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Preparation and characterization of an immunoaffinity column for the selective extraction of azaspiracids

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ABSTRACT

The presence of azaspiracids (AZAs) in shellfish may cause food poisoning in humans. AZAs can accumulate in shellfish filtering seawater that contains marine dinoflagellates such as *Azadinium* and *Amphidoma* spp. More than 60 AZA analogues have been identified, of which AZA1, AZA2 and AZA3 are regulated in Europe. Shellfish matrices may complicate quantitation by ELISA and LC–MS methods. Polyclonal antibodies have been developed that bind specifically to the C-26–C-40 domain of the AZA structure and could potentially be used for selectively extracting compounds containing this substructure. This includes almost all known analogues of AZAs, including AZA1, AZA2 and AZA3. Here we report preparation of immunoaffinity chromatography (IAC) columns for clean-up and concentration of AZAs. The IAC columns were prepared by coupling polyclonal anti-AZA IgG to CNBr-activated sepharose. The columns were evaluated using shellfish extracts, and the resulting fractions were analyzed by ELISA and LC–MS. The columns selectively bound over 300 ng AZAs per mL of gel without significant leakage, and did not retain the okadaic acid, cyclic imine, pectenotoxin and yessotoxin analogues that were present in the applied samples. Furthermore, 90–92% of the AZAs were recovered by elution with 90% MeOH, and the columns could be re-used without significant loss of performance.

1. Introduction

A shellfish poisoning episode 26 years ago led to the discovery of a new group of toxins called azaspiracids (AZAs). The people affected experienced vomiting, severe diarrhea, stomach cramps and general nausea without the presence of known toxins, bacteria or viruses [1]. Since then, AZA1 and a series of analogues have been identified and isolated, most of which share a common C-26–C-40 moiety and a 20,21-diol (Fig. 1) [2–6]. The originally published structures of the AZAs were first revised in 2003 by Nicolaou, et al. [7,8] and again in 2017 by Kenton, et al. [9,10]. The revised structures are shown in Fig. 1 and, so far, more than 60 AZAs have been reported [11].

AZA1 and AZA2, and a range of other AZAs, are produced by *Azadinium* and *Amphidoma* spp. [12–15], whereas the majority of the remaining identified AZAs are shellfish metabolites [16]. AZAs have been found in mussels, clams, oysters, cockles [17–21], and also in brown crabs [22]. Although Europe seems to have the most widespread and frequent occurrence, AZAs have also been found along the coasts of north-west Africa [23], Canada [24], Japan [25], Chile [26,27], China [28] and the USA [29].

Analysis of AZAs is typically performed using a combination of methanolic sample extraction and LC–MS analysis [30]. Given the presence of co-extracted matrix components from algae and shellfish, matrix effects are routinely problematic for quantitation of AZAs

Abbreviations: AZA, azaspiracid; CRM, certified reference material; CNBr, cyanogen bromide; DTX, dinophysistoxin; ELISA, enzyme-linked immunosorbent assay; FDMT, freeze dried mussel tissue; IAC, immunoaffinity chromatography; IgG, immunoglobulin G; LC–HRMS, liquid chromatograph–high-resolution mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; OA, okadaic acid; PBS, phosphate-buffered saline; PTX, pectenotoxin; YTX, yessotoxin.

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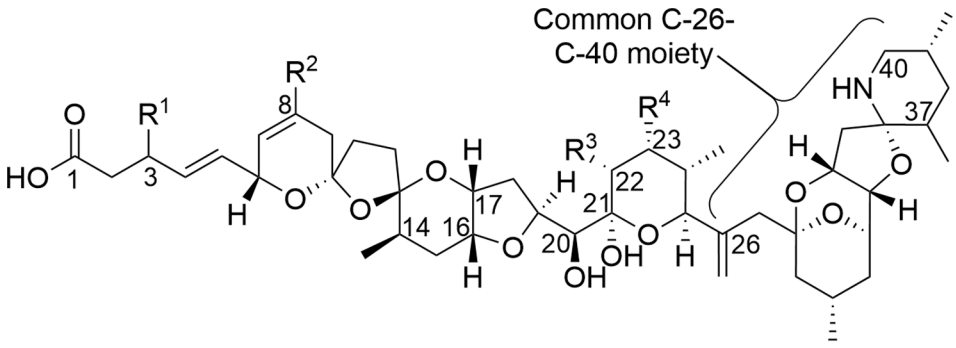

Name	R^1	R^2	R^3	R^4	C-37	*m/z* $[M+H]^+$
AZA1	H	H	CH_3	H	*S*	842.5049
37-*epi*-AZA1	H	H	CH_3	H	*R*	842.5049
AZA2	H	CH_3	CH_3	H	*S*	856.5206
AZA3	H	H	H	H	*S*	828.4893
AZA4	OH	H	H	H	*S*	844.4842
AZA5	H	H	H	OH	*S*	844.4842
AZA6	H	CH_3	H	H	*S*	842.5049
AZA7	OH	H	CH_3	H	*S*	858.4998
AZA8	H	H	CH_3	OH	*S*	858.4998
AZA9	OH	CH_3	H	H	*S*	858.4998
AZA10	H	CH_3	H	OH	*S*	858.4998

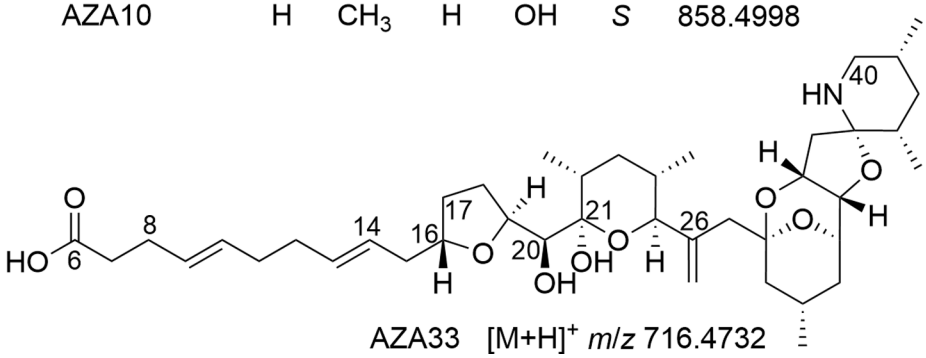

Fig. 1. Structures of selected azaspiracids, showing atom-numbering and the common C26–C40-moiety.

