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A mussel (Mytilus edulis) tissue certified reference material for the marine biotoxins azaspiracids

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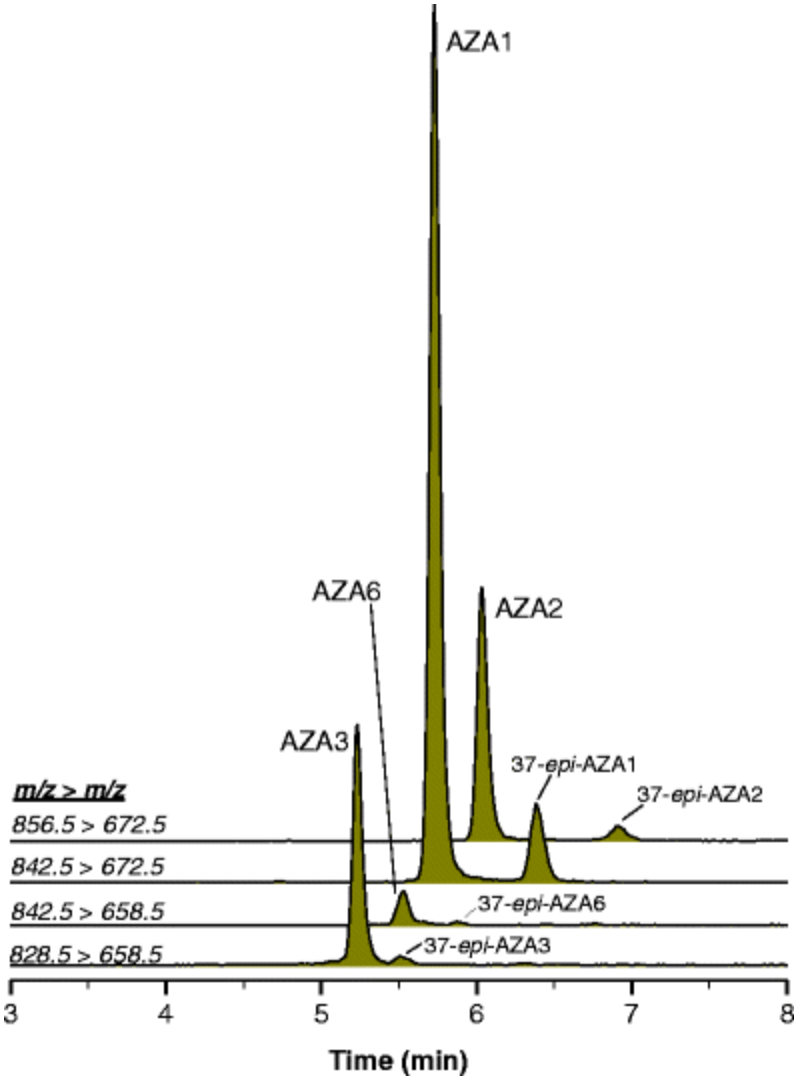
Abstract :

Azaspiracids (AZAs) are lipophilic biotoxins produced by marine algae that can contaminate shellfish and cause human illness. The European Union (EU) regulates the level of AZAs in shellfish destined for the commercial market, with liquid chromatography-mass spectrometry (LC-MS) being used as the official reference method for regulatory analysis. Certified reference materials (CRMs) are essential tools for the development, validation, and quality control of LC-MS methods. This paper describes the work that went into the planning, preparation, characterization, and certification of CRM-AZA-Mus, a tissue matrix CRM, which was prepared as a wet homogenate from mussels (Mytilus edulis) naturally contaminated with AZAs. The homogeneity and stability of CRM-AZA-Mus were evaluated, and the CRM was found to be fit for purpose. Extraction and LC-MS/MS methods were developed to accurately certify the concentrations of AZA1 (1.16 mg/kg), AZA2 (0.27 mg/kg), and AZA3 (0.21 mg/kg) in the CRM. Quantitation methods based on standard addition and matrix-matched calibration were used to compensate for the matrix effects in LC-MS/MS. Other toxins present in this CRM at lower levels were also measured with information values reported for okadaic acid, dinophysistoxin-2, yessotoxin, and several spirolides.

