Quantitative analysis of azaspiracids in *Azadinium spinosum* cultures

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Abstract

Azaspiracids (AZAs) are secondary metabolites of *Azadinium spinosum* that can accumulate in shellfish and cause food poisoning when consumed. We describe here an analytical procedure for the determination of AZAs in cultures of *A. spinosum* with a focus on the formation of AZA methyl esters as artefacts during extraction and sample pre-treatment. *A. spinosum* cells were collected from bioreactor cultures using centrifugation or filtration. Different extraction procedures were evaluated for formation of methyl ester artefacts, yield, and matrix effects. Filtration of cultures using glass-fibre filters led to increased formation of methyl esters, and centrifugation is recommended for recovery of cells. The extraction solvent (MeOH, Acetone, MeCN) did not significantly affect the yield of AZAs as long as the organic content was 80% or higher. However, the use of MeOH as extraction solvent led to increased formation of methyl esters. AZA1 recovery over two successive extractions was 100% at the 95% confidence level for acetone and MeOH. In standard-addition experiments, no significant matrix effects were observed in extracts of *A. spinosum* or *A. obesum* up to a sample size of 4.5×10^9 μm^3. Moreover, experiments carried out to clarify the formation and structure of methylated AZA analogues led to the description of two AZA methyl esters and to the correction of the chemical structures of AZA29–32.

Key words

Extraction procedure, Extraction artefact, Matrix effects, LC-MS/MS, Azaspiracid methyl ester, Dinoflagellate, Liquid chromatography–mass spectrometry

Introduction

Harmful algal blooms are widespread throughout the world, frequently causing problems to public health through consumption of contaminated shellfish and, amongst these, azaspiracid shellfish poisoning is the most recently identified syndrome. In 1995, a human intoxication occurred in the Netherlands after consumption of mussels from Ireland (Killary Harbour), with symptoms typical for diarrhetic shellfish poisoning (DSP). The incident caused diarrhea, nausea, vomiting and stomach cramps in consumers; however, only very low levels of OA-group toxins were found in mussels [1]. A new toxin named azaspiracid (AZA) (now referred to as Azaspiracid-1 (AZA1) (Fig. 1)), was identified three years later [2], with its structure being revised after synthetic studies [3]. AZAs 4-5 were subsequently isolated from contaminated mussels and their structures established using mass spectrometry and NMR spectroscopy [4,5]. Additional AZAs have since been identified and structures proposed based on mass spectrometry, and the group now comprises 32 analogues, including several hypothetical compounds and artefacts [6,7]. Since their initial discovery, AZAs have been found in Europe, Africa and more lately in America and in Japan [8-14].

Even though consumption of AZA-contaminated shellfish has caused public health problems since 1995, it was not until 2003 that AZAs were detected in plankton, namely in the dinoflagellate *Protoperidinium crassipes* [15]. However, a question rapidly arose over whether this organism was an actual AZA producer or whether it was a predator which accumulated the toxin from another organism [16,17]. As *P. crassipes* is a heterotrophic dinoflagellate [18] capable of accumulating phycotoxins [19], and since culturing did not result in AZA production (Tillmann and Krock, unpublished data in [20]), research focussed on possible prey of this species. During a cruise in 2007, a dinoflagellate source of AZAs (strain 3D9), was discovered [21]. This organism was found to contain AZA1 and AZA2 in the field, and produced *de novo* AZA1 and -2 in axenic culture [22,20]. The organism, a small

(12–16 µm length and 7–11 µm width) peridinin-containing photosynthetic dinoflagellate with a thin theca, was formally described by Tillmann et al. [20] and named *Azadinium spinosum*. This species was the type-species for a new genus, and was soon joined by two non-AZA-producing species: *Azadinium obesum* (2E10) [23], a somewhat larger organism (13–18 µm length, 10–14 µm width); and *Azadinium poporum*, which is similar in size to *A. spinosum* but has a slightly lower mean cell length:width ratio (11–16 µm length and 8–12 µm width) [24]. Interestingly, since the morphological description of *A. spinosum*, strains of this organism have also been reported from Mexico [25], Argentina [26], Italy and France (personal communication, R. Siano and E. Nézan, Ifremer, France) and, as to be expected, from Ireland [27]. As the organism appears to be widespread in many oceans, and since AZAs have been reported from many locations, azaspiracid poisoning should be considered of global concern. We have therefore cultivated this organism to better understand the ecophysiology of *Azadinium,* its toxin production and the kinetics of AZA accumulation in shellfish. Finally, quantitative knowledge of the AZA-production by *A. spinosum* is also essential information for the sustainable production of toxins for toxicology experiments and instrument calibration.

In previous studies, the cellular quota of AZAs was highly variable, ranging from 5–40 fg.cell^{-1} [20,27], while in our own studies we found up to 100 fg.cell^{-1}. Such differences may arise from differences in either culture conditions or in analytical procedures, including extraction, recovery in sample pre-treatment, or matrix effects in the final determination using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Consequently, studies on environmental and nutritional factors affecting *A. spinosum* growth and toxicity will require standardised analysis of cells and culture media for their toxin content. Therefore, we decided to investigate the analysis of AZAs from cultures of *A. spinosum*.

Like other lipophilic toxins, AZAs are typically extracted from phytoplankton or contaminated bivalves with organic solvents [28]. Historically, acetone has been used to extract lipophilic toxins from shellfish for the mouse bioassay, whereas MeOH and mixtures of MeOH–water have been used for extraction prior to LC-MS/MS analysis [29,16]. These procedures result in crude extracts that typically cause matrix effects (signal enhancement or reduction) in LC-MS/MS analysis [30]. Matrix effects have been reported in quantitation of AZAs in mussels by LC-MS/MS using different technical approaches [31], and possible solutions were proposed [32,33]. Therefore, matrix effects also need to be evaluated in analytical procedures for quantitation of AZAs in phytoplankton.

A novel AZA analogue observed by Krock et al. [22], and provisionally denoted as “AZAX”, was detected in methanolic extracts of *Azadinium* cultures, and possessed a molecular ion corresponding to that of AZA1 methyl ester. However, the relative retention time of AZAX did not appear to match that originally reported for AZA1 methyl ester [34] (denoted as AZA30 by these authors). Therefore, clarification of the structures and mechanisms of formation of methylated AZA artefacts was required.

The determination of AZA metabolites, and the identification of artefacts formed during analytical procedures, is important for the subsequent assessment of metabolism in shellfish, other aquatic organisms, and mammalian systems. Thus, the present study describes the development of a quantitative analytical method for the determination of AZAs in cultures of *A. spinosum*, clarifies the structures of methylated derivatives of AZAs, and explains the formation of AZA methyl esters as artefacts from the extraction of *A. spinosum* cells with MeOH, and of AZA methyl ketals as artefacts of storage in MeOH.

Materials and methods

Culture condition and cell count

Two species of *Azadinium* were used: the producer of AZA1 and -2, *A. spinosum* (clone 3D9) and the non-AZA-producing species *A. obesum* (clone 2E10). Both strains were grown using K modified medium [35], without NH_4Cl and with Na_2SeO_3 (10^{-8} M) , at 18 °C with a photon flux density of 200 µmol.m^{-2}.s^{-1} and a photoperiod of 16 h of light and 8 h of dark, in a 2.5 L or 100 L chemostat . Algae were sampled at steady state in continuous culture. Cell densities and cellular volume were determined using a particle counter (Multisizer 3 Coulter counter, Beckman).

Reagents

Methanol (MeOH), acetone, acetonitrile (MeCN), ethanol (EtOH), and dichloromethane (DCM) were obtained as HPLC grade solvents from JT Baker and Sigma Aldrich. Formic acid (Puriss quality), ammonium formate (Purity for MS), methanol-d_4 (99.8%), *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, di(ethylene glycol)ethyl ether and 9-anthraldehyde were obtained from Sigma-Aldrich. Milli-Q water for HPLC was produced in-house using a Milli-Q integral 3 system (Millipore).