[31–34]. To reduce the matrix interferences, a pretreatment step for sample enrichment and clean-up may be necessary. The most common solvents used for shellfish toxin extraction are MeOH–water mixtures [34,35]. Alternative approaches based on specific chemical interactions with the analyte can sometimes be used, and in the case of AZAs the reversible binding between boronic acids and the 20,21-diol present in most AZAs can be exploited to reduce matrix effects [31]. However, there are some AZAs that do not contain the *vic*-diol moiety [11], and poly-hydroxylated AZAs may be hard to release from boronic acids polymers [31].

Immunoaffinity chromatography (IAC) is a form of liquid chromatography that uses antibodies bound to a stationary phase to achieve a high degree of selectivity for target analytes. This specific binding, used to capture the analyte in question from complex sample matrices, can be controlled by changing the pH or applying an organic solvent. Thus, IAC has become a popular tool for sample preparation, in both routine analytical and research laboratories, in applications such as mycotoxins, where a range of commercial IAC columns is available [36]. IAC potentially enables "all-in-one" extraction, purification and enrichment of target compounds, providing high sensitivity in the final analysis step using methods like LC–MS, while reducing or eliminating matrix effects. Despite these advantages, IAC has not been widely applied to the analysis of algal toxins.

Shellfish samples usually contain relatively low concentrations of AZAs in a complex tissue matrix containing large amounts of other compounds such as lipids, proteins, and carbohydrates which may be co-extracted. IAC clean-up exploits immunochemical selectivity for the purification and preconcentration of multiple structurally related analytes on immobilized antibodies. In this study, the recognized antigens were compounds containing the C-26–C-40-substructure of AZAs [37]. By using these specific antibodies, we hoped to selectively extract molecules containing this substructure from complex matrices, such as shellfish extracts.

Here we report the preparation and characterization of IAC columns for the selective concentration and clean-up of AZAs in shellfish samples. The experiments showed that AZAs reversibly bound to antibodies attached to sepharose gel. These columns provided excellent recovery of AZAs from shellfish extracts, and removed much of the matrix, including most of the color, okadaic acid, pectenotoxin (PTX), yessotoxin (YTX), and cyclic imine analogues present in the extracts.

2. Materials and methods

2.1. Materials

CNBr-activated sepharose 4B, the HiTrap desalting-column and HiTrap protein G HP-column were from GE Healthcare (Chicago, IL, USA). Isolute SPE accessories such as single fritted reservoirs, frits, Luer tip caps and column caps were from Biotage (Uppsala, Sweden). Maxisorp immunoplates (96 flat-bottom wells) were from Nunc (Roskilde, Denmark), poly(vinylpyrrolidone) 25 (PVP) was from Serva Electrophoresis (Heidelberg, Germany), donkey–anti-sheep IgG (H + L) horseradish peroxidase conjugate (anti-sheep–HRP) was from Agrisera (Vännäs, Sweden), and the HRP-substrate K-blue Aq. was from Neogen (Lexington, KY, USA). Ovine antibodies to AZAs (NVI8122-11b) were obtained as described by Samdal, et al. [38]. AZA1 was from the Marine Institute, Ireland [39], NRC CRM-FDMT1 was from the National Research Council Canada (Halifax, NS, Canada) [40], and mussels (*Mytilus edulis*) were harvested at Bruckless (Donegal Bay, Ireland) in 2005 [39]. Excised hepatopancreatic tissue from the Bruckless mussels (2 g) was homogenized with 5 mL of MeOH using an Omni-prep homogenizer (Omni Int., Kennesaw, GA, USA), the homogenate was centrifuged, and the supernatant filtered through a Titan3 cellulose syringe filter (0.45 μm; ThermoFisher Scientific, Rockwood, TN, USA) and then a Millipore 0.45 μm PVDF spin filter (Merck Millipore, Cork, Ireland). All other inorganic chemicals and organic solvents were of reagent grade or better.

2.2. Buffers

IgG binding buffer was 20 mM sodium phosphate, pH 7.0, IgG eluting buffer was 0.1 M glycine adjusted to pH 2.6 with 0.2 M HCl, and 1 M Tris HCl pH 9.0 was prepared for neutralization of collected IgG-fractions. The IgG coupling buffer was 0.1 M sodium carbonate, pH 8.7, containing 0.5 M NaCl. The blocking buffer was 1.0 M ethanolamine, pH 8.0. The IAC washing/regeneration buffers were 0.1 M Tris HCl (pH 8.0, 0.5 M NaCl), and 0.1 M acetate (pH 4.0, 0.5 M NaCl). A detailed description of IAC buffer preparation is available in the Supplementary data file.

For ELISA, the plate-coating buffer was carbonate buffer (50 mM, pH 9.6). Phosphate-buffered saline (PBS) contained NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (8 mM), and KH_2PO_4 (1.5 mM), at pH 7.4; ELISA washing buffer (PBST) was 0.05% Tween 20 in PBS, and the sample buffer was 10% MeOH (v/v) in PBST, and antibody buffer was 1% PVP (w/v) in PBST.

2.3. Purification of IgG

Serum from sheep 8122 (NVI8122-11b), immunized as described in [38], was filtered (0.45 μm Millipore filter), then purified with respect to IgG using a 5 mL HiTrap Desalting-column followed by a 1 mL HiTrap protein G HP-column, according to the manufacturer's instructions.