Graphical abstract



Keywords : Azaspiracids, Seafood safety, Matrix certified reference material, Liquid chromatography-mass spectrometry, Matrix effects

1 Introduction

In 1995, several people developed a gastrointestinal illness following consumption of mussels (*Mytilus edulis*) harvested off the coast of Ireland [1]. The illness was eventually attributed to azaspiracids (AZAs), a very potent class of previously unreported marine biotoxins [2,3]. The structure of AZA1 was reported in 1998 [2] and then revised in 2004 [4]. Subsequently, much work has been completed on the identification and structural elucidation of a host of AZA analogues and isomers [4-9]. The structures of AZA1-10 are shown in Fig. 1. AZAs have been found in many locations including Western Europe [10-13], North West Africa [14], North America [15] and in Japan [16]. AZAs are produced by *Azadinium spinosum*, a small dinoflagellate first isolated from the North Sea [17,18], which was later found in Western Europe [19]. The major AZA structural variants are produced either directly by *Azadinium spinosum*, through metabolism in shellfish, or by conversion during processing of shellfish [19-21].

The regulated AZAs are resistant to cooking [22], making it essential that shellfish are strictly monitored to ensure the safety of consumers and the production industry. Only AZA1-3 have been found to be of toxicological importance [2,8] and the regulatory limit of 160 mg/kg (expressed as AZA1 toxic equivalents) has been set by the European Union [23]. A report by the European Food Safety Authority suggested that these limits should be reduced [24].

The traditional mouse bioassay does not provide the required sensitivity, selectivity or accuracy to adequately implement the regulatory limit for AZAs [25]. An amendment to the EU regulation regarding testing methods [26] has designated liquid chromatography-mass spectrometry (LC-MS) as the official reference method for determination of AZAs in shellfish. A number of alternative methods for AZA analysis have also been reported [27,28].

Reference materials (RMs) are important for the development and quality control of analytical methods such as LC-MS. Certified reference material (CRMs) are essential for validation and assessment of analytical determinations and for establishing accuracy and traceability. Considerable efforts have been made in the production of AZA calibration standards [29,30] and the availability of pure standard CRMs for AZA1-3 for instrument calibration for several years has facilitated the implementation of LC-MS methods for regulatory monitoring programs. Up until this point, however, there has not been a matrix CRM available for AZAs. Indeed, the availability of matrix CRMs for all classes of shellfish toxins has been somewhat limited with the National Research Council providing mussel tissue CRMs for domoic acid and okadaic acid. More recently an oyster tissue CRM for paralytic shellfish toxins has been produced by CEFAS [31]. Matrix CRMs are designed to assess the performance of entire analytical methods, including: testing of extraction methods; assessment of matrix effects associated with a particular method; and evaluation of the accuracy of the final measurement or determination step. Ideally, matrix CRMs for seafood toxins should be prepared from naturally contaminated tissues.

This paper describes the planning, preparation and certification of a mussel (*Mytilus edulis*) matrix CRM for AZAs. The homogeneity and stability of the candidate CRM was assessed. Extraction, calibration and LC-MS analysis methods were developed to ensure the highest degree of accuracy when assigning values to the CRM, and uncertainties were assigned following standardized ISO procedures. Finally, CRM-AZA-Mus was screened for the presence of other common lipophilic toxins.