Spiking experiments were carried out using AZA1 purified by P. Hess in collaboration with M. Satake in Japan (2001), according to published procedures [5]. AZA1 calibrants for LC-MS/MS analysis were dilutions of either certified AZA1 (CRM-AZA1, National Research Council Canada (NRCC), Halifax, Canada), or using the above AZA1 purified in Japan (calibrated against CRM-AZA1).

LC-MS/MS analysis

The samples were analysed by LC-MS/MS using an Agilent 1100 LC coupled to a triple quadrupole mass spectrometer (API 2000, Applied Biosystems), a UFLCxr (Shimadzu) coupled to a triple quadrupole hybrid mass spectrometer Q-trap (API 4000QTRAP, Applied

Biosystems), and an Agilent 1200 HPLC coupled to an Agilent 6540 QTOF instrument equipped with an electrospray ionization source for quantitation and accurate mass spectral analysis of AZAs.

Liquid chromatography

HPLC was carried out using BDS-Hypersil C8 (50 × 2 mm, 3 µm and 150 × 2.1 mm, 3 µm), MOS-Hyperclone C8 (50 × 2 mm, 3 µm) and Hypersil-Gold C18 (50 × 2 mm i.d., 2 µm) silica-based reversed phase columns (Thermo Scientific). Injection volumes were 5 µL. The A and B mobile phases were 100% water and acetonitrile/water (95:5, v/v) respectively, both containing 2 mM ammonium formate and 50 mM formic acid.

The 50 mm BDS-Hypersil column was eluted isocratically at 250 µL.min^{-1} (75% B) at 20 °C for 5–10 min, depending on which analogues were analysed. The MOS-Hyperclone C8 column was used in gradient elution mode (200 µL.min^{-1} at 20 °C) starting with 70% B rising to 100% B at 2.5 min, held for 4.5 min, decreasing to 70% B over 6 min, and held for 5 min until the next run.

The 150 mm BDS-Hypersil C8 column was used in gradient elution mode (200 µL.min^{-1} at 30 °C) for acquisition of accurate mass data, starting with 25% B rising to 100% B at 12 min, held for 8 min, decreasing to 25% B over 1 min, and held for 10 min until the next run.

The Hypersil Gold C18 column was eluted with a gradient for determination of AZAs and AZA ADAM derivatives, starting with 62.5% B rising to 100% B at 4 min, held for 5 min, decreasing to 62.5% B over 0.5 min, and held for 5 min until the next run.

Mass spectrometry

Multiple reaction monitoring (MRM) and fragmentation experiments were performed in positive ion mode under the conditions given in Table 1. Selected ion monitoring (SIM) was performed in negative mode. The following MRM transitions were monitored: AZA1, *m/z* 842.5→824.5, 842.5→672.5; AZA2 and AZA1 methyl ester, *m/z* 856.5→838.5,

856.5→672.5; AZA2 methyl ester, *m/z* 870.5→852.5, 870.5→672.5; AZA1 d_3-methyl ester, *m/z* 859.5→841.5, 859.5→672.5; AZA2 d_3-methyl ester, *m/z* 873.5→855.5, 873.5→672.5; AZA1 methyl ketal (AZA30), *m/z* 856.5→824.5; 856.5→672.5; AZA2 methyl ketal (AZA32), *m/z* 870.5→838.5; 870.5→672.5; AZA1 (9-anthryl)methyl ester, *m/z* 1032.6→672.5; AZA2 (9-anthryl)methyl ester and AZA1 methyl ketal (9-anthryl)methyl ester, *m/z* 1046.6→672.5; and AZA2 methyl ketal (9-anthryl)methyl ester, *m/z* 1050.6→672.5. The following $[M-H]^-$ ions were monitored in SIM mode: AZA1, *m/z* 840.5; AZA2, AZA1 methyl ester and AZA1 methyl ketal, *m/z* 854.5; AZA2 methyl ester *m/z* 868.5. Quantitation was carried out using external calibration against AZA1, with Analyst 1.5 software (Applied Biosystems).

Accurate mass data were acquired on an Agilent 6540 QTOF operated in positive mode, with full-scan analysis over *m/z* 100–1000 at 1 scan/s and targeted MS/MS analysis at 5 scans/s. Capillary and fragmentor voltages were 4000 V and 220 V, respectively. The Jet Stream Technology source was set at 300 °C with a drying gas flow at 8 L/min and a sheath gas flow of 12 L/min at 400 °C. Three collision energies (30, 50 and 70 V) were applied to the precursor ions to study fragmentation pathways.

Identification of AZA1 and -2 methyl esters and structure confirmation

(a) The effect of heat treatment of the filters containing *A. spinosum* samples on the formation of AZA methyl esters was tested to determine whether their formation was enzyme-catalysed. Triplicate aliquots of *A. spinosum* culture (10 mL) were filtered using GF/C filters and the filters (with cells) were placed in a 1.5 mL Eppendorf tubes. Two types of heat treatment were tested to suppress enzymatic activity: (i) microwaving for 2 min at 800 W, or; (ii) placing in a water bath at 100 °C for 30 min. A control, without heat treatment, was prepared in parallel. Each sample was then extracted with MeOH (5 × 0.5 mL) (described below).

(b) The formation of AZA methyl esters was studied during extraction and reconstitution with MeOH and deuterated MeOH (CD_3OD). The procedure in Fig. 2 was used with the following

solvents: (a) extraction with MeOH, reconstitution with MeOH; (b) extraction with MeOH, reconstitution with CD_3OD; and (c) extraction with CD_3OD, reconstitution with CD_3OD.

(c) Treatment with diazomethane, which derivatises carboxylic acids as their methyl esters, was used to synthesise AZA1 and -2 methyl esters. A methanolic extract from *A. spinosum* (0.5 mL) containing AZA1 and -2 was added to the outside tube of an Aldrich diazomethane generator with System 45 connection, and 1 mL MeOH and 1.5 mL Et_2O were added. Diazomethane was generated in the inner tube of the apparatus and allowed to react *in situ* with the extract, following the manufacturer's protocol [36]. After reacting for 45 min at 0 °C with occasional swirling, the extract was transferred to a glass vial, evaporated to dryness under a stream of N_2, and the residue dissolved in MeOH (1 mL) for LC-MS analysis.

(d) A sample containing AZA30 and -32 was obtained from an experiment studying storage of AZA1 and -2 standards in MeOH (NRCC).

(e) Samples containing AZA1 and -2 methyl esters, and AZA30, and -32 (AZA1 and -2 methyl ketals), were treated with sodium periodate as described by Rehmann et al. [7], then analysed by LC-MS/MS or by LC-MS in negative ion SIM mode. The same samples were also derivatized with 9-anthryldiazomethane, which derivatizes carboxylic acids as their (9-anthryl) methyl esters, and analysed by LC-MS in positive ion MRM mode as described by McCarron et al. [37].

Protocols for the determination of extra- and intra-cellular portion of AZAs

Procedures for AZA extraction evaluated in this study were based on the following standard protocols.

Samples (10 mL) were collected from *A. spinosum* cultures and centrifuged (2500 *g*, 20 min, 4 °C) in 15 mL centrifuge tubes. The culture supernatant was collected for liquid–liquid extraction as described below, and the pellet was re-suspended in 500 µL of solvent and bath-sonicated (10 min) after transferring to a 1.5 mL Eppendorf tube. After sonication, the aliquot was centrifuged (15000 *g*, 10 min, 4 °C). The supernatant was transferred to a 5 mL glass tube

and gently evaporated under nitrogen on a heating block at 35 °C. This process was repeated so that the pellet was extracted three times in total (the number of repetitions varied as a function of the experiment, but three successive extractions were generally used) and, following evaporation of the combined supernatants from each step, the residue was reconstituted in 500 or 1000 µL MeOH–H_2O (9:1 v/v). Subsequently, the sample was filtered with a NANOSEP MF centrifugal device (PALL, 0.2 µm, 1.5 mL vial with filter insert) (15000 *g*, 5 min, 4 °C) and transferred to an HPLC vial with insert (Fig. 2).