2.4. Preparation of immunoaffinity columns

CNBr-activated Sepharose 4B (5.0 g) was suspended in 15 mL 1 mM HCl and additives were removed by washing with HCl (1 mM; 1 L) in a cartridge (200 mL) connected to a vacuum chamber and a peristaltic pump over 1.5 h. Excess liquid was removed, and purified IgG (63.7 mg) was mixed with the gel in IgG-coupling buffer (pH 8.7, 25 mL) and gently mixed end-over-end overnight at 4 °C in a 50 mL Falcon tube. The mixture was centrifuged at 48 *g* for 5 min at 4 °C, and the supernatant retained for protein analysis. After coupling, the gel was washed twice with 25 mL coupling buffer, centrifuged as above, and the supernatant collected for protein analysis. To block any remaining active sites, the gel was added to 1 M ethanolamine (25 mL; pH 8.0), and gently mixed end-over-end for 2 h. After blocking, the gel was centrifuged at 48 *g* and the supernatant collected for protein analysis. The antibody-coupled gel was distributed equally into 10 single-fritted polypropylene cartridges (8 mL), giving approximately 1.5 mL gel per cartridge. A frit was placed at the top of the gel to keep it in place. Regeneration after use was performed by washing the IAC column with three alternating cycles of 3 mL each of acetate buffer (pH 4.0) and 3 mL Tris HCl (pH 8.0). For storage, the column was flushed with 20% EtOH and kept at 4 °C. Purified IgG and all washing and blocking fractions from the preparation of the IAC columns were quantitated for protein using a BioRad-kit (Hercules, CA, USA), based on the method by Lowry et al. [41], to determine the amount of protein coupled to the gel.

2.5. Mussel extracts

Sample 1: This was the main eluted fraction containing AZAs from a boric acid gel column of a Bruckless mussel hepatopancreas extract (referred to previously as Elute Fraction 1, see Fig. 5C of Miles, et al. [31]).

Sample 2: Bruckless mussel hepatopancreas extract [39] was prepared by extraction of 1.7 g homogenized hepatopancreas with 2 × 10 mL of MeOH and adjusted to 25 mL. Aliquots of the extract were filtered through 0.2 μm filters before use.

Sample 3: CRM-FDMT1 [42] (0.35 g) was reconstituted with 1.65 mL water by vortex-mixing (30 s) and bath ultrasonication (1 min). MeOH (5 mL) was added, the mixture was vortex-mixed (2 min, 2500 rpm), centrifuged (10 min, 3950 *g*), and the supernatant decanted into a test tube. The pellet was extracted twice more with MeOH in the same manner, and the combined extracts were concentrated to 4.5 mL under a stream of nitrogen at 40 °C and filtered (0.45 μm PVDF).

2.6. Testing and optimization of the IAC columns.

Prior to use, the IAC columns were equilibrated to room temperature, excess liquid was run out of the columns, and 5 mL PBS was passed through to equilibrate the columns before sample application.

Sample 1 was diluted 30-fold to 3 mL in PBS with 10% MeOH and applied to an IAC column. A stopcock was used to control the flow rate to ~ 1 drop/s. The liquid passing through the column, when the sample was applied to the column, is referred to as "wash 1", as the applied volume was considerably greater than the column-volume and could therefore have removed significant amounts of unretained compounds. The column was then washed with 3-mL of H_2O (wash 2). Elution was performed with a stepwise gradient starting with 10% MeOH (5 × 1 mL), then successively with 20% MeOH (5 × 1 mL), 30% MeOH (5 × 1 mL), 40% MeOH (5 × 1 mL), 50% MeOH (5 × 1 mL), 60% MeOH (5 × 1 mL), 70% MeOH (5 × 1 mL), 80% MeOH (5 × 1 mL), 90% MeOH (5 × 1 mL), and finally 100% MeOH (5 × 1 mL), with 1-mL fractions collected. After use, the column was regenerated and flushed with 20% EtOH for storage at 4 °C.

Sample 2 was diluted 30-fold to 3 mL in PBS with 10% MeOH and applied to an equilibrated IAC column as described above. After wash 2, performed as for Sample 1, the column was eluted with 90% MeOH (3 × 3 mL) to yield Elutes 1, 2 and 3 (3 mL each), and the column was regenerated as described above. A regenerated and equilibrated IAC column was also tested similarly to determine whether the IAC columns could be re-used. Sample 2 was also applied in water (i.e., without PBS) containing 10% MeOH, as above, to an un-used AZA-IAC column to test the performance of the columns when the sample was applied without buffer.

Sample 3 was diluted to 28.7 mL with PBS to give ~10% MeOH, and 5 mL was applied to an equilibrated IAC column. After wash 2, performed as for Sample 1, the sample on the column was eluted with 85% MeCN containing 1% HCO_2H (3 × 3 mL), and Elutes 1, 2, and 3 (3 mL each) were collected. The column was then regenerated as described above.

