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Effects of Heating on Proportions of Azaspiracids 1–10 in Mussels (*Mytilus edulis*) and Identification of Carboxylated Precursors for Azaspiracids 5, 10, 13, and 15

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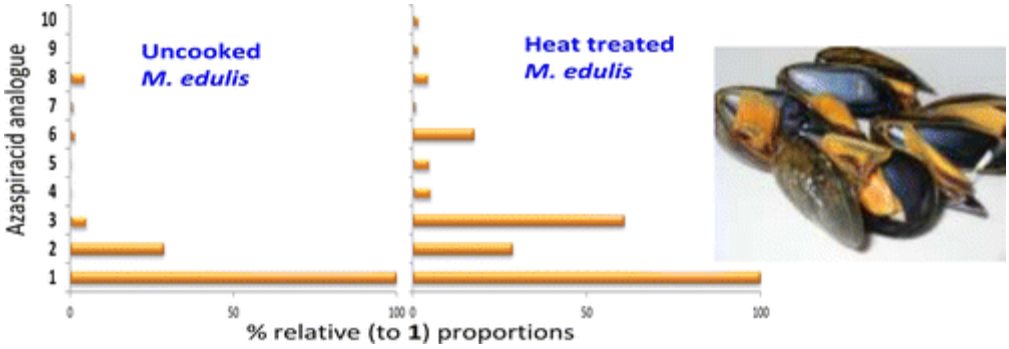
Abstract :

Azaspiracids (AZAs) are marine biotoxins that induce human illness following the consumption of contaminated shellfish. European Union regulation stipulates that only raw shellfish are tested, yet shellfish are often cooked prior to consumption. Analysis of raw and heat-treated mussels (Mytilus edulis) naturally contaminated with AZAs revealed significant differences (up to 4.6-fold) in AZA1–3 (1–3) and 6 (6) values due to heat-induced chemical conversions. Consistent with previous studies, high levels of 3 and 6 were detected in some samples that were otherwise below the limit of quantitation before heating. Relative to 1, in heat-treated mussels the average (n = 40) levels of 3 (range, 11–502%) and 6 (range, 3–170%) were 62 and 31%, respectively. AZA4 (4) (range, <1–27%), AZA5 (5) (range, 1–21%), and AZA8 (8) (range, 1–27%) were each ~5%, whereas AZA7 (7), AZA9 (9), and AZA10 (10) (range, <1–8%) were each under 1.5%. Levels of 5, 10, AZA13 (13), and AZA15 (15) increased after heating, leading to the identification of novel carboxylated AZA precursors in raw shellfish extracts, which were shown by deuterium labeling to be precursors for 5, 10, 13, and 15.

Graphic abstract



Keywords : azaspiracid, decarboxylation, hydroxylation, chemical conversion, heating, mass spectrometry, metabolism

INTRODUCTION

AZAs were first identified following a poisoning incident in which several people became ill in the Netherlands after consuming mussels (*Mytilus edulis*) harvested off the West coast of Ireland.[1,2,3] Since that time more than 30 analogues have been observed in shellfish,[4,5,6] phytoplankton,[7,8,9] crabs[10] and a marine sponge.[11] AZA1–10, (**1–10**),[2,3,12–14] and 37-*epi*-**1**[15] have been isolated from shellfish and their structures elucidated through a combination of NMR spectroscopy, LC-MS and chemical reactions. Further analogues AZA33 and -34 were isolated from bulk cultures of *A. spinosum.*[9] Only **1**, **2** and **3** are currently regulated in raw shellfish.[16] Compounds **1** and **2** are produced by the dinoflagellate *Azadinium spinosum*.[17] Many of the other analogues have been shown to be shellfish metabolites[18–21] and a metabolic pathway for some of the AZAs described has been proposed.[18,21]

Oral administration of AZAs induces chronic effects in mice[22] and damage to internal organs.[23] In vitro, AZAs are cytotoxic to mammalian cell lines[24] and teratogenic to fish embryos.[25] To date, the mode of action has not been identified. AZAs have been shown to be K^+ channel blockers,[26] however, the concentrations required are two-fold those for cytotoxicity. The current regulatory limit is in part based on intraperitoneal mouse studies performed following the initial isolation of **1**–**3**.[2,3] These studies indicated that **2** and **3** were more toxic than **1** and toxic equivalent factors are applied to results to reflect the difference in toxicity.[27] However, recent oral and intraperitoneal mouse studies have contradicted these results showing that **1** is more toxic than **2** and **3**.[28] Furthermore, an oral mouse study on **6** was performed for the first time showing that it is slightly less toxic than **1**.[28] In vitro, the order of potency was **2** > **6** > **8** ≈ **3** > **1** > **4** ≈ **5** using the Jurkat T lymphocyte cell assay.[14]

Studies, comparing the analysis of raw and cooked mussels, have shown significant differences in concentrations. Levels of AZAs were found to increase 2-fold in tissues that were cooked due to loss

of water from the matrix.[29] A similar study also reported the same effect for the OA group toxins,[30] while additional work on the OA group toxins reported significant increases (up to 150 %) which could not be accounted for due to a concentration effect alone but was additionally due to increased extraction of toxins following heat treatment.[31] Further studies on AZAs revealed that levels of **3**, **4**, **6** and **9** increased when samples were heat treated due to decarboxylation of AZA17, -21, -19 and -23 respectively,[18] however the scale of these increases was not fully evaluated. Levels of the 37-epimers of AZAs were also found to increase after application of heat, with levels increasing to as much as 16% that of the parent analogue.[15]

Here we evaluate the current regulatory methods used for the detection of AZAs in shellfish by accurately quantitating and comparing the toxin profiles in both raw and heat treated mussels. We additionally describe new AZA analogues and subsequently amend the previously proposed metabolic pathway in *M. edulis*.