The supernatant from centrifugation of the algal culture was transferred to a 15 mL tube and 2 mL of DCM added. The mixture was homogenized (1 min vortexing), centrifuged (2500 *g*, 10 min, 4 °C), and the organic phase transferred to a 15 mL glass tube. The supernatant was extracted three times in this manner, and the resulting DCM extract evaporated under nitrogen on a heating block at 35 °C and the residue was reconstituted and filtered as above (Fig. 2).

The above extraction protocol was used to evaluate the following aspects of *A. spinosum* extraction:

(a) Effect of sample size, and residence time of *A. spinosum* in a 15 mL centrifuge tube prior analysis, on intra- an extra-cellular AZA content. The aliquots were preserved with neutral Lugol and immediately observed using a Nageotte cell-counting chamber. During the experiment, the aliquots were maintained at room temperature (18 ± 2°C).

(b) Influence of procedures for separation of algal cells from the culture medium (filtration and centrifugation) on intra- and extra-cellular AZA content.

(c) Effect of extraction solvent on yield and artefact formation.

(d) Effect of algal matrix on recovery on the standard procedure.

Matrix effects in LC-MS/MS analyses of culture extracts

Matrix effects were evaluated using the following approaches:

(a) AZA1 addition to a constant amount of algal matrix. *A. obesum* culture medium (10 mL) was extracted using the standard procedure (Fig. 2) with MeOH–H_2O (9:1) or acetone–H_2O

(9:1) (v/v). Reconstitution was carried out in triplicate using an AZA1 solution and MeOH, to obtain AZA1 concentrations ranging from 5.3–213 ng.mL^{-1}.

(b) The same extraction was applied as for (a) above, but with *A. spinosum* culture medium.

(c) Matrix addition to a constant AZA concentration. Samples of a culture of *A. obesum* (58000 ± 1000 cells.mL^{-1}, 83.3 × 10^6 µm^3.mL^{-1}) (0.62, 3.1, 6.2, 10, 21, 50 mL) were extracted in triplicate (Fig. 2) using MeOH–H_2O (9:1) or acetone–H_2O (9:1 v/v) and reconstituted with 40 µL of AZA1 solution and 460 µL of MeOH–H_2O (9:1 v/v) to give an AZA1 concentration of 42.7 ng.mL^{-1}.

Statistical analysis

All data are expressed as mean ± standard deviation. Depending on the data, statistical analyses consisted either of multifactorial analysis of variance, one-way analysis of variance (ANOVA), or a Kruskal–Wallis test, followed, when necessary, by a Fisher's least significant difference procedure or a box-and-whisker plot. Differences were considered significant at $p<0.05$. Statistical analyses were carried out using Statgraphics Centurion XV.I (StatPoint Technologies, Inc.). Before each ANOVA analysis or Kruskal–Wallis test, normality and equality of variance were tested to decide which tests were going to be used.

Results and discussion

Identification of AZA1 and -2 methyl esters and structure confirmation

A late-eluting LC-MS peak in *A. spinosum* extracts, provisionally denoted as AZAX, was previously identified as a possible isomer of AZA2 (or a methylated AZA1 analogue), based on retention time and mass [20,21]. The present work demonstrated the artefactual formation of methylated derivatives of AZA1 and AZA2 during extraction of *A. spinosum* cultures, and procedures that reduce their formation. However, the mechanism of formation and identities of these analogues were unclear.

Enzymatic activity can modify the chemical structure of toxins when extracting phytoplankton [38]. To test whether this might be the case for AZAs, the filters containing *A. spinosum* were heated in a water bath or microwave before extraction. Neither procedure reduced the formation of methylated analogues of AZA1 nor -2, indicating that enzymatic activity of the alga is not responsible for the formation of the methylated analogues.

Brondz et al. [39] reported that extractions of natural products with MeOH may produce methyl esters of fatty acids or other molecules with a carboxylic acid groups. Methyl esters of AZAs (now known to be methyl ketals, see below) have also been reported as storage artefacts [7], and the proportion of these was reduced when acetone, MeCN or DCM were used (Table 2 and 3).

To confirm that the two methylated analogues (subsequently identified as AZA1 and AZA2 methyl esters) were artefacts of the extraction/reconstitution process, and to determine at which stage formation occurred, extraction and reconstitution were carried out using MeOH or CD_3OD. The formation of methylated analogues took place mainly during extraction but also, to a lesser degree, during reconstitution (Fig. 3). Therefore, these two analogues were now clearly identified as artefacts from extraction with MeOH, and were suspected to be AZA1 and -2 methyl esters. Methyl esters of AZA1–3 and 6, denoted as AZA29–32, were reported by Rehmann et al. and were identified as artefacts of storage in MeOH [7]. Specimens containing AZA30 and -32 as artefacts of long term storage in MeOH from NRCC were analysed by LC-MS/MS (Fig. 4, 5 and 6). The retention times and mass spectra (API-4000, linear ion trap and Agilent 6540 QTOF) of AZA30, -32 were different to those of the two methylated AZA-extraction artefacts observed in the present study (Fig. 4 and 6). High resolution mass spectrometry was consistent with the chemical formulae of the molecular structures and fragments (Table 4 and Electronic Supplementary Material). The observation that the methylated extraction artefacts from the present study had identical retention times

and mass spectra to semisynthetic AZA1 and AZA2 methyl esters (produced by treatment with diazomethane) unambiguously identified these artefacts as the methyl esters (Fig. 4 and 5).

Thus, AZA30 and -32 are methylated derivatives of AZA1 and -2, respectively, but are not methyl esters. Rehmann et al. [7] reported an initial loss of 32 amu in the mass spectra of AZA30 and -32, suggesting the loss of MeOH. However, we observed only initial loss of 18 amu (H_2O) in the mass spectra of authentic AZA1 and -2 methyl esters (Table 4 and Electronic Supplementary Material). A plausible hypothesis is that AZA30 and -32 are 21-methyl ketals formed by exchange at the 21-OH hemi ketal of AZA1 and AZA2, respectively, with MeOH. This proposal is consistent with the observed initial losses of H_2O from AZA1 and -2 and their methyl esters, and of CH_3OH from the corresponding methyl ketals (Fig. 4 and 7). To test this hypothesis, samples containing AZA1 and -2, their methyl esters, and AZA30 and -32 were treated with periodate, which oxidatively cleaves the 20,21-diol in AZAs to form a lactone derivative (Fig. 7) under mild conditions [7,40]. LC-MS/MS analysis showed complete conversion of AZA1 and -2, and of their methyl esters, to the lactone, whereas no detectable reaction occurred with AZA30 and -32, indicating that the latter compounds have been modified in the 20,21-diol moiety (Fig. 7).

LC-MS analysis in negative ion SIM mode [5] established the presence of a free carboxyl in AZA30 and -32, and the absence of a free carboxyl in AZA1 and -2 methyl esters. Only AZAs with free carboxylic acid groups would be negatively ionised in electrospray MS, and it was found that no signal was obtained for AZA1 and -2 methyl esters, while AZA1, -2, -30 and -32 were detected (Electronic Supplementary Material). Additionally, derivatization with ADAM produced (9-anthryl)methyl ester derivatives of AZA1, -2, -30 and -32 (yields >98%), whereas the methyl esters of AZA1 and AZA2 were unaffected (Fig. 1 and Electronic Supplementary Material). These results strongly support the hypothesis that AZA30 and -32

are the methyl ketals of AZA1 and -2, respectively, and are not methyl esters as originally reported by Rehmann et al. [7], and it seems likely that this is also the case for AZA29 and -31 (reported as methyl esters of AZA3 and -6 by Rehmann et al. [7]). The methylation artefacts from extraction of *A. spinosum* cultures are unambiguously identified as AZA1 and AZA2 methyl esters, and the “AZAX” observed in methanolic extracts by Krock et al. [22] appears to be confirmed as AZA1 methyl ester (“AZAX” mass spectrum observed by Krock et al. is presented in the Electronic Supplementary Material).

Evaluation of extraction protocols of azaspiracid from *A. spinosum*

Particulate and dissolved toxins

After the aliquots were sampled from the bioreactor, intra- and extra-cellular toxin contents were determined in triplicate immediately or after resting periods, with different solvents, with an aliquot of culture (10 mL) taken to assess morphological changes for each of the resting periods.