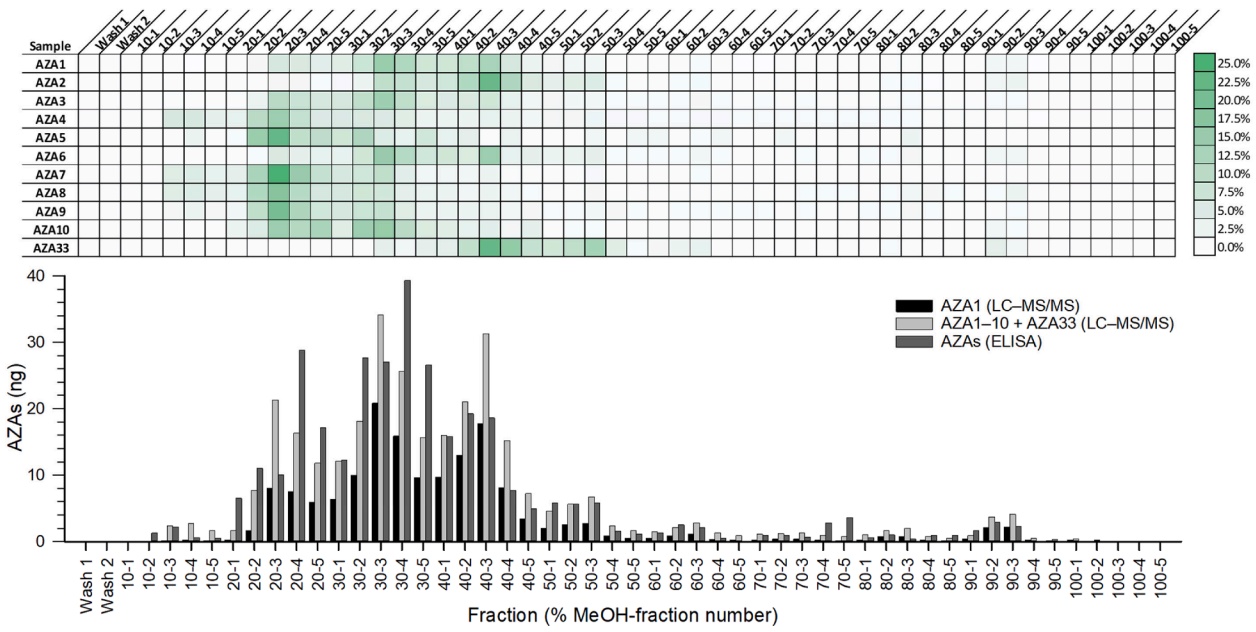

Fig. 2. Initial experiment to investigate the distribution of AZAs in eluted IAC-fractions (1-mL) with increasing percentages of MeOH after application of Sample 1. Fraction numbering refers to the percentage of MeOH and sub-fraction number, e.g., 10–1 refers to the first 1-mL fraction eluted with 10% MeOH, and 10–2 refers to the second 1-mL fraction eluted with 10% MeOH, etc. Top, a heatmap showing the percentages in each fraction of the total AZA1–10 and AZA33 detected by LC–MS/MS in the collected fractions. The total amount of each AZA-analogue is set to 100% and the fractions marked darker green are those containing the most of that particular analogue, and bottom, the amount (ng) of various AZAs in each fraction as analyzed by ELISA (total AZAs), AZA1 (by LC–MS/MS), and sum of AZA1–10 and AZA33 (by LC–MS/MS).

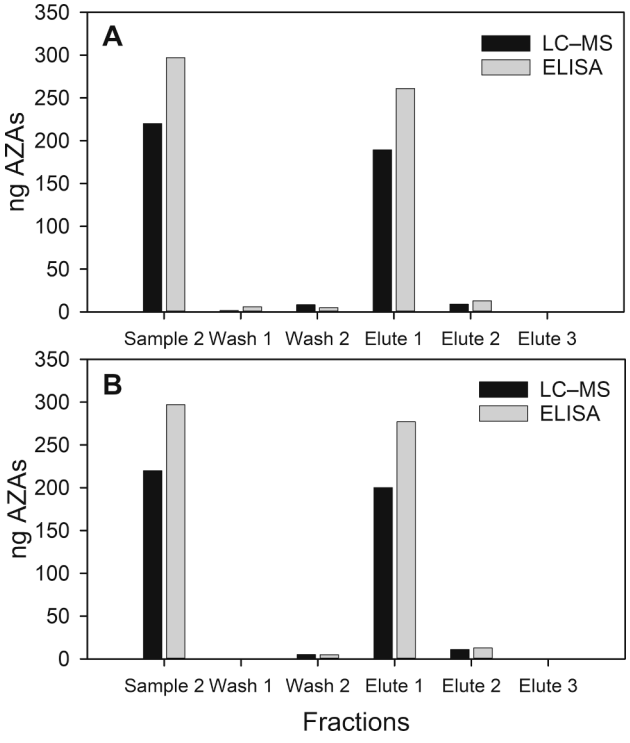

Fig. 3. Quantitation of AZAs by ELISA, and the sum of AZA1–10 and AZA33 by LC–MS/MS, in applied Sample 2 (extract from Bruckless mussel hepatopancreas), wash 1 (load volume plus first wash), wash 2, and three successive elutions with 90% MeOH (Elutes 1–3). A) Result from using a freshly prepared AZA-IAC column, and; B) the result obtained from re-using the same IAC column as in A after the column regeneration procedure.

2.7. AZA-ELISA

The concentration of AZAs in each sample or fraction was determined by indirect competitive ELISA [43] based on antibodies (AgR367-11b), raised using a synthetic AZA-fragment (C-26–C-40) [37], and the plate-coating antigen OVA–cdiAZA [43]. Other AZA-ELISA reagents were as described by Samdal, et al. [43], using AZA1 from the Marine Institute (Ireland) as a secondary standard (92% cross-reactivity relative to CRM-AZA1). The AZA1 standard in MeOH (1.3 μg/mL) was diluted in PBST and ELISA sample-buffer, to give a MeOH concentration of 10%. Serial three-fold dilutions of the AZA1 standard were performed with sample buffer, giving 10 standards from 0.011 to 213 ng/mL. Serial dilutions of standards and samples were performed in duplicate. Absorbances were measured at 450 nm using a SpectraMax i3x plate reader (Molecular Devices, Sunnyvale, CA, USA). All incubations were performed at ~20 °C. Assay standard curves were calculated using 4-parameter logistic treatment of the data using SoftMax Pro 6.5.1.

2.8. LC–MS/MS analysis

Quantitative LC–MS/MS analysis of AZAs was performed on a Waters Acquity UPLC coupled to a Xevo G2-S QToF monitoring in MS^E mode (m/z 100–1200), using leucine encephalin as the reference compound. The cone voltage was 40 V, the collision energy was 50 eV, the cone and desolvation gas flows were set at 0 and 600 L/h, respectively, and the source temperature was 120 °C. Analytical separation was performed on an Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm; Waters, Wexford, Ireland). Binary gradient elution was used, with phase A consisting of water and phase B of MeCN (95%) in water, with both containing 2 mM ammonium formate and 50 mM formic acid. The gradient was from 30 to 90% B over 5 min at 0.3 mL/min, held for 0.5 min, and returned to the initial conditions and held for 1 min to equilibrate the system. The injection volume was 2 μL and the column and sample temperatures were 25 °C and 6 °C, respectively. AZA1−3 were quantitated using CRMs [44], while AZA4−10 were quantitated with reference materials as described by Kilcoyne, et al. [6].

Table 1
Amounts of AZAs detected (ng) in Sample 2 (Bruckless mussel HP extract) applied to an AZA-IAC column, and in fractions obtained from washing followed by elution of the IAC column with 90% MeOH. Quantitation for AZA1–10 and AZA33 was by LC–MS/MS and for total AZAs by AZA-ELISA.

