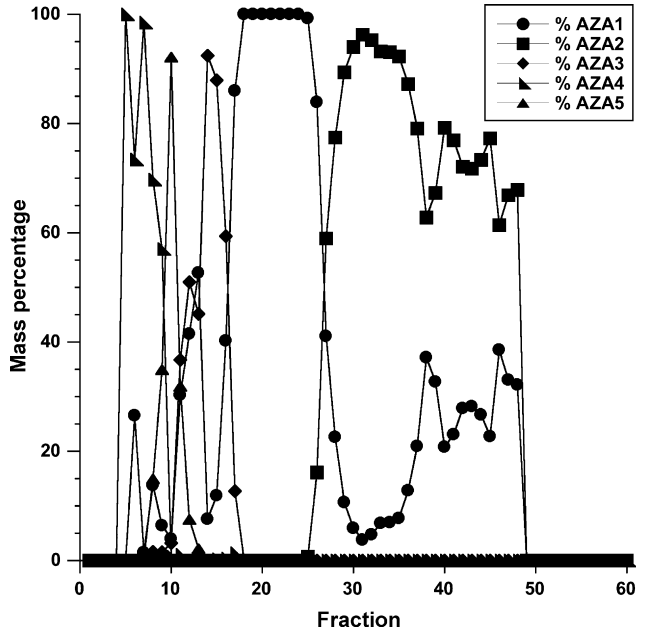


Fig. 2. Mass percentage of AZAs analogs in fractions collected using in preparative HPLC, with a mobile phase flow of 2.5 ml/min. The mass percentage of each AZA analog was calculated in fractions obtained in the assay described in Table 6.

Table 7
AZAs recovery in four injections in preparative HPLC with a mobile phase flow of 2.5 ml/min

	Injection a	Injection b	Injection c	Injection d	Media	S.E.M.
AZA1 (ng)	58,542	59,426	71,188	56,579	61,434	3,305
AZA2 (ng)	9,603	9,837	11,218	8,002	9,665	659
AZA3 (ng)	1,657	1,454	1,915	1,529	1,639	101
AZA4 (ng)	375	366	354	466	391	26
AZA5 (ng)	1,310	1,581	1,370	1,786	1,512	108

Repeatability of the method described in Table 6 and Fig. 2 was determined by four injections with the same characteristics. Total amount of each analog was calculated in each injection and compared with the other ones.

Finally we verify the repeatability of the protocol doing four sample injections. Before this we dilute sample D to decrease the viscosity and achieve that the injected samples were more homogeneous than in the previous experiments. Table 7 shows AZAs amount in the fractions collected after four injections (a–d) of diluted sample D, with a high repeatability. Separation between analogs is shown in Fig. 3. Time distribution of the analogs varies between different injections, but the curve profile of each one remains constant.

4. Discussion

Although initially AZAs contamination of shellfish was a problem located in a determinate geographical extension [1,2], nowadays analogs of this toxin appear in different coasts and can become a worldwide problem. Investigations about their mechanism of action and toxicity are reduced due to limited availability of standards and certified materials. In this paper we study the large-scale isolation of different AZAs analogs from a contaminated