In laboratory culture, AZAs produced by *A. spinosum* were clearly intra-cellular. When cells were gently separated from the culture medium either by filtration or centrifugation (no cells were detected under the inverted microscope in the filtrate or supernatant), the majority of toxins were found in the particulate fraction (95%). A significant loss of intra-cellular AZAs, with a concomitant increase in extra-cellular AZAs, was observed with increasing time between sampling and centrifugation. Apparently, increased residence time of cells in medium outside the bioreactor led to handling stress of the cells, which in turn resulted in a substantial loss of cell bound toxins to the dissolved phase (Electronic Supplementary Material). A significant loss of intra-cellular toxins (> 10 %) occurred when cells were stressed for more than 60 min and a maximum proportion of extra-cellular toxins of 24% was

observed after 5 h. Therefore, it is recommended that samples are centrifuged immediately when the cell quota of AZAs is being determined.

Microscopical observation showed that, under these stress conditions, an increasing number of cells without theca occurred, although cells generally kept their integrity (Electronic Supplementary Material). It is a common reaction among dinoflagellates to adverse conditions that cells leave their theca (*ecdysis*), and is often connected with the formation of temporary cysts [41]. This type of dinoflagellate cyst normally is round and surrounded by a cell wall. However, this has not yet been observed for *A. spinosum*. The reason for the increase in extra-cellular toxins is not clear; shedding of the cells outer layer including thecal plates and their membrane vesicles might be associated with a pulsed toxin loss or extruded protoplasts may have a higher exudation rate. However, the possibility that total disintegration of a small portion of the cells contributed to the increase of extra-cellular toxins cannot be excluded.

Effect of centrifugation and filtration on toxin recovery and profile

An initial experiment was carried out in triplicate with MeOH as extraction solvent to evaluate AZA yield after filtration of 10 mL of *A. spinosum* culture on GF/C glass microfiber filters (25 mm diameter), or after centrifugation (2500 *g*, 4 °C, 20 min). Both filtrate and supernatant were kept for liquid–liquid extraction with DCM (Fig. 2). Five successive extractions were carried out on each filter or pellet, to ensure complete toxin recovery. A second trial was carried out under the same conditions using either MeOH or acetone as extraction solvent using three successive extractions.

The total amount of AZAs obtained after 5 successive extractions using MeOH was not significantly different when using either filtration or centrifugation as a method to separate cells from culture medium. If only three extractions with either MeOH or acetone were carried out, the yield was significantly lower ($P<0.05$) when using filtration. Moreover, the

AZA ratios were different for filtration compared to centrifugation (Table 2). High levels of AZA1 and -2 methyl esters were obtained with filtration. Glass microfiber filters contain silica and are known to catalyse some reactions, which may possibly explain the methylation of AZA1 and -2 observed in these experiments. If filtration is necessary, it is recommended to study other types of filters with *A. spinosum* (*i.e.* polycarbonate filters).

Influence of extraction solvent composition

To determine the procedure with the best extraction yield and minimal formation of artefacts (methylated AZAs), the extraction procedure described in Fig. 2 was applied using a variety of solvents and solvent compositions. Four experiments were carried out: (a) 100% MeOH, acetone, MeCN, EtOH, or DCM, (b) MeOH–H_2O, 10:0, 9:1, 8:2, 7:3, 6:4 (v/v), (c) Acetone–H_2O, 10:0, 9:1, 8:2, 7:3 (v/v), (d) MeCN–H_2O, Acetone-H_2O, MeOH-H_2O, (10:0, 9:1 (v/v) each).

Between MeOH, acetone, MeCN, EtOH and DCM, no significant differences were observed on AZA1, AZA2 and total AZA contents. Nonetheless, significant differences were observed in the content of AZA1 methyl ester (Table 3), with increased formation of this derivative when extracting with MeOH or EtOH (in the following order MeOH = EtOH > acetone > MeCN > DCM). No ethyl analogue was observed when extracting with EtOH. It is not entirely clear how this methyl analogue formation occurs.

The formation of the methyl esters of AZA1 and -2 was variable from one experiment to the next and concentrations of AZA1 methyl ester may range from 3 to 15% (Table 2, 3, and data not shown) when using MeOH as extraction solvent and centrifugation as separation technique.

As expected, the formation of AZA1 methyl ester is significantly reduced when extracting with acetone, MeCN and DCM, however, detectable traces are still formed with these solvents used in extraction. This observation led us to hypothesize that reconstitution in

MeOH by itself may lead to formation of AZA methyl esters. This hypothesis has been confirmed using deuterated MeOH (see previous section).

It is common to add some water to an organic solvent to increase the extraction yield or to minimise the extraction of lipids which could lead to matrix effects [29]. However, in the present study, no statistical differences were observed between 100, 90 and 80% MeOH or acetone. Nonetheless, below a ratio of 7:3 organic solvent–H_2O, the yield decreased and was significantly lower than with MeOH.

Extraction with acetone was considered to be most appropriate, as it reduces the formation of AZA methyl esters and is easier to handle thanks to its ease of evaporation and low toxicity. However, acetone extracts may also result in more complex crude extracts, as acetone is a good solvent for extracting lipids and pigments [42]. This was an additional reason to evaluate matrix effects in further trials.

Effect of sample size

The effect of sample size on the extraction yield was studied following the standard extraction procedure (Fig. 2) using acetone–H_2O (9:1 v/v) as extraction solvent. The *A. spinosum* culture used for this experiment had a cell concentration of 161000 ± 1000 cells.mL^{-1}, corresponding to a biovolume of 92.6×10^6 $\mu m^3.mL^{-1}$. The following sample volumes were used: 0.62, 3.1, 6.2, 10, 31, 50 mL.

The extraction yield differed significantly as a function of sample size. Yields were somewhat higher in the middle of the studied range (3.1–31 mL). More extraction cycles or higher solvent-to-sample ratios could potentially be used to increase AZA yield for the large sample size (50 mL). However, the procedure was not suitable for small amounts of biomass (<1–2 mL), potentially reflecting that small losses become significant when handling low amounts of toxin. Alternative protocols may need to be developed for samples below 200000 cells, such as the procedure described during the identification of *A. spinosum* [20].

Recovery and yield after successive extractions in presence or absence of matrix

Extraction yield and recovery were tested following the standard procedure with one to five successive extractions with either MeOH–H_2O or acetone–H_2O (9:1 v/v), with or without matrix as follows:

(a) without matrix: AZA1 (40 µL) solution (0.53 µg.mL^{-1}) was transferred into 15 mL centrifuge tubes, and extracted with 500 µL of organic solvent water mixtures. The extract was reconstituted in 500 µL MeOH–H_2O (9:1 v/v). A control in triplicate with 40 µL of AZA1 solution and 460 µL of MeOH–H_2O (9/1 v/v) was used to estimate the recovery.

(b) with algal matrix: Aliquots (10 mL) of *A. obesum* culture (50000 ± 2000 cells.mL^{-1}, 65.2×10^6 μm^3.mL^{-1}) were centrifuged in 15 mL centrifuge tubes. After decanting the supernatant, AZA1 (40 µL) solution was added to the pellet, extracted as described above. The extract was reconstituted in 500 µL MeOH–H_2O (9:1 v/v). For controls, *A. obesum* culture (10 mL) was extracted using MeOH–H_2O or acetone–H_2O (9:1 v/v) in triplicate (Fig. 2). The control extracts were evaporated to dryness and subsequently taken up with 460 µL MeOH–H_2O (9:1 v/v) and 40 µL of AZA1 solution.

Two successive extractions were sufficient in all cases (100% of recovery at 95% confidence level) while three consecutive extractions reduced the deviations observed for triplicate samples. The presence of matrix did influence recovery. Without matrix, one extraction was almost sufficient to recover all AZA, whereas two successive extractions were necessary when *A. obesum* matrix was present. No significant differences were observed between acetone and MeOH on AZA recovery in the presence, or absence, of matrix when two or three successive extraction cycles were carried out.