Fraction/Sample	LC–MS												ELISA	ELISA/ LC–MS
	AZA1	AZA2	AZA3	AZA4	AZA5	AZA6	AZA7	AZA8	AZA9	AZA10	AZA33	Total		
Sample 2	107.0	25.7	34.1	22.5	4.3	6.6	4.1	3.6	9.2	1.7	1.0	219.9	297	1.4
Wash 1	0.8	0.0	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	6	4.1
Wash 2	3.3	0.4	1.1	2.0	0.4	0.1	0.5	0.4	0.2	0.1	0.0	8.4	5	0.6
Sum Wash 1 + 2	4.1	0.4	1.3	2.5	0.4	0.1	0.5	0.4	0.2	0.1	0.0	10.0	11	1.1
Elute 1	90.1	24.7	31.2	17.8	3.2	6.3	2.8	2.5	8.0	1.5	1.2	189.3	261	1.4
Elute 2	3.9	1.1	1.5	0.9	0.2	0.4	0.1	0.1	0.5	0.1	0.0	8.9	13	1.4
Elute 3	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0	0.0
Sum Elute 1–3	94.1	25.8	32.8	18.8	3.4	6.7	2.9	2.7	8.5	1.6	1.2	198.5	274	1.4
Recovery (%)[a]	88	100	96	83	79	101	71	74	93	96	114	90	92	

[a] Expressed as sum of AZAs present in Elute 1–3 relative to the amount applied in Sample 2.

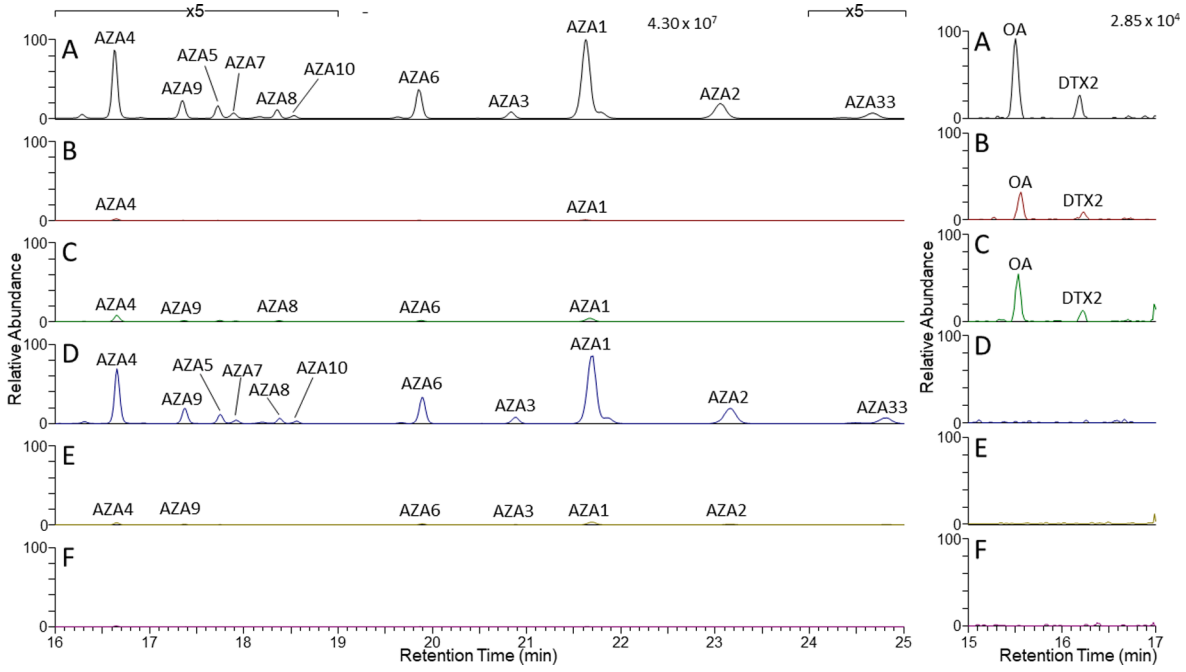

Fig. 4. LC–HRMS chromatograms of fractions from Sample 2 (Bruckless mussel HP extract, see Fig. 3) on an AZA-IAC column. Chromatogram: A) applied sample; B) load/wash 1; C) wash 2, and; D–F), three successive elutions of the IAC column with 90% MeOH ("Elute 1" to "Elute 3", respectively). Full-scan LC–HRMS chromatograms of AZAs (left-hand panels) were extracted for the exact *m/z* values (Fig. 1) for: $[M + H]^+$ of AZA1–10 and AZA33 (note that the 5-fold expansions for peaks eluting from 16–19 and 24–25 min, to highlight minor AZAs); and for the sum of $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$ and $[M + K]^+$ of OA/DTXs (right-hand panels). The AZA chromatograms are scaled to the maximum peak height for AZA1 in the applied sample (4.30×10^7), while the OA/DTX chromatograms are scaled relative to the peak height for OA in the applied sample (2.85×10^4).

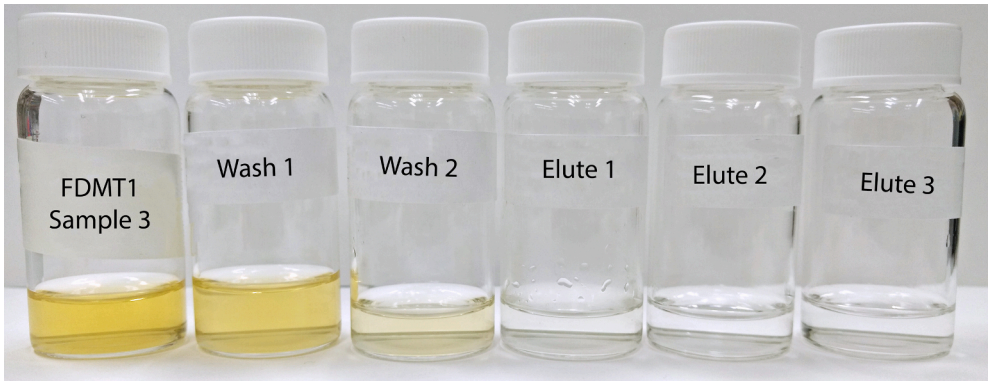

Fig. 5. The extract of NRC CRM-FDMT1 (Sample 3), 5 mL of which was applied to the AZA-IAC column (left), and the five fractions obtained from elution of the IAC-column. The five fractions are, from left to right: "Wash 1" (after load; 5 mL); Wash 2 (3 mL), and Elute 1–3 (from the three successive 3-mL elutions of the IAC column with 85% MeCN containing 1% HCO_2H). Extracted ion chromatograms from the applied extract are shown in Fig. 6A and S2, while the corresponding chromatograms from "Elute 1" are shown in Fig. 6B.