MATERIALS AND METHODS

Chemicals All solvents (pesticide analysis grade) were from Labscan (Dublin, Ireland). Distilled H_2O was further purified using a Barnstead nanopure diamond UV purification system (Thermo Scientific, Waltham, MA). Formic acid (>98%), ammonium formate and deuterated MeOH (CH_3OD, >99.5 atom-% D) were from Sigma–Aldrich (Steinheim, Germany). AZA CRMs for **1**–**3** were obtained from the National Research Council (Halifax, NS, Canada).[32] Non-certified calibrant standards for **4**–**10** were prepared as described previously.[14]

Analysis of Raw and Heat Treated Mussel Tissues. AZA-contaminated raw samples, tested as part of the routine monitoring programme in Ireland, were selected for analysis. The shellfish were shucked and homogenised before extraction. Tissue samples were weighed (2 g) in duplicate into

50 mL centrifuge tubes with one set placed in a water bath (Grant Ltd) and heated to 90 °C for 10 min, then allowed to cool. The samples were extracted by vortex mixing for 1 min with 9 mL of MeOH, centrifuged at 3,950 g (5 min), and the supernatants decanted into 25 mL volumetric flasks. The remaining pellet was further extracted using an Ultra Turrax (IKA) for 1 min with an additional 9 mL of MeOH, centrifuged at 3,950 × g (5 min), and the supernatants decanted into the same 25 mL volumetric flasks, which were brought to volume with MeOH. The samples were then passed through Whatman 0.2 μm cellulose acetate filters into HPLC vials for analysis by LC-MS/MS.

Raw Hepatopancreas Extract. 5g of homogenised hepatopancreas (dissected from AZA contaminated *M. edulis*, collected from the Northwest of Ireland in 2005) was extracted with MeOH by vortex mixing for 1 min with 4 mL of MeOH, centrifuged at 3,950 g (5 min), and the supernatant decanted into a 10 mL volumetric flask. The remaining pellet was further extracted using an Ultra Turrax (IKA) for 1 min with an additional 4 mL of MeOH, centrifuged at 3,950 × g (5 min), and the supernatant decanted into the same 10 mL volumetric flask. The sample was passed through a Whatman 0.2 μm cellulose acetate filter into a HPLC vial for analysis. A 500 μL of the extract was placed in a water bath heated to 90 °C for 10 min, then allowed to cool.

Deuterium Incorporation. Two 500 μL aliquots of the hepatopancreas extract were transferred to HPLC vials and evaporated under N_2 without the use of heat. One of the dried residues was dissolved in 500 μL of CH_3OD and the other was dissolved in 500 μL of MeOH. Both aliquots were heated in a water bath at 70 °C for 10 min. The samples were evaporated under N_2 without the use of heat. The residues were then re-dissolved in 500 μL of MeOH and analyzed by LC–MS.

LC-MS Experiments.

Method A. Analysis was performed on a model 2695 LC instrument (Waters, Manchester, UK) coupled to a triple-stage quadrupole (TSQ) Ultima instrument (Micromass, Manchester, UK) operated in selected reaction monitoring (SRM) mode, with the following transitions: **5** *m/z*

844.5→808.5/362.3, AZA44, **11** *m/z* 888.5→808.5/362.3, **10** *m/z* 858.5→822.5/362.3, AZA45, **12** *m/z* 902.5→822.5/362.3, **13** *m/z* 860.5→824.5/362.3, AZA46, **14** *m/z* 904.5→824.5/362.3, **15** *m/z* 874.5→838.5/362.3, AZA47, **16** *m/z* 918.5→838.5/362.3. The cone voltage was 60 V and the collision voltage was 40 V, the cone and desolvation gas flows were set at 100 and 800 L/h, respectively, and the source temperature was 150 °C.

Binary gradient elution was used, with phase A consisting of water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid). The column used was a 50 mm × 2.1 mm i.d., 3 μm, Hypersil BDS C8 column with a 10 mm × 2.1 mm i.d. guard column of the same stationary phase (Thermo Scientific, Waltham, MA). The gradient was from 30−90% B over 8 min at 0.25 mL/min, held for 5 min, then held at 100% B at 0.4 mL/min for 5 min, and returned to the initial conditions and held for 4 min to equilibrate the system. The injection volume was 5 μL and the column and sample temperatures were 25 °C and 6 °C, respectively.

Method B. Analysis was performed on an Acquity UPLC coupled to a Xevo G2-S QToF (Waters, Manchester, UK) operated in MS^e mode, scanning from 100−1200 *m/z*. Leucine enkephalin was used as the reference compound. The cone voltage was 40 V, collision energy was 50 V, the cone and desolvation gas flows were set at 100 and 1000 L/h, respectively, and the source temperature was 120 °C.

The column used was a 50 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH C18 (Waters, Wexford, Ireland), using the same mobile phase described in method A. The gradient was from 30–90% 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 volumes were 2 μL and 5 μL and the column and sample temperatures were 25 °C and 6 °C, respectively.

Method C. Carboxylated precursors were monitored using the same instrument and UPLC conditions as was used for method B, scanning in MS/MS mode for the following ions: *m/z* 844.4 (**5**), 888.5 (**11**), 858.5 (**10**), 902.5 (**12)**, 860.5 (**13**), 904.5 (**14**), 874.5 (**15**) and 918.5 (**16**).