2 Material and Methods

2.1 Chemicals and reagents

HLPC grade acetonitrile, glass-distilled methanol (MeOH), hydrochloric acid (HCl) and sodium hydroxide (97%) (NaOH) were obtained from Caledon (Georgetown, ON, Canada). Formic acid (>98% ACS grade) was obtained from EMD (Gibbstown, NJ, USA). Ammonium formate (99%), ammonium acetate (99%), pepsin (porcine gastric mucosa, ≥2,500 units/mg protein), ethoxyquin, oxytetracylcine, erythromycin and ampicillin were obtained from Sigma-Aldrich (Oakville, ON, Canada). Bakerbond C8-bonded silica (40 μm) was obtained from Mallinckrodt Baker Inc. (Philipsburg, N.J., USA). All water used was purified with a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA) (18.2 MΩ cm at 25 °C; <1 ppb TOC).

Certified calibration solutions of AZA1-3, okadaic acid (OA), dinophysistoxin-1 (DTX1), DTX2, 13-desmethylspirolide-C (13-desMe-SPX-C), pectenotoxin-2 (PTX2), and yessotoxin (YTX) were provided by the National Research Council of Canada (NRCC, Halifax, NS, Canada). A blank mussel tissue CRM (CRM-Zero-Mus) was also provided by NRCC.

2.2 Heat treatment studies

Uncooked mussels harvested in Bantry Bay (2001, Southwest Ireland) were removed from freezer storage (-20°C) and defrosted before shucking. Whole flesh was removed and homogenized using a Waring blender. Aliquots (2 g) were transferred to 5 ml glass reaction tubes (Wheaton, Millville, NJ, USA). The tubes were sealed tightly with PVDC lined screw caps and aliquots (n=3) were heated for 10 min in an oil bath at temperatures ranging from 50 °C to 150 °C in 10 °C increments.

To determine if AZA decarboxylation was complete in the stock mussel tissues a separate trial was conducted in which a sample of the steam cooked Bruckless mussel tissue (Donegal, Ireland, 2005) was allowed to thaw and then placed in an oven at 95 °C for 20 min. Prior to heating the tissue temperature was 9.5 °C and afterwards it was 80 °C.

2.3 Preparation of CRM-AZA-Mus

The AZA-contaminated mussels were retrieved from Bruckless, Donegal, Ireland in September 2005. The mussels were steam cooked immediately after harvesting, vacuum packed in 1 kg lots and stored at -20 °C until use. AZA-free mussels were harvested from New London Bay, Prince Edward Island, Canada, in April 2005. These mussels were also steam cooked prior to storage at -20 °C.

AZA-contaminated (6.58 kg) and AZA-free tissues (13.2 kg) were combined by passing through a Comitrol 3600 food cutter (Urschel Laboratories Inc., Valparaiso, IN, USA) equipped with 020-020 blades a total of three times. The Comitrol was rinsed with 1 kg of water after the second and third passes and this was combined with the homogenate. The Comitrol was then fitted with 010-010 cutting blades and the homogenate was passed through while mixing in an additional 5.26 L of water. Stabilisers (6.5 g each of ethoxyquin, erythromycin, oxytetracycline and ampicillin) were mixed with 3.58 L of water and then added to the homogenate before two final passes through the Comitrol (010-010) with an additional 2.47 L of water. The material was collected in two separate 25 L containers and the moisture content determined to be 85% using an OHAUS moisture determination balance (Cole-Parmer, IL, USA). Both containers of homogenate were blended using a Polytron™ (Kinematica, Lucerne, Switzerland) (5,000 rpm, 10 min) before degassing and storing overnight at 4 °C. The next day the tissues were degassed