Evaluation of matrix effects on LC-MS/MS analysis

Matrix effects were assessed for AZA1 using MeOH and acetone extracts of *A. obesum* (Fig. 8a and b, respectively), applying the standard addition method as described in Fux et al.

[31]. Negligible effect (+1.4%) was observed when using MeOH, and more significant signal suppression (−8.4%) was observed with acetone. For *A. spinosum*, suppression effects of −7.7% and −6.4% were measured for MeOH and acetone extracts, respectively (Fig. 8c and d).

Following the approach used by Fux et al. (28) matrix effects were also assessed by varying matrix strength while maintaining a constant AZA1 concentration (Fig. 8e). No significant matrix effects were detected with different amounts of biomass, as all values measured were within the precision of the experiment.

Previously, matrix effects were considered significant at values greater than 10% enhancement or suppression [31,43], due to the repeatability of an analytical method with LC-MS/MS. Following this arbitrary limit of significance, the effects observed here for *A. spinosum* analysis can be considered insignificant. It should be stressed that the analysis of AZAs from *A. spinosum* at this scale typically deals with much less matrix content compared to shellfish. From large-scale extraction experiments (data not shown), it is estimated that the strongest matrix crude extract using MeOH in our study contained ca. 2 $mg.mL^{-1}$, which is at the lowest value of the range evaluated for a shellfish matrix by Fux et al., [31].

Conclusion

These results highlight the importance of carefully studying sample preparation, extraction procedures and solvent choice for assessing the recovery of the method and possible matrix effects.

Based on the results of this study, the following procedures are recommended for the analysis of AZA-1 and -2 in *A. spinosum*:

- Sample and immediately separate the cells from the culture medium by centrifugation.
- Extract AZAs with acetone or MeCN; acetone is most appropriate as it reduces both the formation of methyl analogues and is easy to handle thanks to its ease of

evaporation and low toxicity. MeOH is inappropriate due to possible artefact formation.

- Two to three successive extractions are suggested to ensure high extraction yield.
- No significant matrix effects were observed during LC-MS/MS analysis with acetone or MeOH under the conditions tested.

This work clarifies the formation of AZA artefacts during extraction of *A. spinosum*, describes mass spectral fragmentation of two AZA methyl esters, and corrects the chemical structures of AZA29–32. Furthermore, the procedure developed allows quantitation of AZAs in algal cultures and thus will facilitate the optimisation of processes aimed at the preparative isolation of AZAs required for the sustainable supply of AZAs for instrument calibration.

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Tables

Table 1 Operating conditions for the two mass spectrometers (AU = arbitrary units)

Operating conditions	API 2000	API 4000 Q-trap
Curtain gas	19 AU	30 AU
Temperature	350°C	450°C
Gas 1	30 AU	50 AU
Gas 2	50 AU	50 AU
CAD gas	5 AU	Medium
Ion spray voltage	5800 V	5500 V
Declustering potential	140 V	116 V
Entrance potential	10 V	10 V
Collision cell	50 & 70 V	41 & 69 V
Cell exit potential	35 V	12 & 16 V

Table 2 Percentage of AZA analogue as a function of the harvesting (filtration or centrifugation) and extraction (MeOH or acetone) procedure for 3 consecutive extraction cycles

	AZA1 (%)	AZA2 (%)	AZA1 methyl ester (%)	AZA2 methyl ester (%)
Filtration & MeOH	49	18	31	3
Centrifugation & MeOH	71	26	3	0
Filtration & Acetone	55	20	23	2
Centrifugation & Acetone	73	25	2	0

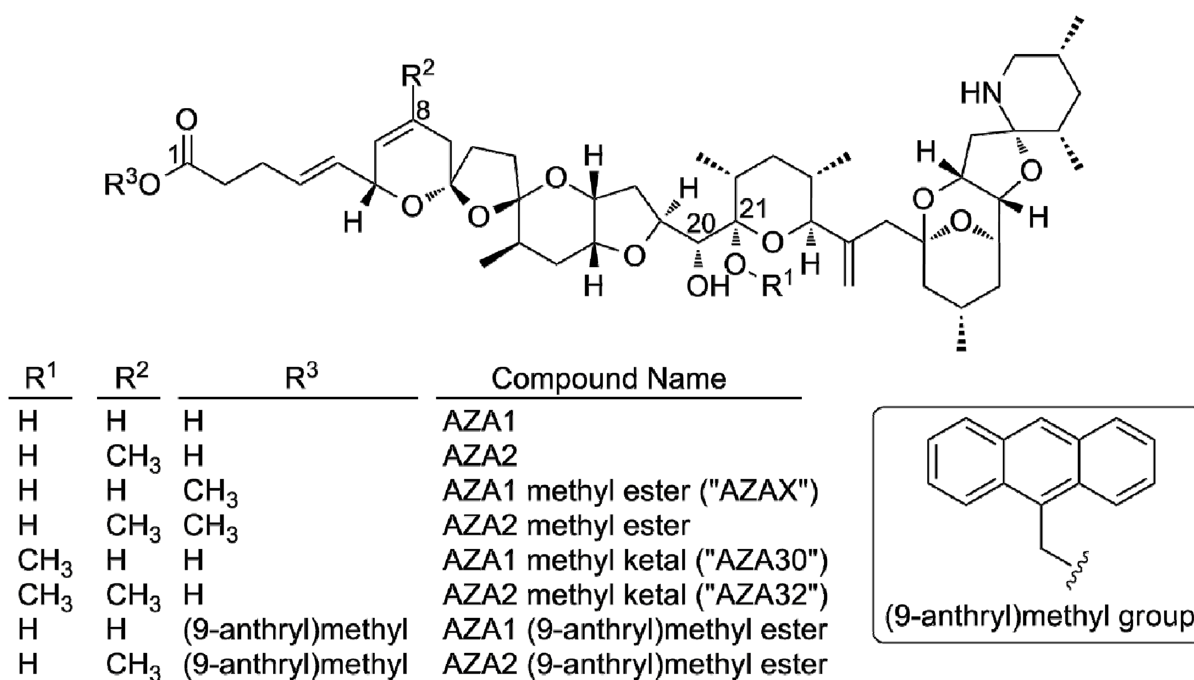
Table 3 Yield of AZA ($fg.cell^{-1}$) with extraction solvent from *A. spinosum* pellets after centrifugation. Values with different letters are statistically different at $P<0.05$

Solvent	AZA1	AZA2	AZA1 methyl ester	Total
MeOH	85±8	25±2	3.7±0.3 (d)	114±10
Acetone	87±5	24±1	2.4±0.3 (c)	113±6
MeCN	95±5	26±1	0.8±0.1 (b)	122±7
EtOH	94±10	26±2	3.7±0.2 (d)	124±11
DCM	82±2	22±1	0.3±0.1 (a)	104±3

Table 4 High resolution LC-MS and LC-MS/MS data (measured *m/z* and Δ (ppm)) for AZA1, its methyl ester (extraction artefact from *A. spinosum*, and semi-synthetic), and AZA30 (AZA1 methyl ketal). Fragment ions correspond to Fig. 1