RESULTS AND DISCUSSION

Proportions of 1–10 in Raw and Heat Treated Mussels. To determine the relative importance of **1**–**10**, raw shellfish contaminated with AZAs were heated to simulate cooking (with no water loss). The analysis of cooked mussels most accurately reflects what is ingested by the consumer, and additional differences have been reported between the analysis of raw and cooked shellfish (*M. edulis*) in terms of concentrations.[29−31] Compounds **3**, **4**, **6** and **9** are produced by heat-induced decarboxylation of AZA17, -21, -19 and -23 respectively (Figure 1), and are not normally present in significant amounts in uncooked mussels.[18] Compounds **5** and **10** were proposed to be direct bioconversion products of **3** and **6** respectively.[21,20] LC-MS showed that **1**–**3** (regulated) and **6** were the predominant analogues in heat treated mussels (Table 1, Figure 2). There was huge variation in the levels of the analogues **3**−**10** (Figure 2), possibly due to differing rates of metabolism in the mussels. Time of harvesting may also be significant as mussels harvested directly following an intense bloom will likely have higher proportions of **1** and **2** than if they were harvested at some time after the bloom (due to metabolism). The average levels (relative to **1**) of **3** and **6** were 62% (range 11−502%) and 31% (range 3−170%) respectively. The average levels of **4**, **5** and **8** were each ~ 5%, while **7**, **9** and **10** were each under 1.5% (Figure 2). Figure 3 shows an LC-MS/MS chromatogram of a heat treated sample with significant levels of **3**, **4** and **6** that were not present in significant quantities in the raw sample. A feeding study (in which *M. edulis* was fed with *A. spinosum*)[21] showed that metabolism of **1** and **2** to AZA17 and -19, respectively, was detectable after 3 h, with levels of these metabolites increasing up to 2 days and then remaining constant to the end of the experiment (4 days). Relative to **1**, the proportions of AZA17 and -19 reached a maximum of 145%

and 55% respectively while the analogues **4**, **5** and **7**–**10** accounted for ~ 58% in total. However, that study was performed under laboratory conditions, and the high levels of AZA accumulation observed in naturally contaminated mussels[33] was not replicated.

In a recent study, **6** was found to be 7-fold more cytotoxic than **1**,[14] whereas a mouse oral dosing study found it to be only slightly less toxic than **1**.[28] Nonetheless, these results highlight the degree to which AZA-toxicity can be underestimated in routine monitoring programs where uncooked shellfish are tested. Previously, total levels of AZA analogues other than **1**–**3** were reported to comprise less than 5%,[6] however this study indicates that the analogues **4**–**10** comprise on average 13% (ranging from 5% to 24%) of the total AZAs (**1**–**10**) in heat treated mussels. Further analysis of AZA contaminated mussels using an ELISA method showed the total concentration of AZAs was significantly higher than the regulated toxins (AZA1−3) detected by LC-MS/MS.[34] All of the six formal risk assessments for AZAs[35] have been based on a poisoning event in 1997[36] and only take into account the analogues AZA1, -2 and -3. However, it is now clear that other analogues must have also been present at the time. Different toxin profiles have been reported from other countries, where **2** is more abundant than **1**,[11,37−39] and the shellfish from these locations are therefore likely to contain higher levels of **6**, **9** and **10**. In such circumstances, these analogues may have greater significance.

Identification of Novel Carboxylated Analogues. Previously **5** and **10** were suggested to be formed via C-23 hydroxylation of **3** and **6**, respectively.[21] In the present study, however, levels of **5** and **10** increased significantly after heat treatment (Figures 2 and 3). This suggested that **5** and **10** are, as previously demonstrated for **3**, **4**, **6**, and **9**,[18] produced via heat-promoted decarboxylation of the corresponding 22-carboxy-precursors (AZA44 and AZA45, respectively). In the heating process, enzymes responsible for hydroxylation would have been destroyed, so it is unlikely that the observed increase in **5** and **10** after heating were due to enzymatic hydroxylation of **3** and **6**, respectively. To test this hypothesis, LC-MS/MS analysis of AZA contaminated *M. edulis* samples for AZA44 (*m/z* 888) and AZA45 (*m/z* 902) was performed. Analogous carboxylated precursors for **13** and **15** were

also anticipated, so the possible presence of AZA46 (*m/z* 904) and -47 (*m/z* 918) was also investigated. It was expected that concentrations of these postulated analogues would be low, so a concentrated *M. edulis* hepatopancreas extract containing high levels of AZAs was analysed. The precursor compounds AZA44–47 (**11**, **12**, **14** and **16**) were observed in the hepatopancreas extract, however the presence of the analogues **3**, **6**, **4**, **5**, **9**, **13** and **15** (Figure 4) also indicated that some decarboxylation had already occurred prior to extraction. Following the application of heat (90 °C, 10 min) the carboxylated precursors (AZA17, AZA19, AZA21, AZA23, **11**, **12**, **14** and **16**) could no longer be detected in the extract, and there was a corresponding increase in the intensities of the peaks corresponding to their 22-decarboxylation products (**3**, **6**, **4**, **9**, **5**, **10**, **13** and **15**, respectively). Because this experiment was performed on filtered methanolic solutions, enzymatic catalysis is unlikely to be directly involved in the transformation.

Accurate mass measurements (Table 2) were consistent with the proposed structures of AZA44–47. The carboxylated and decarboxylated analogues displayed similar fragmentation patterns, with the carboxylated precursors showing an increase in mass of 44 Da. The spectra of the carboxylated precursors displayed an initial water loss, followed by a loss of 44 Da due to loss of the 22-carboxylic acid group as CO_2 (Table 2, Figures 5 and 6). Relative to their decarboxylated products, a smaller RDA fragment at *m/z* 674.4 is observed, in addition to the absence of the *m/z* 408.3 fragment that is characteristic[6] to the C-23 hydroxylated analogues (Figures 5 and 6). To confirm that **5**, **10**, **13** and **15** are formed following decarboxylation of AZA44, -45, -46 and -47 respectively, an experiment on the hepatopancreas extract was performed to show incorporation of deuterium following heat treatment in the presence of deuterated MeOH. Uptake of deuterium was observed for all analogues with increases in the + 1 Da isotope, that was not observed for **1** and **2**. The uptake of deuterium and conversion to known and established structures provides very strong structural evidence for AZA44 and -45 (Supporting information). As the structures for AZA13 and -15 have not yet been fully characterized, the proposed structures for AZA46 and -47 remain tentative. The

189 available evidence is consistent with the pathway shown in Figure 7, with oxidative metabolism at
190 C-3, C-13 and on the 22-Me, and slow (but accelerated by heating) decarboxylation of the resulting
191 22-carboxy group.