under vacuum and N_2 was bubbled through the homogenate prior to re-combining. The homogenate was then transferred to a container equipped with a mechanical stirrer (Lightnin Labmaster Mixer, L1U1O-A200, Cole Parmer, IL, USA) set at 750 rpm. This container was in-turn placed in larger container with ice in order to avoid heating and evaporation of water from the homogenate during the dispensing step. A Masterflex dispensing pump (Cole Parmer) was calibrated to dispense 8 g (7.9 - 8.2 g) of homogenate into 10 mL polypropylene bottles. Filled bottles were purged with argon before hermetically sealing with strips of trilaminate foil using a MKIII heat-sealing machine (SiS Ltd., Chadwicks of Bury, UK). The bottles were then labeled, screw capped and stored at -20 °C. The bottles were transported on dry-ice to the Agriculture and Agri-Food Canada facility in Ste-Hyacinthe (Quebec, Canada) for gamma irradiation. The irradiation dose received by all bottles was in the range of 14.3 to 19.4 kGy (γ-irraditon, source cobalt 60) [32]. The bottles were returned frozen to the NRCC in Halifax for sealing (12ASD Heat Sealer, Sentinal Packaging Inc., Hyannis, MA, USA) inside trilaminate lightproof foil pouches (Retort Pouches, ISE USA Inc). A total of 3697 bottles were pouched and then stored at -80 °C. The production process for CRM-AZA-Mus is summarized in Fig. 2.

2.4 Sample preparation

2.4.1 Method A (homogeneity and stability studies)

The entire content of a CRM-AZA-Mus bottle (8.0 ± 0.2 g) was transferred to a 50 mL centrifuge tube, weighed and extracted in 3 steps with 30 mL aliquots of MeOH for each step. A multi tube vortex mixer (Model: DVX 2500, VWR, West Chester, PA, USA) was used for the first extraction step (2500 rpm, 2 min) prior to centrifuging (3000 *g*, 10 min) and decanting the liquid phase. A Polytron^{TM} (10,000 rpm, 1 min) was used for the second extraction step. Vortex

mixing was used again for the final extraction step. The decanted extracts from all three steps were combined in a 100 mL volumetric flask and the contents were made to volume with MeOH. Aliquots of the final extracts were passed through 0.45 µm regenerated cellulose filters (Millipore Corp, Billerica, MA, USA) prior to analysis (note: all extracts prepared for this work were filtered in this manner).

2.4.2 Method B (certification by liquid solid extraction (LSE))

Aliquots (8 g) were extracted in 50 mL centrifuge tubes in 4 steps with 22 mL aliquots of MeOH for each step. Vortex mixing was used for the first extraction step (see above), followed by centrifugation (3950 *g*, 10 min) and decantation. The second and third extraction steps used an Omni prep homogenizer (Omni Int., Kennesaw, GA, USA) (10,000 rpm, 3 min). Vortex mixing was used again for the final step. The extracts from all four steps were combined in 100 mL volumetrics and brought to volume with MeOH.

2.4.3 Method C (certification by matrix solid phase dispersion (MSPD))

An MSPD [33] procedure was developed for extraction of AZAs. An aliquot (0.5 g) was weighed into a polystyrene weigh boat. The tissue was ground with 2.0 g of Bakerbond C8-silica using a glass mortar and pestle. Once the sample was ground thoroughly with the silica, it was transferred to a 7 mL glass SPE column pre-packed with 0.25 g of clean C8-silica placed between two PTFE frits. The sample was packed carefully into the tube, ensuring no voids, and a piece of glass wool was placed on top. The SPE column was attached to a vacuum manifold, equipped with 10 mL volumetric flasks for collection. The mortar and pestle were rinsed with 3 mL of MeOH, and the rinse transferred to the MSPD tube. The MSPD tube was then eluted

drop wise under controlled vacuum and eluted with additional MeOH until the collection flask was filled almost to volume. The sample was made to 10 mL using MeOH.

2.4.4 Method D (minor analog profiling and protein binding work)

Samples (2 g) were extracted with a two-step procedure using 4.5 mL aliquots of MeOH. A hand-held variable speed Omni prep homogenizer was used (15,000 rpm, 3 min) for both steps. The resulting supernatants were combined in a 10 mL volumetric flask and the sample was made to 10 mL using MeOH.

2.4.5 Ester hydrolysis

To hydrolyse esters of the OA group toxins 1 mL aliquots of CRM-AZA-Mus extract (prepared with Method D) were placed in an HPLC vial, 125 µL of 2.5 N NaOH was added, and the mixture was placed in a water bath at 76 °C for 40 min. The samples were cooled and neutralized with 125 µL of 2.5 N HCl before filtration prior to LC-MS analysis for OA, DTX1 and DTX2.