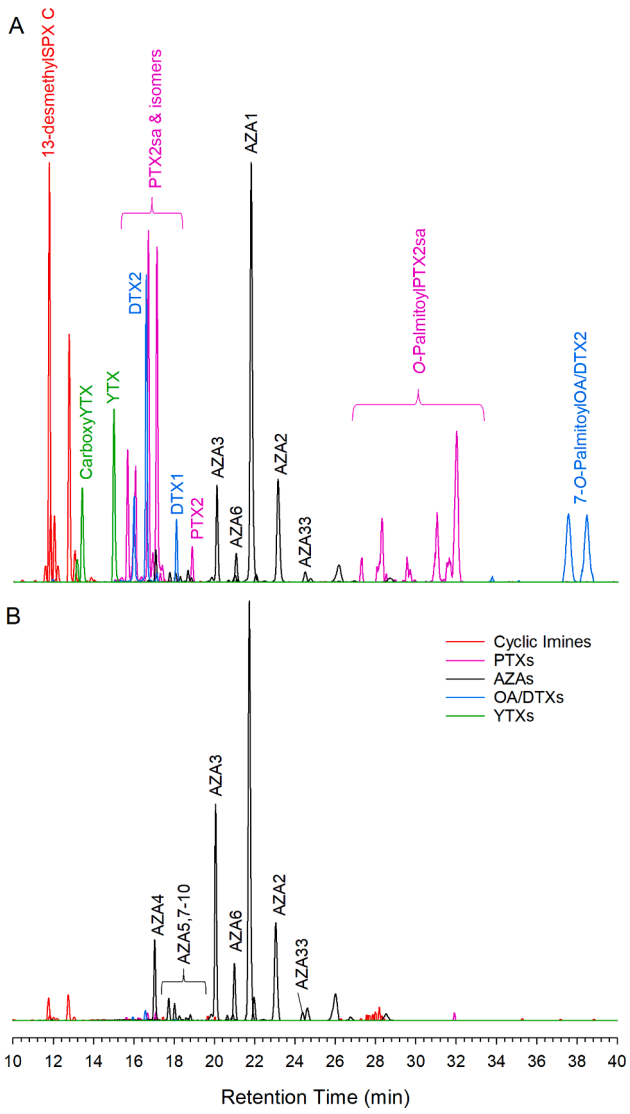

Fig. 6. Extracted ion LC–HRMS chromatograms of the extract from NRC CRM-FDMT1 (Sample 3) for algal toxins known to be present [46]: A) before application to the IAC column, and; B) in the Elute 1 fraction from the IAC column (see Fig. 5). AZAs are shown in black, cyclic imines in red (SPX = spirolide), PTXs in pink (sa = seco acid), OA/DTXs in blue, YTXs in green, and the identities of selected peaks are indicated. Each toxin (except for AZAs) is displayed with a different relative vertical scale (zoom) to ensure peak visibility, but the same zoom was used for both chromatograms to allow direct comparison of the relative degree of retention of each toxin by the IAC column. A list of the extracted exact masses is given in Table S4, along with the scaling factors that were applied to each toxin class in the displayed chromatograms, and the retention times of identified analogues.

2.9. LC–HRMS

Qualitative LC–HRMS and LC–HRMS/MS analyses were performed on a Q Exactive HF Orbitrap mass spectrometer equipped with a heated electrospray ionization interface (ThermoFisher Scientific, Waltham, MA, USA) using an Agilent 1200 G1312B binary pump, G1367C autosampler and G1316B column oven (Agilent, Santa Clara, CA, USA) connected to a Poroshell SB-C18 HPLC column (150 × 2.1 mm i.d., 2.7 µm; Agilent, Santa Clara, CA, USA) held at 30 °C. Analyses were performed with mobile phases A and B of water and 95% MeCN, respectively, each of which contained both ammonium formate (2 mM) and formic acid (50 mM). Gradient elution (0.275 mL/min) was from 5 to 100% B over 20 min, followed by a hold at 100% B (25 min), a return to 5% B over 1 min and a hold at 5% B (10 min) to equilibrate the column. The mass spectrometer was operated in positive ion mode and calibrated from *m/z* 74–1922. The spray voltage was 3 kV, the capillary temperature was 350 °C, and the sheath and auxiliary gas flow rates were 35 and 10 units respectively. The mass spectrometer was operated in all-ion fragmentation (AIF) mode (full-scan: scan range *m/z* 500–1200, resolution 120,000, AGC target 3×10^6, max IT 200 ms; AIF: scan range *m/z* 93–1200, resolution 60,000, AGC target 3×10^6, max IT 200 ms, and collision energy 65 eV) to obtain alternating full-scan and all-ion fragmentation spectra.

3. Results and discussion

The affinity and specificity of antibodies can be used to develop sensitive and specific rapid assays for trace analytes in difficult sample matrices. The same advantageous features of antibodies can also be applied to create IAC columns that selectively concentrate the same analytes from such samples, facilitating their analysis by instrumental methods such as LC–MS. Such an approach combines the advantages of antibody specificity and affinity to selectively concentrate groups of analytes and remove matrix effects, with the benefits of instrumental analysis such as the ability to identify and quantitate individual analogues. The AZA-antibodies were obtained during the production of the highly specific AZA-antisera successfully used in the development of sensitive immunoassays for AZAs [38,43,45].