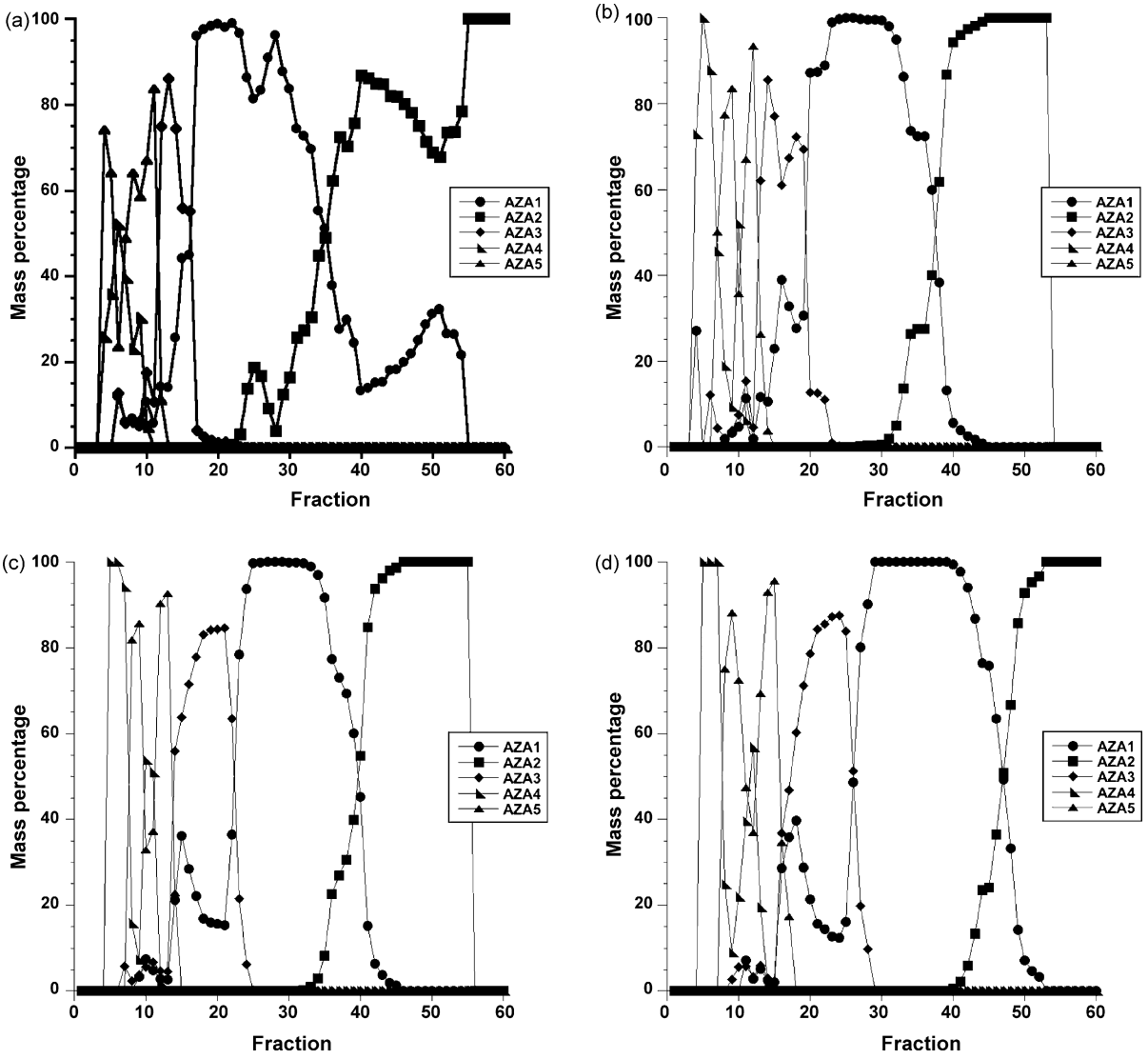


Fig. 3. Mass percentage of AZAs analogs in fractions collected in four injections in preparative HPLC with a mobile phase flow of 2.5 ml/min. The mass percentage of each AZA analog was calculated in fractions obtained in the assays described in Table 7. a: injection a, b: injection b, c: injection c, d: injection d.

Table 8
Amounts and concentrations of toxins in HP and meat + liquid calculated from data obtained during the extraction procedure

	HP (μg)	Meat + liquid (μg)	HP (μg/kg)	Meat + liquid (μg/kg)
AZA1	41,364	125,796	15,909	6,818
OA	2,093	3,894	805	211
DTX-2	369	0	142	0

Samples A (digestive glands of mussels) and B (the sixth part of the rest of the mussel meat and the interstitial liquid collected) were extracted and partitioned with various solvents. Finally two ethyl acetate layers (samples 8 and 9) were collected for each one of the initial samples and measured by LC/MS, containing AZA1, OA and DTX-2. With these results we calculated the amounts and the concentrations of toxins in the hepatopancreas (HP) and in the rest of the mussel meat and the interstitial liquid (meat + liquid) of the initial mussel sample, considering that the masses of these two parts were 2.6 kg and 18.45 kg, respectively.

mussel sample (with AZAs and DSP toxins), which will be used in basic investigations.