2.5 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analyses for this work were performed on an Agilent 1200 LC system (Palo Alta, CA, USA) connected to a Q-TRAP API4000 mass spectrometer (AB-Sciex, Concord, ON, Canada), equipped with a turbospray ionization source.

2.5.1 Method 1 (homogeneity and stability)

A 50 × 2.1 mm i.d. column packed with 3 µm BDS Hypersil-C8 (Thermo Scientific, Waltham, MA, USA) was used with a binary mobile phase of water (A) and acetonitrile:water 95/5 (B), both containing 2 mM ammonium formate and 50 mM formic acid. Gradient elution was from 30-100% B over 8 min, at 250 µL/min at 20 °C, with 5 µL injections. The MS was operated in positive selected reaction monitoring (SRM) mode: AZA1 (842.5 → 672.5 & 362.3); AZA6 (842 → 658.5 & 362.3); AZA2 (856.5 → 672.5 & 362.3); AZA3 (828.5 → 658.5 & 362.3). The collision energies (CEs) for AZA1-3 were set to 65 and 75 eV for the 672.5/658.5 and 362.3 fragment transitions respectively, and the declustering potential (DP) was 50 eV for all. The source temperature was 350°C and the ion-spray voltage was 5500.

2.5.2 Method 2 (certification measurements)

A 50 mm × 2.1 i.d. column packed with 2.5 µm Luna C18(2) HST (Phenomenex, Torrence, CA, USA) was eluted with a binary mobile phase of water (A) and acetonitrile/water (95/5) (B) each containing 5 mM ammonium acetate (pH 6.8). A gradient was run from 25-100% B over 5 min at 350 µL/min at 15 °C, with 5 µL injections. The MS was operated in positive ion SRM mode as described for AZA1-3 in the homogeneity and stability section above. This procedure was validated in-house for AZA determinations.

2.5.3 Method 3 (analysis of additional AZA toxins)

Additional non-certified AZA analogs were measured using Method 2 with some modifications. The gradient was run from 25-100% B over 20 min at 250 µL/min at 15 °C. Analysis was in positive ion SRM mode screening for all AZA analogs as reported by Rehmann et al. [7].

2.5.4 Method 4 (analysis of additional lipophilic toxins)

CRM-AZA-Mus was screened for a range of lipophilic toxins using a previously reported method [35].

2.5.5 Method 5 (analysis of spirolides)

A 50 × 2.1 mm i.d. column packed with 2.5 µm Luna C18(2) HST eluted with a binary mobile phase of water (A) and acetonitrile/water (95/5) (B), each containing 5 mM ammonium acetate (pH 6.8). A gradient was run from 25-75% over 12 min at 300 µL/min, maintaining the column at 20 °C, with 5 µL injections. The MS was operated in positive ion SRM mode: 13-desMe-SPX-C, SPX-G (692.5 → 164.1); 13-desMe-SPX-D, SPX-B, PnTX-G (694.5 →164.1); SPX-D (706.6 → 164.1); SPX-C (708.5 → 164.1); unknowns (710.6 → 164.1, 722.5 →164.1, 782.6 → 164.1). CEs and DPs were 70 and 80 eV respectively. The source temperature was 300 °C and the ion-spray voltage was 5000.

2.6 Calibration and quantitation procedures

Two different quantitation procedures were used to deal with matrix effects in the LC-MS/MS measurements: standard addition and matrix-matched calibration.