3.1. Preparation and characterization of IAC columns

Polyclonal antibodies can often be used directly from serum without prior purification steps, such as in an ELISA, due to their specificity. Because serum contains not only antibodies but also albumins, antigens, hormones, and electrolytes etc., it is desirable to purify the antibodies prior to coupling them to solid supports, to avoid coupling too many other serum proteins to the support. Therefore, anti-AZA IgG was purified using sheep IgG's affinity to Protein G. Protein G binds preferentially to the Fc portion of IgG, but can also bind to the Fab region, and this binding can be reversed by lowering the pH. The purified IgG-fraction was immediately neutralized and immobilized on CNBr-activated sepharose 4B (the solid support material).

To investigate whether the anti-AZA-antibodies were immobilized on the gel, coupling efficiency was evaluated. Of the 63.7 mg of IgG applied to the CNBr-activated sepharose 4B, only trace amounts of protein were measured in the washing and blocking fractions from the IAC column preparation (below quantitation limits), suggesting that essentially all the IgG had been coupled to the sepharose.

3.2. Performance of the IAC columns

3.2.1. Loading/coupling conditions

To make the conditions favorable for the antibodies to bind the antigen, PBS was used to dilute the samples. Because the antigen was a lipophilic algal toxin, and because most extraction methods for lipophilic algal toxins from shellfish use MeOH (usually 80, 90 or 100%), the sample was applied with 10% MeOH in PBS. Since this procedure appeared to work well (Fig. 2), this protocol was adopted for subsequent samples. However, to test whether the loading could be done without the PBS, one experiment was performed loading the sample in 10% MeOH in water. This resulted in similar binding to that obtained with 10% MeOH in PBS (Table S3), suggesting that the use of a buffer for loading may not be necessary for some sample types.

3.2.2. Washing conditions

Shellfish extracts often come with a complex matrix that can interfere with analytical methods, and some of these compounds could be retained on the gel via nonspecific adsorption due to hydrophobic, ionic, and other interactions with the gel. To eliminate such nonspecific

binding of compounds other than specifically bound AZAs from the samples, water was tested for simplicity. Initial experiments (Fig. 2) showed that washing with water worked well, with the added advantage that it also removed buffers and salts from the IAC column that might otherwise interfere with subsequent analyses and was therefore adopted as the IAC column-washing procedure.

3.2.3. Elution conditions

Most analytical methods, such as LC–MS and the AZA-ELISA, are optimized for samples in MeOH–water, therefore MeOH was initially chosen for eluting the IAC columns after sample application and washing. Since MeOH was potentially harmful to the antibodies on the column, initial experiments were performed with incrementally increasing concentrations of MeOH to determine the lowest concentration of MeOH necessary for eluting the AZAs from the IAC column, followed by 100% MeOH to completely elute the column. The IAC columns were then immediately equilibrated with an IgG-compatible regeneration buffer system, to minimize the exposure time of the immobilized antibodies to high concentrations of MeOH.

To test whether there were any differences between AZA-analogues in how they were retained by and eluted from the IAC column, Sample 1 was applied to the column, washed with water, and then eluted with a stepwise gradient of MeOH–water, with 1-mL fractions collected. The total amounts of AZAs in nanograms by ELISA, AZA1 by LC–MS/MS, and the sum of AZA1–10 and AZA33 by LC–MS/MS, in the collected fractions are shown in Fig. 2 (and Fig. S1). The heatmap (Fig. 2) shows that AZA1 starts to elute in 20% MeOH (3rd fraction). An increase in elution of AZA1 was seen with 30% MeOH in the 3rd fraction and again in the 3rd fraction of 40% MeOH, and by fraction 40–4, 85% of AZA1 had eluted. In contrast, AZA2 needed a higher concentration of MeOH for elution, which began at fraction 30–3. By fraction 50–3, 88% of AZA2 had eluted. AZA3 and AZA6 followed almost the same pattern as AZA1, while the more polar AZA4, AZA7 and AZA8 started to elute in fraction 10–3, and almost all these hydroxylated AZA metabolites were eluted by fraction 30–3. AZA9 and AZA10 started to elute in fraction 20–2, with fraction 20–3 containing the highest concentration of these hydroxylated AZA metabolites. Almost all (82%) of AZA9 had eluted by fraction 40–5. AZA33 was more strongly retained, beginning to elute with 40% MeOH and was almost completely eluted (88%) by fraction 50–4. Fig. 2 shows that, in total, most of the AZAs were eluted by fraction 50–4, although small amounts continued to elute up until fraction 90–3. For all AZA analogues except AZA4, 5 and 7, fractions 90–2 and 90–3 contained an additional pulse of AZAs constituting between 1.4 and 4.8% of that specific AZA analogue (median 2.7%, mean 2.8%) (Fig. 2). Consequently, subsequent elution of samples from the IAC columns was conducted with a single portion containing a high proportion of organic solvent (either 90% MeOH (3 mL), or 85% MeCN containing 1% HCO_2H (3 mL) followed by immediate equilibration with buffer to stabilize the immobilized antibodies. In MeOH, azaspiracids are susceptible to slow acid-catalyzed methylation at C-1 and C-21 to form methyl esters and ketals [39]. The feasibility of eluting the IAC columns with acidic MeCN–water was therefore tested as a potential measure to avoid the possibility of these methylation reactions.

The column was then tested with a highly contaminated mussel hepatopancreas extract from Bruckless, Ireland (Sample 2, Fig. 3), containing AZA1–10 and AZA33 (in total 220 ng by LC–MS and 297 ng by ELISA; Fig. 3, Table 1). According to LC–MS/MS and ELISA, only 1% and 2% of the AZAs, respectively, were not retained on the IAC column when the sample was applied (wash 1), and only 2% (by ELISA) and 4% (by LC–MS/MS) was eluted during washing of the column with water (wash 2) (Fig. 3A). Three successive elutions with 90% MeOH (Elute 1–3, Fig. 3) were performed, and 86–88%, 4%, and 0%, respectively, of the AZAs were eluted in the three fractions (Fig. 3A). The recovery of the major AZA analogues (AZA1–4 and AZA9) in Sample 2 varied from 83−100% (Table 1), whereas recoveries of the minor AZA analogues (AZA5, 7, 8, 10 and 33) varied from 71−114%, possibly due to the low levels being close to the LC–MS-method's LOQ.