192 In terms of retention time, distinct differences were observed. AZA44 and -45 eluted ~ 0.5 min
193 earlier than their respective decarboxylated products (**5** and **10**), while smaller retention time
194 differences were observed for AZA46 and -47 compared to **13** and **15** respectively (difference of ~
195 0.3 min) (Figure 4).

196 In summary, analysis of heat-treated mussels from Ireland that were naturally contaminated with
197 AZAs revealed high levels of **3** and **6**. These compounds were not present at significant levels in the
198 uncooked shellfish, highlighting the fact that AZA equivalent values for raw mussels can grossly
199 underestimate the toxicity of the AZAs present (up to 4.6-fold difference for **1**−**3** and **6**). This effect
200 is further compounded by the increase in concentration of these compounds due to water loss during
201 cooking.[29] Levels of **4**, **5** and **7**–**10** were generally low in Irish mussels, and did not contribute
202 significantly to overall toxicity, although the situation may be different for other shellfish species.
203 However, in areas where **2** is the predominant AZA analogue, **6**, **9** and **10** will most likely have more
204 relevance than in Irish mussels. Not only do these results suggest that tissues should be heat-treated
205 prior to analysis, but also that **6** should be included in the regulations to more accurately reflect the
206 toxin profile to which shellfish consumers are exposed. Due to the huge variation in levels of the
207 decarboxylated analogues it is difficult to build in a safety factor that deals with these bioconversions
208 effectively based on the currently regulated toxins. The EU harmonized LC-MS method[40] has been
209 amended to deal with a concentration effect due to the loss of water during cooking for processed
210 samples, however, for the analysis of raw samples a heating step should be included. These measures
211 are necessary to enhance human health protection and prevent loss of valuable processed product due
212 to rejection by importing countries. Such amendments would warrant a review of the current
213 regulatory limit, which should consider the fact that no cases of human intoxications were reported

214 from mussels that were over the regulatory limit following heat treatment.

215 Four additional carboxylated AZA analogues were identified which were shown to be precursors for
216 the analogues **5**, **10**, **13** and **15** and were named AZA44, -45, -46 and -47, respectively.

217

ASSOCIATED CONTENT

Supporting Information

Table showing proportions of **1**–**10** in *M. edulis* samples (n=40); mass spectra of **5**, **10**, **13** and **15** showing incorporation of deuterium; chromatograms of hepatopancreas extract after heating; table showing retention times of decarboxylated AZAs and their precursors.

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Notes

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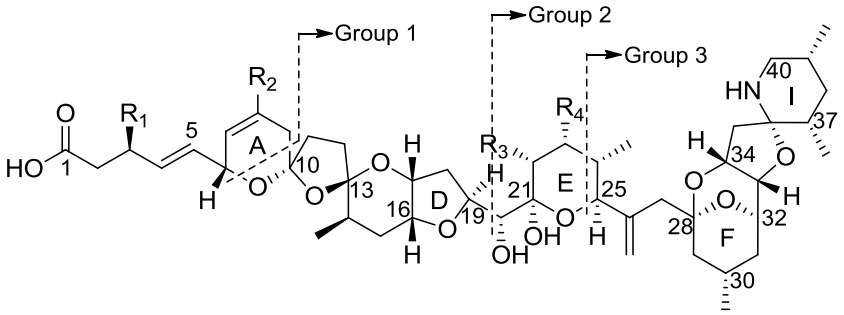
Table 1. Measured Concentrations (μg/g) of **1**−**3** and **6** in Irish *M. edulis* Samples Before and After Heating (method A)

Harvesting location (Irish Atlantic coast)	Harvesting date	Raw					Heated					
		1	**2**	**3**	**6**	***AZA equiv. (1−3)**	**1**	**2**	**3**	**6**	***AZA equiv. (1−3)**	**Ratio of 1–3 + 6 in heated and raw *M. edulis***
West	26/09/2012	0.06	0.02	0.00	0.00	0.10	0.06	0.02	0.07	0.02	**0.18**	2.1
Southwest	27/09/2012	0.16	0.04	0.01	0.00	**0.24**	0.16	0.04	0.06	0.01	**0.30**	1.3
Southwest	27/09/2012	0.10	0.02	0.00	0.00	0.15	0.10	0.02	0.04	0.01	**0.20**	1.4
Northwest	27/09/2012	0.04	0.01	0.00	0.00	0.07	0.04	0.02	0.07	0.02	**0.17**	3.0
West	24/09/2012	0.22	0.05	0.01	0.00	**0.33**	0.20	0.05	0.12	0.03	**0.48**	1.4
West	24/09/2012	0.12	0.03	0.00	0.00	**0.18**	0.10	0.03	0.07	0.02	**0.24**	1.5
Southwest	24/09/2012	0.11	0.03	0.00	0.00	**0.16**	0.09	0.02	0.04	0.01	**0.18**	1.1
West	24/09/2012	0.03	0.01	0.01	0.00	0.07	0.03	0.02	0.14	0.04	**0.25**	4.6
Southwest	26/09/2012	0.08	0.02	0.00	0.00	0.12	0.08	0.02	0.03	0.00	**0.16**	1.3
West	24/09/2012	0.02	0.02	0.01	0.00	0.06	0.03	0.02	0.08	0.02	**0.18**	3.0

*Equivalents of total regulated AZAs (**1**–**3**) calculated following application of the toxic equivalence factors for **2** (1.8) and **3** (1.4) relative to **1**.[23] Values exceeding the EU regulatory limit (0.16 μg/g) are shown in bold text.
Red indicating areas where there is significant change.

Table 2. Accurate Mass Measurements (method B) of **11**, **12**, **14** and **16**.