2.6.1 Standard addition

Two accurate mixed standard solutions were prepared using AZA1-3 calibrant CRMs for spiking the two different types of extracts. Method B extracts (LSE) were spiked with a solution containing 370 ng/mL AZA1, 83 ng/mL AZA2 and 70 ng/mL AZA3. Method C extracts

(MSPD) were spiked with a solution containing 220 ng/mL AZA1, 50 ng/mL AZA2 and 42 ng/mL AZA3. Extracts of CRM-AZA-Mus and CRM-Zero-Mus were spiked by mixing 50 µL of the appropriate spiking solution with 200 µL of the extracts using a Microlab diluter/dispenser (Hamilton Company, Reno, NV, USA). For zero levels, the extracts were spiked with MeOH containing no toxin. Spiking solutions were also mixed with MeOH to prepare matrix-free controls. All samples were prepared in triplicate.

2.6.2 Matrix-matched calibration

A mixed stock solution was prepared using AZA1-3 calibrant CRMs at concentrations of 706, 176 and 142 ng/mL, respectively. A working solution was made by preparing a 3-fold dilution of this stock, which was subsequently used for preparation of a six-level dilution series with the Microlab. Concentrations were in the range of 706 - 3 ng/mL, 176 - 0.7 ng/mL and 142 - 0.6 ng/mL for AZA1-3, respectively. Matrix-matched calibration solutions were prepared by mixing 100 µL aliquots of each level with 400 µL aliquots of CRM-Zero-Mus extract (both LSE and MSPD) using the Microlab. The solutions were also mixed with MeOH to prepare matrix-free calibration standards.

3 Results and Discussion

3.1 Design and preparation of CRM-AZA-Mus

The production of a CRM requires significant planning to ensure fitness for purpose. Analytes of interest must be homogeneously dispersed throughout the material and between bottles. A CRM must also be stable, as potential changes to concentration values during shipping and in storage severely risk the material's usefulness in method development and validation. Homogeneity can be achieved through rigorous blending and mixing of the stock material before and during the bottling stage. Ensuring stability is frequently more challenging, in particular for matrix CRMs, and generally requires more careful research and planning.

Although heat treatment is a common step in stabilization of tissue RMs for shellfish toxins [36,37], previous research on AZA RMs demonstrated problems with heat treatment due to the instability of AZA3 [30]. Heat-treatment was further studied in this work by hermetically sealing raw AZA-contaminated tissue in containers and heating them for 10 min at temperatures ranging from 50-150 °C. As shown in Fig. 3, AZA1 and AZA2 were stable up to 110 °C, with extensive degradation becoming apparent at temperatures above 120 °C. AZA3 showed an increase in concentration from 0.1 mg/kg at room temperature to 0.7 mg/kg at 90 °C. This increase was a result of the AZA1 metabolite, AZA17, which was present in the uncooked mussel tissues, decarboxylating to form AZA3 as the tissues are heated [20]. At temperatures greater than 90 °C, AZA3 was observed to degrade extensively, with complete decomposition measured at 150 °C. This study confirmed that post-bottling thermal processing (>115°C) was not an appropriate method for stabilisation of a CRM to be certified for AZAs. However, because of the presence of AZA17 in the matrix , it was important that some heat treatment was carried out on the tissues to eliminate AZA17 before preparation of CRM-AZA-Mus. After

harvesting, the Bruckless mussels were steamed to assist with shucking. While this process also served to stabilize the AZA3 profile, a trial was performed to determine if decarboxylation was complete. Aliquots (n=3) of the processed tissues were heated at 95 °C for 10 min, and no significant change in AZA3 level was observed indicating that the initial heat treatment applied was sufficient (0.70 and 0.68 mg/kg before and after heating, respectively).

The AZA tissues from Bruckless were highly concentrated (approximately 3.0, 0.67 and 0.70 mg/kg for AZA1-3, respectively). In order to make the CRM more appropriate for end users, in relation to regulatory levels, a 3-fold dilution of the stock tissue was prepared. Whole cooked mussels harvested in Prince Edward Island were used for this dilution as they had no detectable levels of AZAs.

The moisture content of CRM-AZA-Mus was adjusted to 85% in order to be representative of typical unprocessed mussels. During this moisture adjustment step a number of stabilizing agents were added at a concentration of 0.02% (w/w) in the final material. Previous studies have shown that a combination of antioxidant and antibiotics are beneficial for the stability of some shellfish toxins and, importantly, they are beneficial for the stability of the mussel matrix [38].