The initial sample (frozen mussels) was defrosted and divided in two parts: one with the digestive glands and the other one with the rest of the meat and the interstitial liquid, in order to compare toxic profiles in meat and hepatopancreas. We processed two samples: sample A contained all the digestive glands (2.6 kg) and sample B contained a sixth part of the homogenate of meat and liquid (approximately 3 kg from a total of 18.45 kg). There are some papers about the extraction of AZAs from shellfish. In the first toxic episodes [7], these toxins were extracted with acetone and twice with methanol, followed by a partition in hexane and methanol 80%, being AZAs concentrated in the methanolic phase. Other authors showed that two extractions with acetone remove AZAs from shellfish homogenate efficiently [17], followed by a partition in water and ethyl acetate [11]. Finally, some authors suggest that methanol can be the better extraction solvent with some shellfish matrices [18]. Our first objective was to extract the largest possible amounts of AZAs analogs, so we decided to apply a complete and laborious procedure, the same for the two tested samples. We did three extractions, one with acetone and two with methanol, in order to remove all the AZAs from the shellfish samples. After this, we mixed and evaporated the obtained extracts. The two partitions previously described (hexane/methanol 80% and water/ethyl acetate) were used to separate the largest possible amount of contaminants and the order between them was selected to avoid the mixing of various solvents. As the extract from shellfish contained acetone and methanol and the acetone boiling point is lower than methanol one, the evaporated residue could contain trace amounts of methanol. Therefore, it was dissolved in methanol 80% and partitioned with hexane. This process achieved the elimination of various fat layers and lipophilic components solved in the hexane phases, being all the toxins dissolved in the final methanolic layer, which was vacuum dried. Since this layer contained methanol and water and the water boiling point is higher than methanol one, the evaporated residue could contain trace amounts of water. So, it was dissolved in water and partitioned with ethyl acetate. With this process, hydrophilic contaminants were eliminated in the aqueous layer and the toxins (AZA1, OA and DTX-2) were separated in the two ethyl acetate layers (samples 8 and 9) obtained for each one of the initial samples.

Results show that OA concentration in HP is 3.8 times higher than in meat + liquid, whereas AZA1 concentration in HP is only 2.3 times higher and DTX-2 is located in HP. This means that shellfish body distribution is not the same for all toxins, perhaps due to different chemical properties like polarity. This points that the whole body of shellfish must be analyzed in routine AZAs controls, since there is not a high difference between AZA1 concentration in HP and meat + liquid, far away from the fivefold difference measured by some authors [18] and near the percentages found by another [17]. Table 8 summarizes the amounts and the concentrations of toxins in the initial samples calculated from the extraction results, considering that the quantity of toxins in HP is the amount of samples 8 and 9 from A whereas the quantity in meat + liquid is six times the amount of samples 8 and 9 from B.

Samples 8 and 9 obtained after the extraction procedure contained all the initial toxins with contaminants and pigments. Due to this a cleaning procedure was applied to isolate AZAs from the rest of the sample. Literature contains a lot of references about solid phase extractions with different cartridges (silica, diol and C18) and mobile phases (acetone, methanol, chloroform or water) [7,11,25], as well as gel permeation [7] or flash chromatography [11]; all of them are processes applied to clean extracts and obtain purified toxins. With these references and from previous experiences on toxins purification [28], we designed a SPE with silica, using acetone and methanol as mobile phases and achieving the separation between DSP toxins and AZAs analogs. Different eluotropic properties of these solvents on silica, together with solubility characteristics of the toxins make possible the isolation of AZAs analogs in the methanolic phase. This process does not depend on the matrix since separation is achieved with samples from HP and from the rest of the meat and liquid, and can be successfully scaled to bigger amounts of samples. The separation of the two groups of toxins is essential to avoid interferences during the later purification of AZAs analogs, so the SPE designed is one of the most important steps of this study.

AZAs analogs were isolated from the mixture previously obtained (sample D), using a chromatographic process described in various references, each one with its own characteristics [10–12,17,18,22–27]. From these data, different protocols were tested in an analytical column and the one with best results was selected to scale up to the preparative process. Even either methanol or acetonitrile can be used; best results were obtained with methanol. The key of the process was the adjustment of the mobile phase flow in preparative to achieve the highest recovery and purity of AZAs analogs. The % of recovery remains constant between injections with some variations in the time of collection. These problems are being solved equilibrating the column during more time and washing between consecutive injections.

LC/MS is used along the study to determine toxin concentrations in the samples. In this case the chromatographic characteristics employed guarantee the separation of the lipophilic toxins tested and the mass spectrometry method (multiple reaction monitoring) allows the detection of all of them simultaneously. Only interferences between AZA4 and AZA5 can be observed, because the selected mass transitions are the same (844.5 > 826.6/808.7) and their retention times in column are very similar (4 min and 4.50 min, respectively). To solve this problem other transitions, different for AZA4 and AZA5, could be used, at least in fractions in which these are the most abundant toxins.

This paper shows a laborious method that allows obtaining large quantities of AZAs analogs, which can be used in the future in different investigations and/or in the production of certified materials and standards.

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