	$[M+H]^+$		$[M+H-H_2O-COO]^+$		Group 1		Group 2		Group 3	
AZA	Measured mass	Δ ppm	Measured mass	Δ ppm	Measured Mass	Δ ppm	Measured Mass	Δ ppm	Measured Mass	Δ ppm
AZA44 (**11**)	888.4738	-0.2	826.4744	0.3	674.3895	-1.3	446.2901	-1.1	362.2679	-4.4
AZA45 (**12**)	902.4898	0.2	840.4915	2.0	674.3900	-0.6	446.2891	-3.4	362.2695	0
AZA46 (**14**)	904.4700	1.2	842.4667	-2.8	674.3912	1.2	446.2921	3.4	362.2681	-3.9
AZA47 (**16**)	918.4825	-2.2	856.4838	-1.1	674.3897	-1.0	446.2901	-1.1	362.2680	-4.1

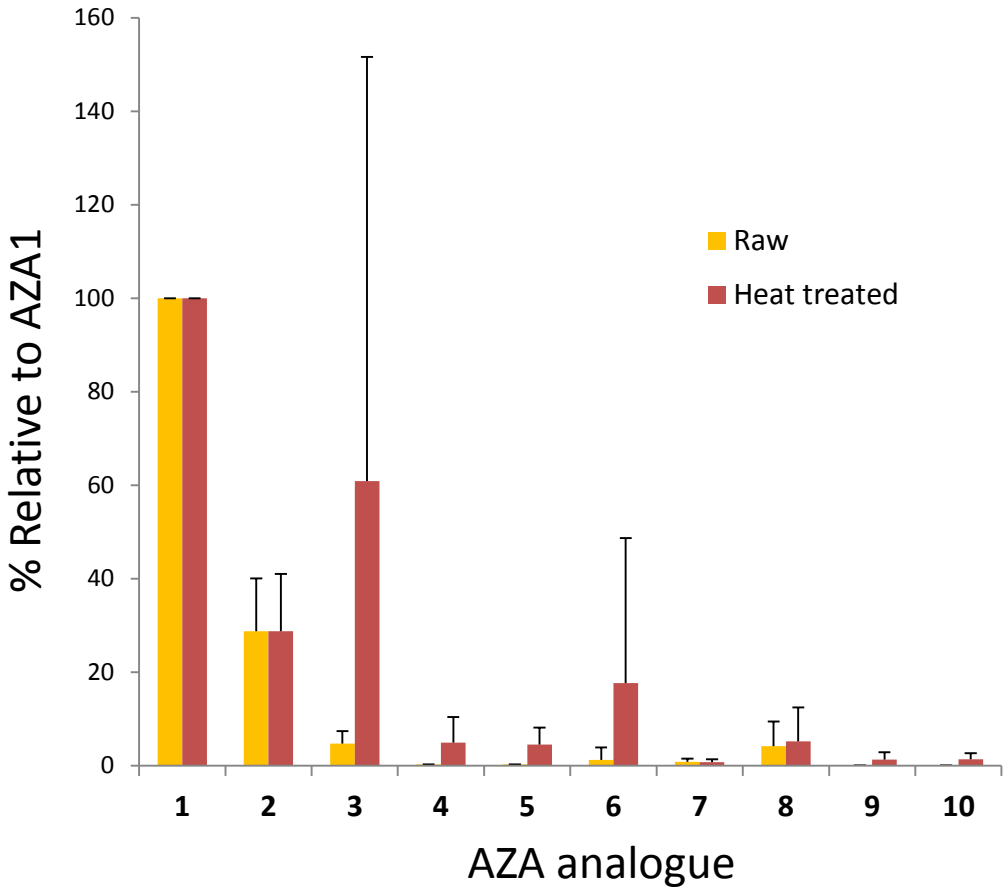


AZA	R_1 (C-3)	R_2 (C-8)	R_3 (C-22)	R_4 (C-23)	$[M+H]^+$ *m/z*	Origin	Status	Decarboxylation product
AZA1 (**1**)	H	H	CH_3	H	842.5	*A. spinosum*	phycotoxin	
AZA2 (**2**)	H	CH_3	CH_3	H	856.5	*A. spinosum*	phycotoxin	
AZA3 (**3**)	H	H	H	H	828.5	shellfish	metabolite	
AZA4 (**4**)	OH	H	H	H	844.5	shellfish	metabolite	
AZA5 (**5**)	H	H	H	OH	844.5	shellfish	metabolite	
AZA6 (**6**)	H	CH_3	H	H	842.5	shellfish	metabolite	
AZA7 (**7**)	OH	H	CH_3	H	858.5	shellfish	metabolite	
AZA8 (**8**)	H	H	CH_3	OH	858.5	shellfish	metabolite	
AZA9 (**9**)	OH	CH_3	H	H	858.5	shellfish	metabolite	
AZA10 (**10**)	H	CH_3	H	OH	858.5	shellfish	metabolite	
AZA11	OH	CH_3	CH_3	H	872.5	shellfish	metabolite	
AZA12	H	CH_3	CH_3	OH	872.5	shellfish	metabolite	
AZA13 (**13**)	OH	H	H	OH	860.5	shellfish	metabolite	
AZA14	OH	H	CH_3	OH	874.5	shellfish	metabolite	
AZA15 (**15**)	OH	CH_3	H	OH	874.5	shellfish	metabolite	
AZA16	OH	CH_3	CH_3	OH	888.5	shellfish	metabolite	
AZA17	H	H	COOH	H	872.5	shellfish	metabolite	AZA3
AZA19	H	CH_3	COOH	H	886.5	shellfish	metabolite	AZA6
AZA21	OH	H	COOH	H	888.5	shellfish	metabolite	AZA4
AZA23	OH	CH_3	COOH	H	902.5	shellfish	metabolite	AZA9
AZA44 (**11**)	H	H	COOH	OH	888.5	shellfish	metabolite	AZA5
AZA45 (**12**)	H	CH_3	COOH	OH	902.5	shellfish	metabolite	AZA10
AZA46 (**14**)	OH	H	COOH	OH	904.5	shellfish	metabolite	AZA13
AZA47 (**16**)	OH	CH_3	COOH	OH	918.5	shellfish	metabolite	AZA15

Figure 1. Structures of AZA1–16, -17, -19, -21, -23, -44, -45, -46 and -47, their protonated masses and origin. Note: Only **1**–**10** have had their structures confirmed by NMR, while AZA17, -19, -21, -

384 23, **11**, **12, 14** and **16** have had their structures confirmed by conversion to analogues with

385 established structures (**3**, **6**, **4**, **9**, **5**, **10**, **13** and **15**, respectively).