Ion		AZA1	AZA1 methyl ester (artefact)	AZA1 methyl ester (semi-synthetic)	AZA30
$[MH]^+$	Formula	$C_{47}H_{72}NO_{12}^+$	$C_{48}H_{74}NO_{12}^+$	$C_{48}H_{74}NO_{12}^+$	$C_{48}H_{74}NO_{12}^+$
	m/z (Δ)	842.5049 (0.0)	856.5209 (0.5)	856.5210 (0.6)	856.5239 (4.0)
$[MH-ROH]^+$ (Fragment 1)	Formula	$C_{47}H_{70}NO_{11}^+$	$C_{48}H_{72}NO_{11}^+$	$C_{48}H_{72}NO_{11}^+$	$C_{47}H_{70}NO_{11}^+$
	m/z (Δ)	824.4946 (0.4)	838.5105 (0.6)	838.5104 (0.5)	824.4929 (1.7)
Fragment 2 (RDA 1)	Formula	$C_{38}H_{58}NO_9^+$	$C_{38}H_{58}NO_9^+$	$C_{38}H_{58}NO_9^+$	$C_{38}H_{58}NO_9^+$
	m/z (Δ)	672.4111 (0.7)	672.4101 (0.7)	672.4112 (0.9)	672.4105 (0.2)
Fragment 3	Formula	$C_{31}H_{48}NO_6^+$	$C_{31}H_{48}NO_6^+$	$C_{31}H_{48}NO_6^+$	$C_{31}H_{48}NO_6^+$
	m/z (Δ)	530.3476 (0.0)	530.3470 (1.1)	530.3466 (1.9)	530.3461 (2.8)
Fragment 4	Formula	$C_{27}H_{44}NO_5^+$	$C_{27}H_{44}NO_5^+$	$C_{27}H_{44}NO_5^+$	$C_{27}H_{44}NO_5^+$
	m/z (Δ)	462.3213 (0.2)	462.3206 (1.7)	462.3218 (0.9)	462.3206 (1.7)
Fragment 5 (RDA 2)	Formula	$C_{22}H_{36}NO_3^+$	$C_{22}H_{36}NO_3^+$	$C_{22}H_{36}NO_3^+$	$C_{22}H_{36}NO_3^+$
	m/z (Δ)	362.2688 (0.6)	362.2691 (0.3)	362.2690 (0.0)	362.2682 (2.2)

Figures



R^1	R^2	R^3	Compound Name
H	H	H	AZA1
H	CH_3	H	AZA2
H	H	CH_3	AZA1 methyl ester ("AZAX")
H	CH_3	CH_3	AZA2 methyl ester
CH_3	H	H	AZA1 methyl ketal ("AZA30")
CH_3	CH_3	H	AZA2 methyl ketal ("AZA32")
H	H	(9-anthryl)methyl	AZA1 (9-anthryl)methyl ester
H	CH_3	(9-anthryl)methyl	AZA2 (9-anthryl)methyl ester

Fig. 1 Structures of AZA1, -2 and their methyl and (9-anthryl)methyl derivatives

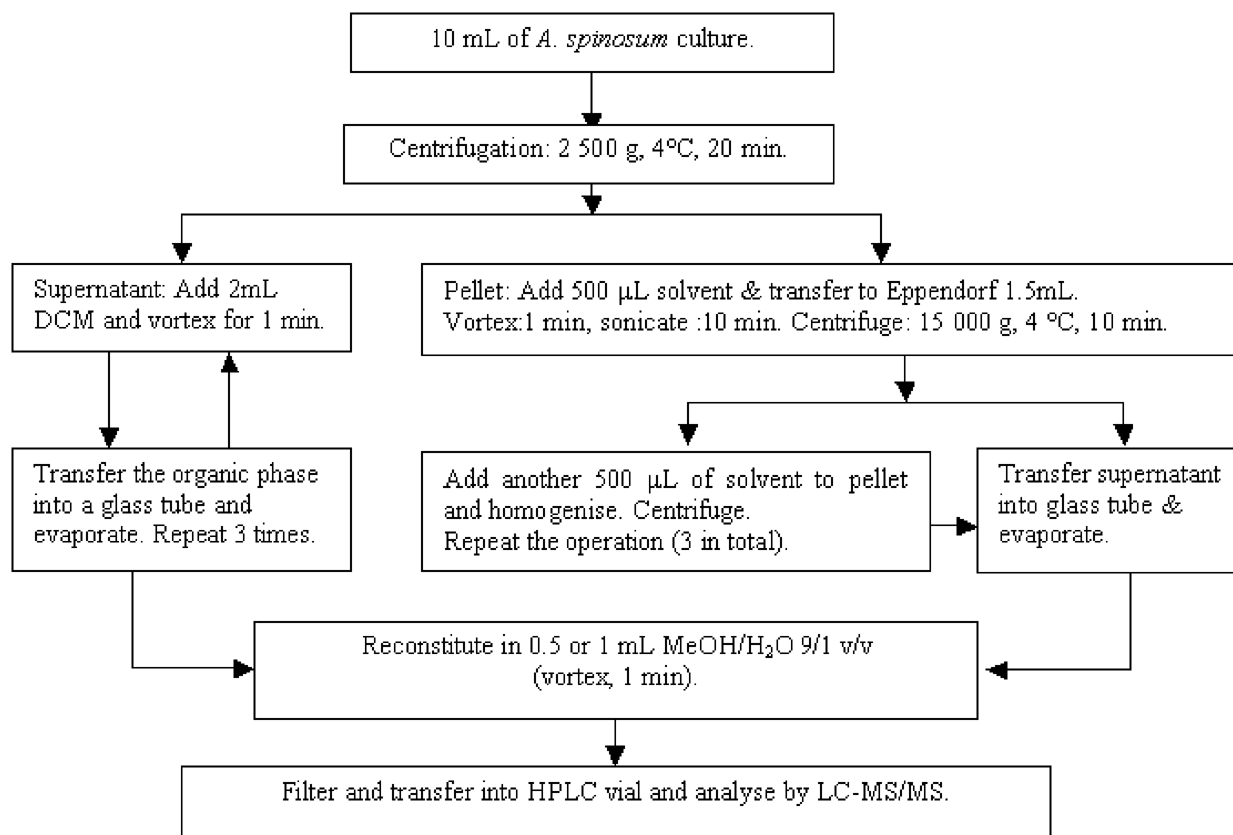


Fig. 2 Sample preparation scheme for extraction of AZAs from *A. spinosum*

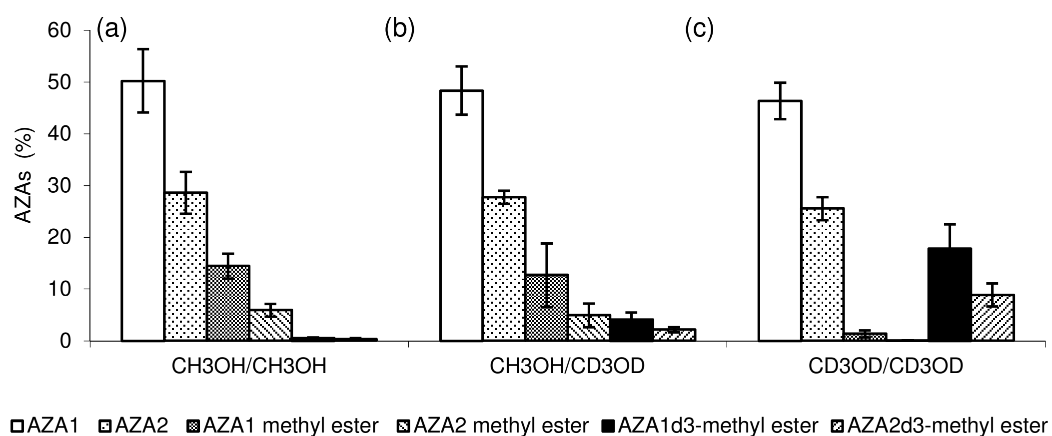


Fig. 3 Percentages of AZA analogues from *A. spinosum* using centrifugation after: (a) extraction and reconstitution with MeOH; (b) extraction with MeOH and reconstitution with CD_3OD, and; (c) after extraction and reconstitution with CD_3OD