3.2.4. Reuse

The possibility of re-using the IAC columns was tested by applying an aliquot of Sample 2 to the same IAC column a second time, after regeneration with the regeneration buffers. According to both ELISA and LC–MS, all the AZAs were retained by the column when the sample was applied (wash 1), and only 2% came off during washing of the column with water (wash 2) (Fig. 3B). In the three successive elutions with 90% MeOH (Elute 1–3), 91–93% and 4–5% of the AZAs eluted in the first and second fractions, respectively, and no AZAs eluted in the third fraction (Fig. 3B and Table S2). Overall, the results were essentially identical to those obtained during the first use of this column (Table 1 and Table S1), indicating that re-using the IAC columns might be feasible for some applications.

3.2.5. Specificity

To investigate whether AZAs might be retained by the IAC column due to non-specific interactions, the Bruckless and FDMT1 extracts, which contain other algal toxins including okadaic and dinophysistoxins (OA/DTXs), were used to evaluate the ability of the AZA-IAC columns to retain AZAs specifically. Fig. 4 shows the LC–MS chromatograms of the main AZAs and OA/DTXs in Sample 2 (Bruckless extract) before application to an IAC column, and in the various fractions eluted from the column (wash 1, wash 2, and the three elution fractions Elute 1–3). The main marine algal toxins identified in Sample 2 were AZA1–10, AZA33, OA, and DTX2. Only minor amounts of AZAs passed through the column when Sample 2 was applied and the IAC column was washed (Fig. 4B and 4C, left-hand panels), whereas OA and DTX2 were not retained and were completely removed during the loading and washing steps (Fig. 4B and 4C, right-hand panels). The column's capacity for binding OA and DTXs was approximately zero, as no OA/DTXs were detectable in any of the subsequently eluted fractions (Fig. 4D–F, right-hand panels), indicating that the retention of AZAs on IAC column was unlikely to be due to non-specific interactions with the immobilized antibody or the sepharose itself. Therefore, it seems likely that the AZAs were bound specifically by the antibodies on the IAC column.

Similar results (Figs. 5 and 6) were obtained with an extract of CRM-FDMT1 (Sample 3), a matrix CRM designed to mimic homogenized whole mussels contaminated with moderate concentrations of a wide variety of marine algal toxins [42], including a range of AZAs, OA/DTXs, cyclic imines, PTXs and YTXs [46]. Due to the potential for acid-catalyzed formation of methyl esters and ketals of AZAs during storage of azaspiracids in MeOH [16], in this experiment, we also tested whether acidic MeCN could be used for eluting AZAs from the IAC column. In this case, we found that the color from the original extract passed straight through the IAC column when the sample was applied and the column washed (Wash 1 and Wash 2, Fig. 5), whereas the subsequently eluted fractions (Elute 1–3, Fig. 5) were essentially colorless. Furthermore, LC–HRMS analysis (Fig. 6) again showed selective capture and release of AZAs on the IAC column, with only trace amounts of the OA/DTXs, cyclic imines, PTXs and YTXs originally present in the FDMT1-extract (Fig. 6A) being detected in the AZA-elution fractions (Fig. 6B). This demonstrated the ability of the AZA-IAC columns not only to recognize and retain a wide range of AZAs, but also to not significantly retain a wide range of other non-AZA polyether toxins of algal origin, indicating a high level of specificity for AZAs. This specificity is not unexpected, given that the antibodies used in the IAC columns were raised against antigens containing only the relatively conserved C-26–C-40 moiety (Fig. 1) of the AZA skeleton [37]. These antibodies were raised in parallel with those used in the ELISA-format [38,43] and have already been demonstrated to have broad specificity for the AZA family of marine biotoxins. However, the performance of the IAC columns highlights the AZA-antibodies' lack of affinity for other classes of marine algal toxins that might co-occur with AZAs in shellfish. This experiment also demonstrated that acidic MeCN was as effective as

90% MeOH for eluting AZAs from the IAC column.

3.2.6. Capacity

Roughly estimating the levels of major AZAs in CRM-FDMT1, reported as either certified values for AZA1–3 or non-certified information values for AZA4–10 [42], and noting the presence of many other AZAs in CRM-FDMT1 [46] without reported concentrations, over 2.9 μg of AZA toxins were loaded onto the IAC column from Sample 3. Analysis of individual elution steps indicated the IAC column was overloaded by approximately three times its capacity as evidenced by approximately equal signal for major AZAs in Wash 1, 2, and Elute 1 fractions (Fig. S2). Based on IAC results from Sample 2 (300 ng on-column) and the extent to which Sample 3 (~3 μg total AZAs applied) was overloaded, the capacity of the AZA-IAC columns appears to be between 300 and 1000 ng of total AZAs, on-column.

4. Conclusions

Immunoaffinity columns were prepared with IgG-antibodies obtained from sheep immunized with a synthetic hapten containing a highly conserved substructure of the AZA toxin group, conjugated to a carrier protein. Experiments using partially purified mussel hepatopancreas extract, crude mussel hepatopancreas extract, and a mussel tissue certified reference material demonstrated selective column binding (greater than 90%) and elution of AZAs. The results also showed that the AZA-IAC columns were able to selectively retain a wide range of AZAs, whereas other algal toxins such as OA/DTXs, cyclic imines, PTXs, and YTXs, passed straight through the column. This ability to selectively retain a wide range of AZAs shows that the AZA-IAC columns might be a useful tool for reducing LC–MS sample matrix effects and for purification of larger samples of AZAs, although these potential applications were not investigated in this study.

CRediT authorship contribution statement

Ingunn A. Samdal: Methodology, Supervision, Investigation, Resources, Visualization, Writing – original draft, Funding acquisition. **Morten Sandvik:** Methodology, Investigation. **Jennie Vu:** Investigation. **Merii S. Sukenthirarasa:** Investigation. **Sinthuja Kanesamurthy:** Investigation. **Kjersti L.E. Løvberg:** Investigation. **Jane Kilcoyne:** Resources, Investigation, Writing – review & editing, Funding acquisition. **Craig J. Forsyth:** Resources. **Elliott J. Wright:** Investigation, Resources, Writing – review & editing. **Christopher O. Miles:** Writing – review & editing, Resources, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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