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387 **Figure 2.** Proportions (% ± SD) of **2**–**10** relative to **1** (method B) in raw and heat treated *M. edulis*

388 (n=40) harvested off the Atlantic coast of Ireland.

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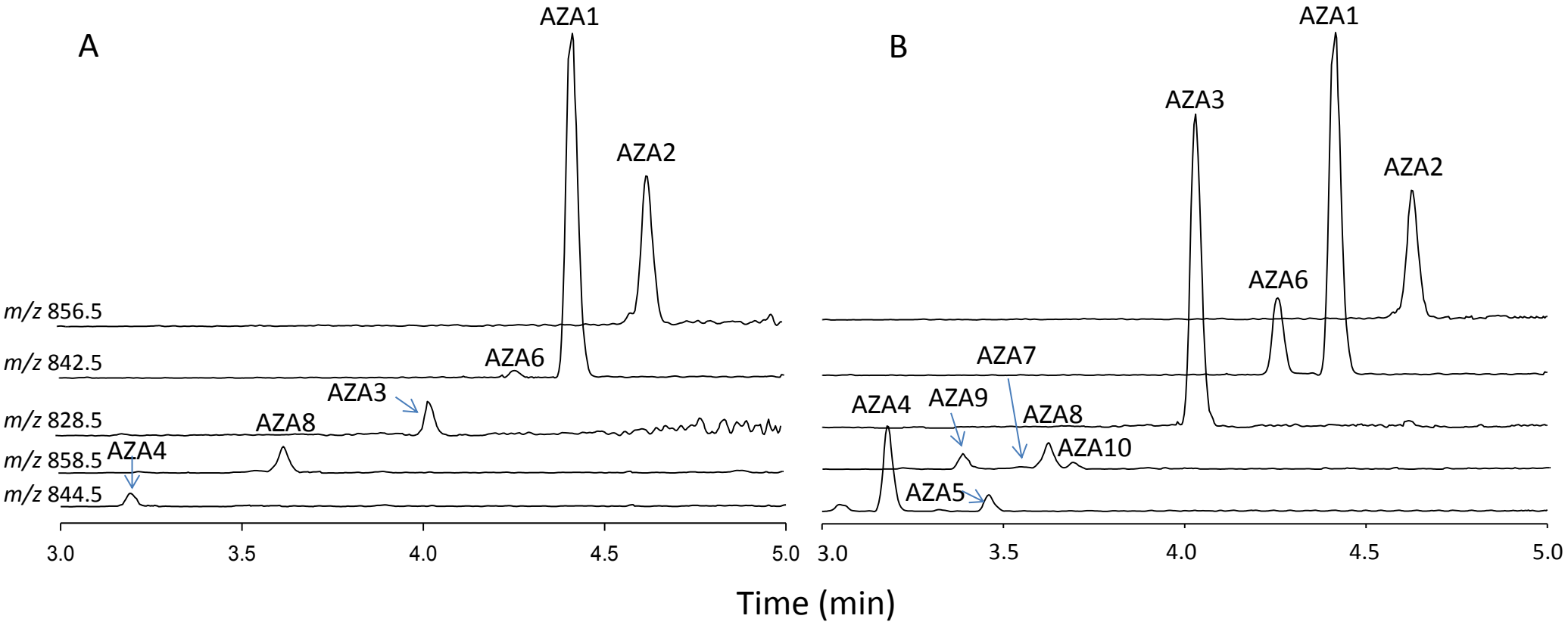


Figure 3. LC-MS (method B) of A) a raw *M. edulis* sample extract (0.7 µg/g AZA equivalents in the raw extract) from the Marine Institute biotoxin monitoring programme and B) the same extract after heat treatment, showing peaks for **1**–**10**.