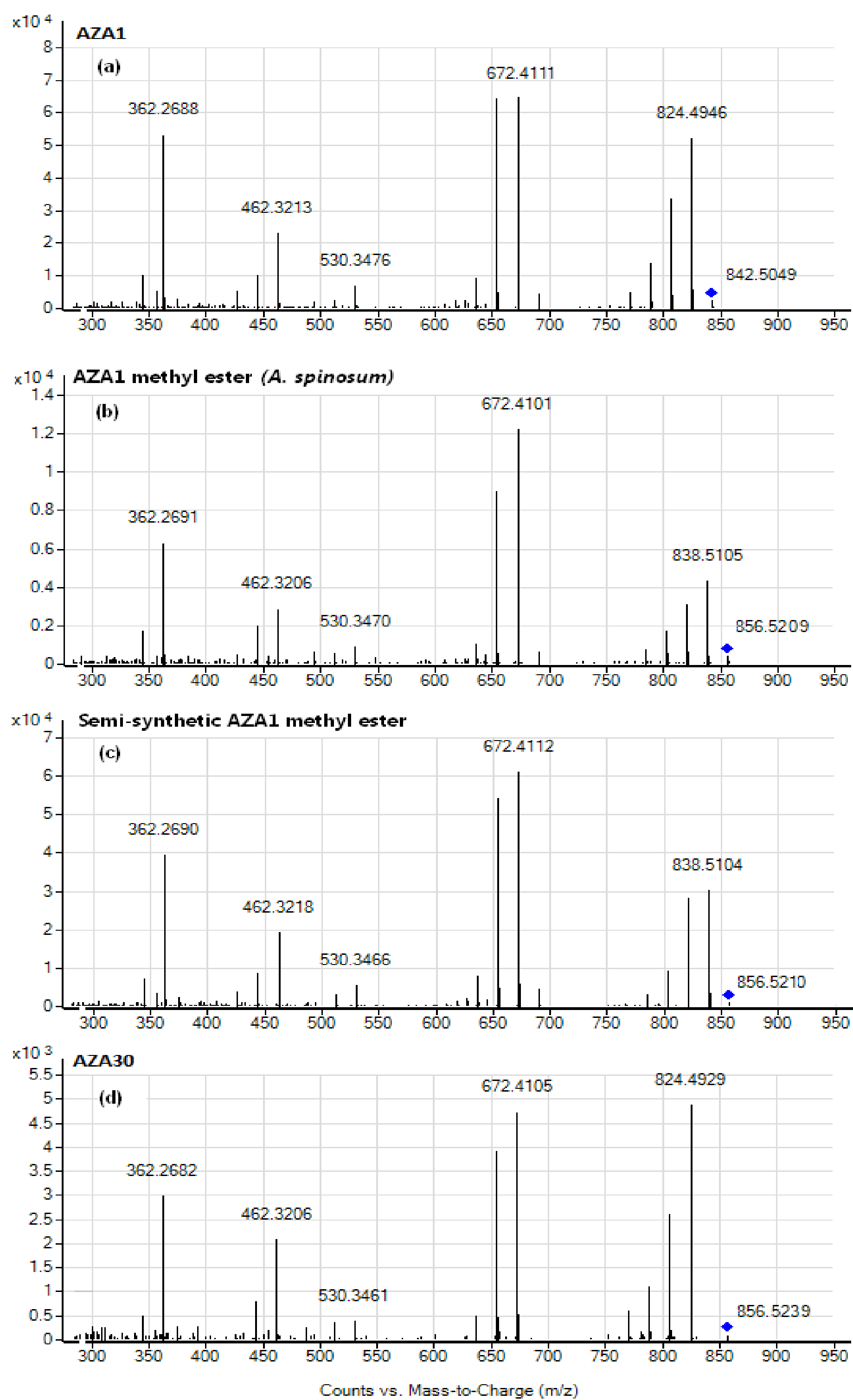


Fig. 4 High resolution mass spectra of: (a) AZA1; (b) AZA1 methyl ester of AZA1 obtained through extraction from *A. spinosum*; (c) semi-synthetic AZA1 methyl ester, and; (d) AZA30 (AZA1 methyl ketal) obtained as an artefact of storage of AZA1 in MeOH

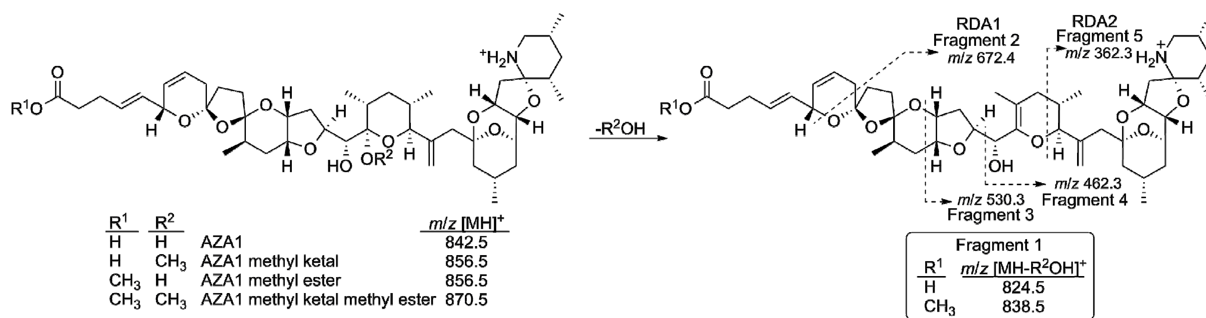


Fig. 5 Structure and *m/z* for $[M + H]^+$ ions of AZA1, AZA1 methyl ketal, AZA1 methyl ester, AZA1 bismethyl ketal ester and MS/MS fragmentation with indicated groups

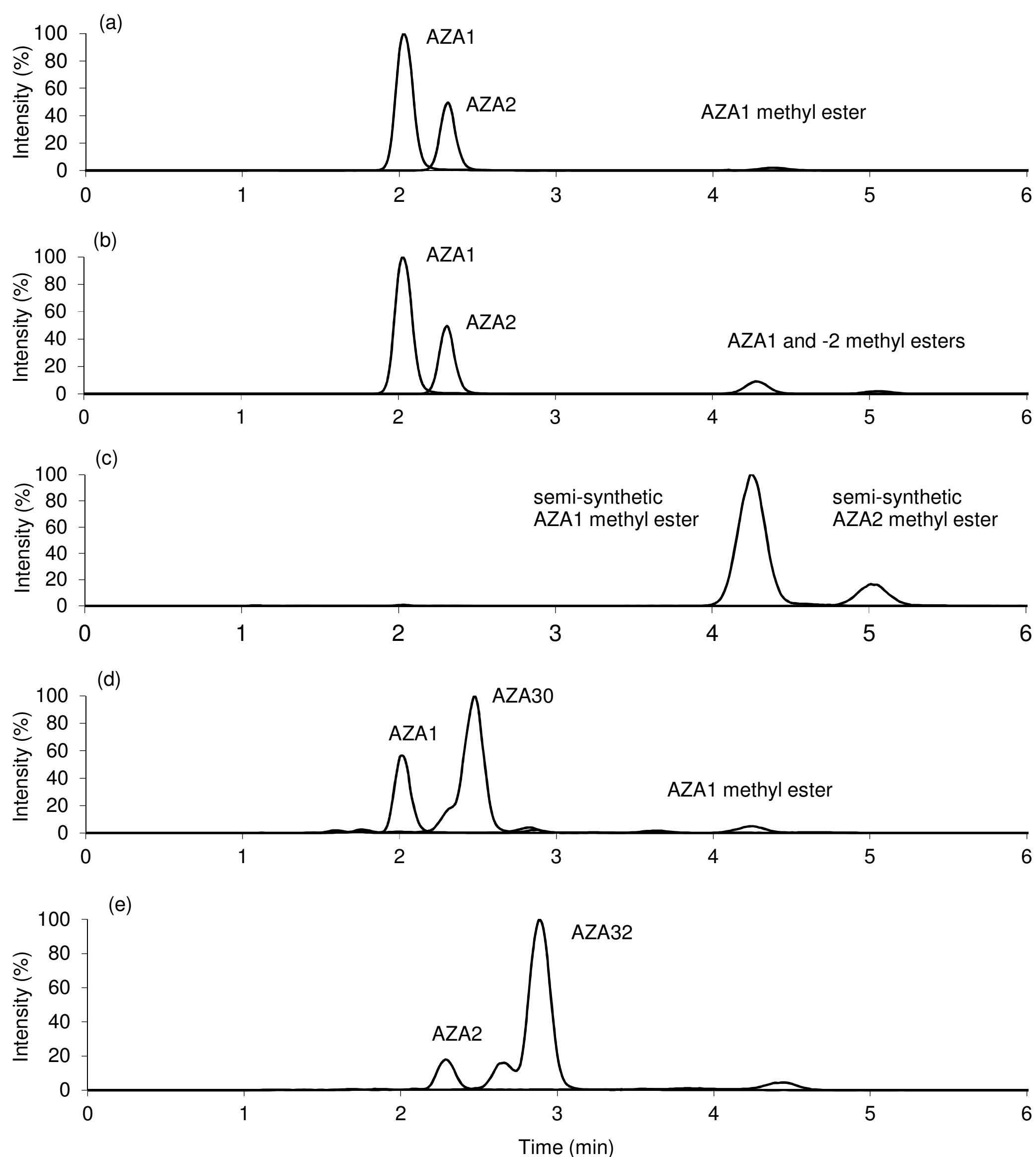


Fig. 6 LC-MS chromatograms of AZAs obtained with an isocratic elution: (a) *A. spinosum* after extraction with acetone; (b) *A. spinosum* after extraction with MeOH; (c) semi-synthetic AZA1 and AZA2 methyl esters produced with diazomethane; (d) AZA1 standard after long-term storage in MeOH, and; (e) AZA2 standard after long-term storage in MeOH

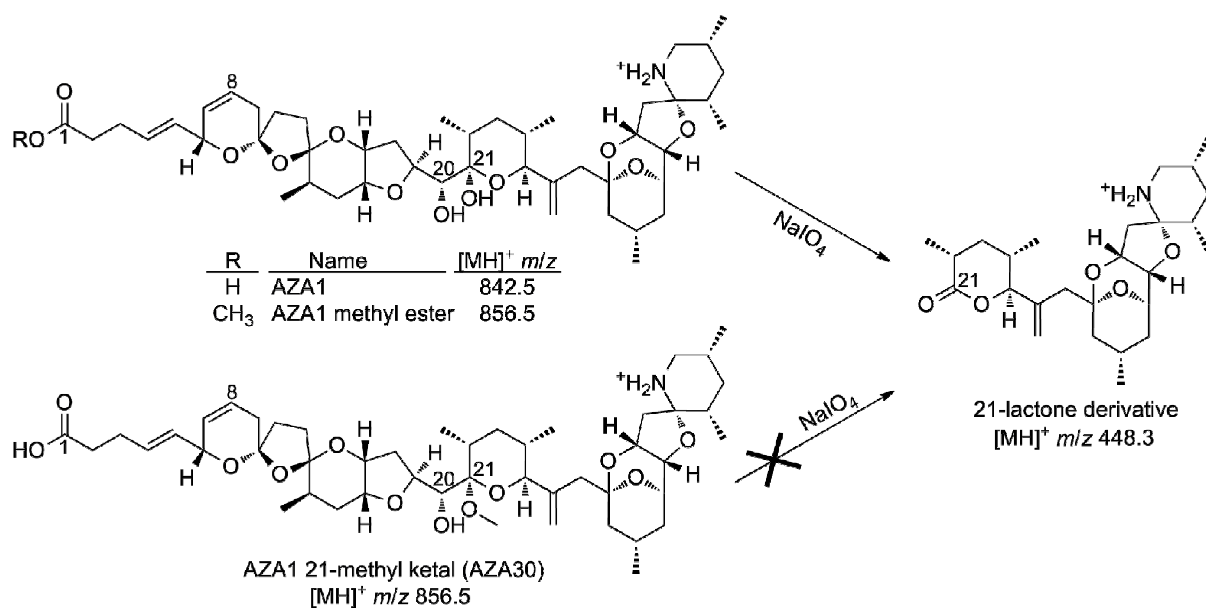


Fig. 7 Fate of AZA1 and its methylated derivatives when treated with sodium periodate, which oxidatively cleaves the 20,21-diol group present in natural AZAs.

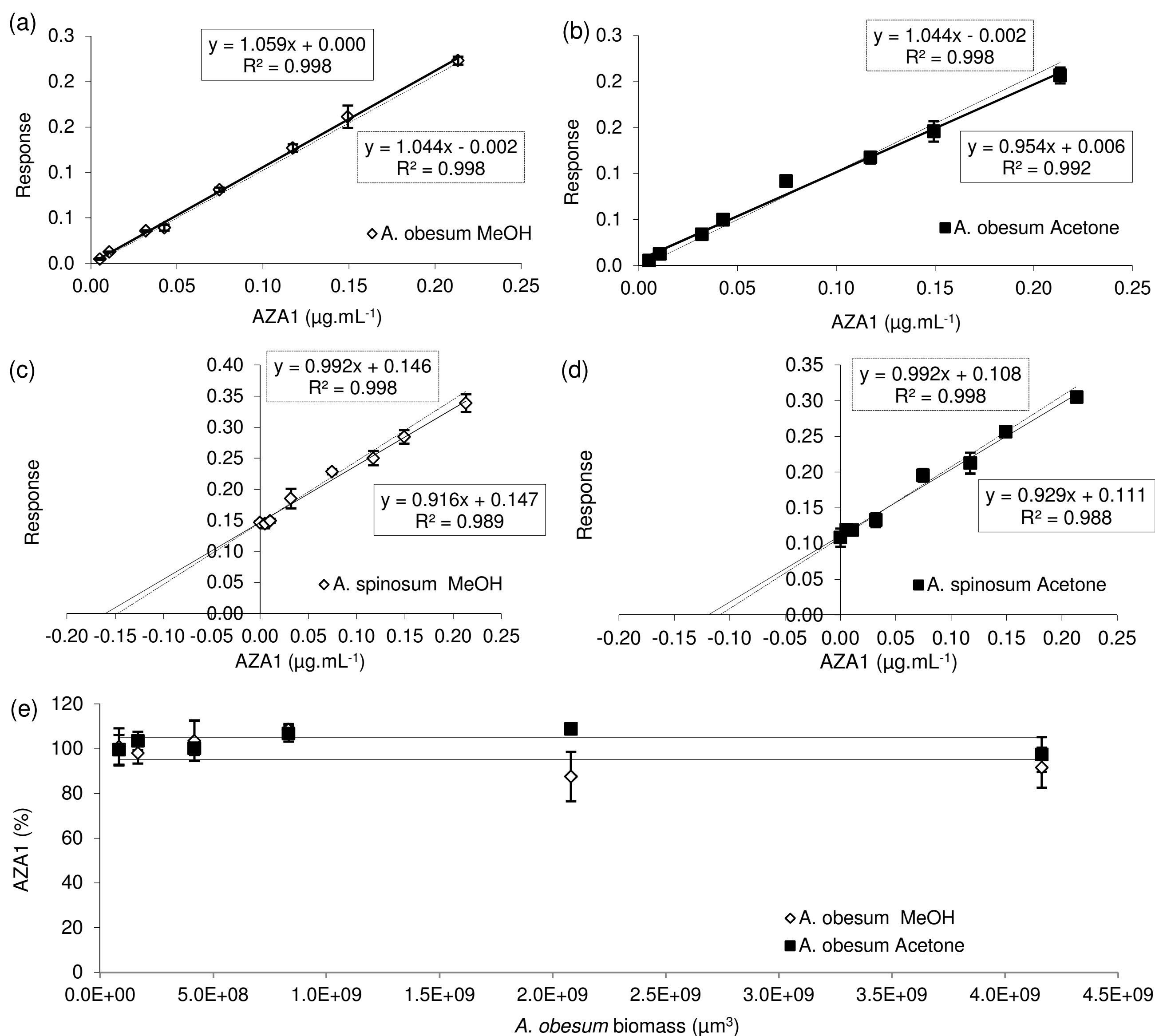


Fig. 8 Slopes, intercepts, correlation coefficients for quantitation of AZA1 in methanolic or acetone extracts of *A. obesum* and *A. spinosum* spiked with standards (a, b, c, d) and as a function of the sample size (e) using LC-MS/MS with isocratic elution. Lines represent the least-squares correlation for quadruplicate injections of spiked matrix-free solutions (a, b, c, d) (dashed lines) and triplicate injection of spiked solutions with matrix (a, b, c, d) (solid lines). The solid lines in (e) represent the 95% confidence interval obtained from triplicate injection of spiked matrix-free MeOH solutions. (notice initial response difference between (c) and (d) was due to different sampling day)