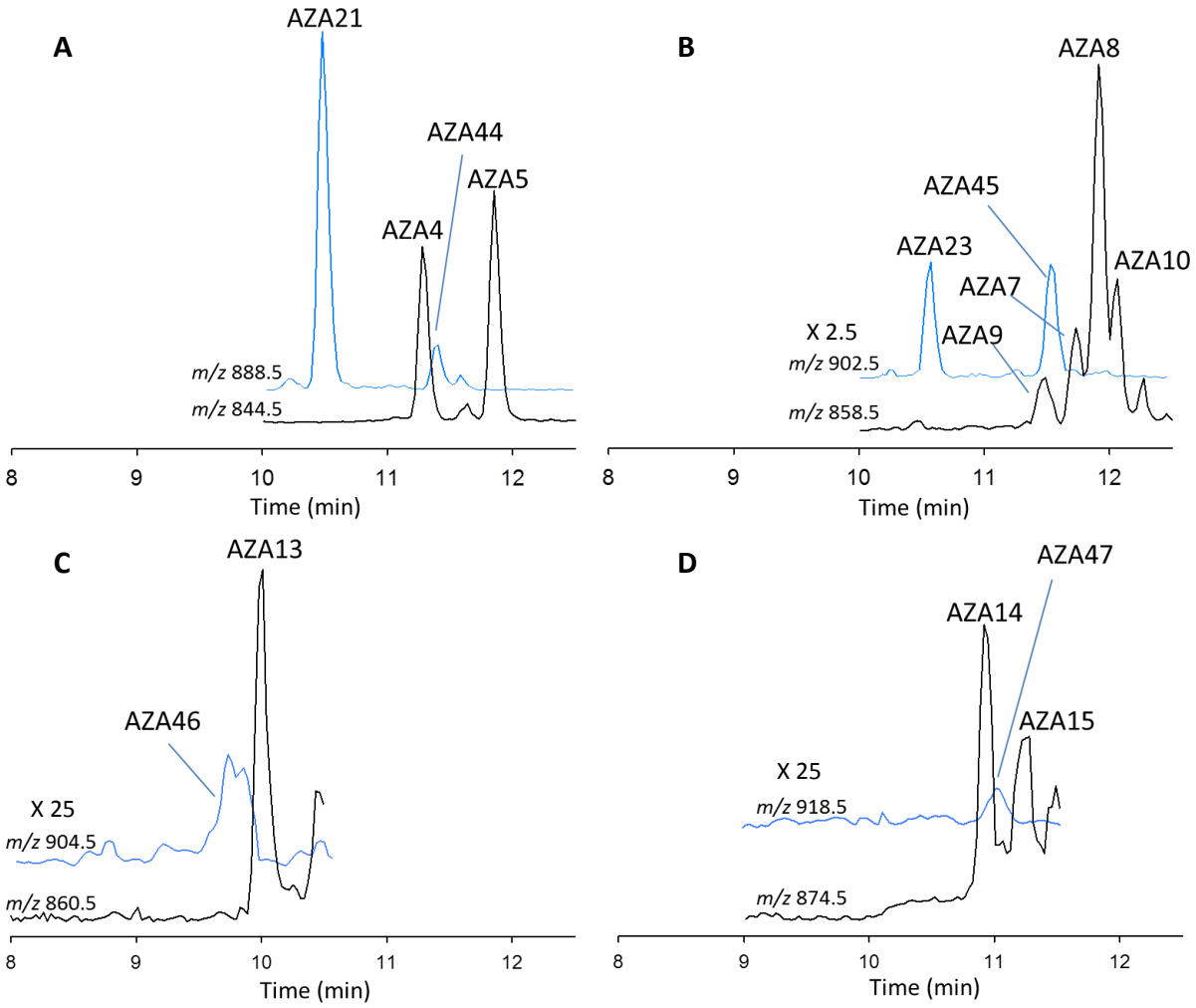


Figure 4. LC-MS analysis (method A) of: A) AZA44 (**11**) and AZA5 (**5**); B) AZA45 (**12**) and AZA10 (**10**); C) AZA46 (**14**) and AZA13 (**13**); and D) AZA47 (**16**) and AZA15 (**15**) in a raw *M. edulis* hepatopancreas extract.

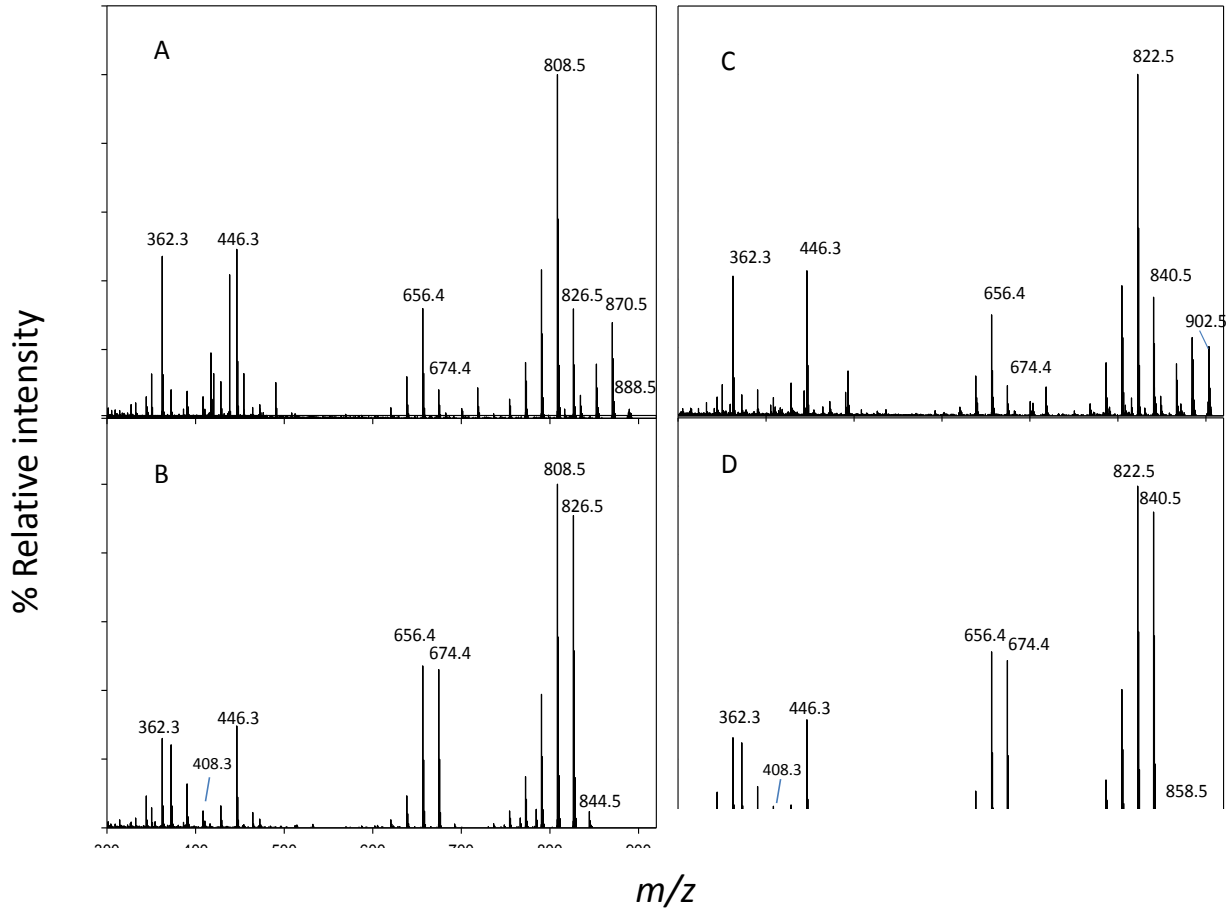
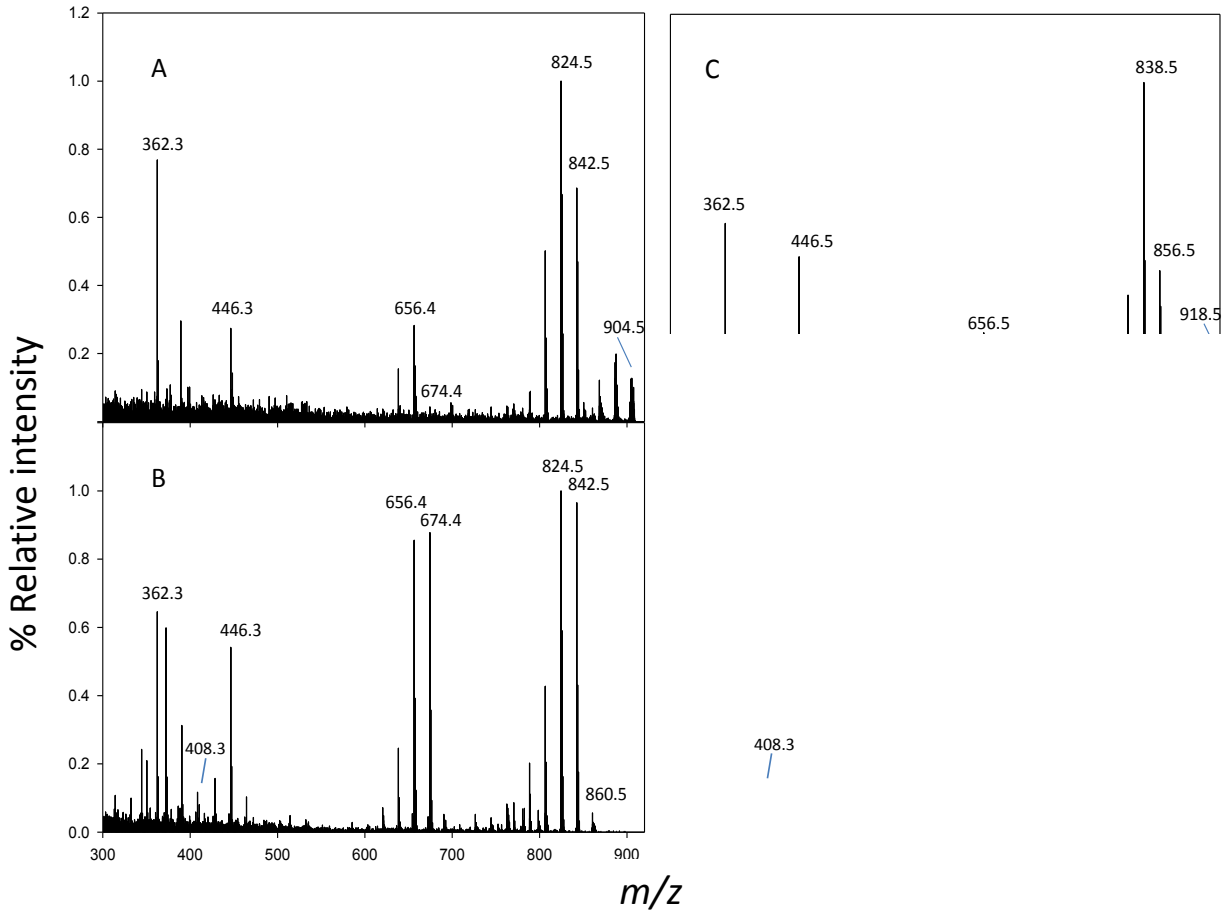


Figure 5. Mass spectra (method C) of: A) AZA44 (**11**) and B) its decarboxylation product AZA5 (**5**); C) AZA45 (**12**) and D) its decarboxylation product AZA10 (**10**).



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426 **Figure 6.** Mass spectra (method C) of: A) AZA46 (**14**) and B) its decarboxylation product AZA13

427 (**13**); C) AZA47 (**16**) and D) its decarboxylation product AZA15 (**15**).

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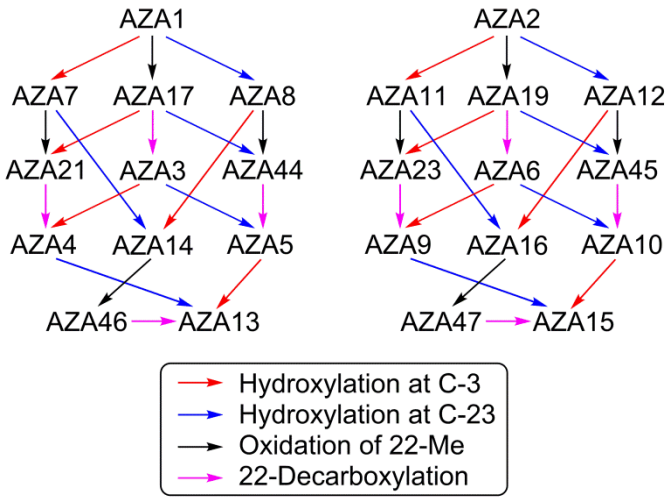

Figure 7. Proposed AZA inter-relationships in *M. edulis*.