The last major consideration in the production of CRM-AZA-Mus was whether to include a final stabilization step. As shown, heat treatment (>115 °C) was not a viable method for the stabilization of AZA in matrix RMs. Freeze-drying has been established as a beneficial procedure for AZA RMs [39,40] but the resources required for preparation of a large scale freeze-dried CRM were not available when CRM-AZA-Mus was produced. The decision to proceed with production of a wet tissue homogenate is strengthened when it is considered that this matrix is very similar to the type of wet shellfish samples generally received as part of routine regulatory monitoring programs. Feasibility studies showed that γ-irradiation was

suitable as a stabilization procedure for use in the preparation of AZA RMs, and it was demonstrated that the treatment was highly effective in eliminating microbial activity in shellfish tissue RMs after they have been bottled [32]. Irradiation was therefore selected as a post-bottling stabilization process. All bottles received a dose in the range of 14 to 20 kGy. The variation in dose received posed no risk to the stability of the AZAs in the mussel matrix [32].

3.2 Homogeneity and stability

The homogeneity of CRM-AZA-Mus was assessed following an approach described by van der Veen *et al.* [41]. Bottles of CRM-AZA-Mus (n = 15) were selected from across the fill series and analysed under repeatability conditions. The percent coefficients of variation (%CVs) were 4.0, 3.2 and 5.1% for AZA1, -2 and -3, respectively. The variation between bottles ($U_{c(bb)}$) comprises between-bottle heterogeneity (S_{bb}) and the variation due to measurement (S_{meas}). The latter was estimated taking 4 sub-samples from a single bottle. Uncertainties due to homogeneity (u_{homo}) were then established according to Equation 1. Relative homogeneity uncertainty values between 2.2 and 3.5% were estimated for AZA1-3 in CRM-AZA-Mus.

Equation 1:
$$S_{bb} = \sqrt{U_{c(bb)}^{\ 2} - S_{meas}^{\ 2}}$$

A large batch of CRM-AZA-Mus was prepared to ensure a continuous supply for a sufficient number of years. It was therefore important to ensure stability of the material over its lifetime. An isochronous stability study was run at -12, 4, 18 and 37 °C using time points of 14, 67, 144 and 267 days, maintaining reference samples at -80 °C (Table 1). All toxins were stable over 14 days at temperatures up to +20 °C, suggesting limited risk during shipping and short-

term handling of CRM-AZA-Mus. Nevertheless, chilled packing will be used as a precaution against fluctuating transport conditions during shipping. All three analytes were found to be stable over the entire duration of the study at -12 and +4 °C. As previously observed, AZA3 was the least stable analog, with approximately 30% degradation after 267 days at +18 °C. At the stress temperature of 37 °C, AZA3 is nearly completely degraded by the end of the study period, while AZA1 and AZA2 show significant degradation. For comparison, excellent stability of AZAs was observed in a freeze-dried mussel tissue CRM prepared for multiple groups of lipophilic toxins, in which no detectible degradation was observed after 1 year for AZA1-3 at temperatures up to +20 °C [40]. While the freeze-dried matrix clearly provides increased stability over a wet tissue matrix good stability was observed for CRM-AZA-Mus and appropriate transport and storage conditions have been established. Stability monitoring of CRM-AZA-Mus is ongoing. Uncertainties due to stability were established by calculating the uncertainty of the slope (μ_b) for the -20 °C stability data using Microsoft Excel regression statistics (data plotted as -20°C response (relative to reference temperature) against time points). Uncertainties due to stability (μ_{stab}) were calculated according to Equation 2. Relative values for AZA1-3 were 1.3, 2.3 and 0.6%, respectively, and were calculated based on an assigned 1 year shelf life (t_{stab}).

Equation 2: $$\mu_{stab} = \mu_b * t_{stab}$$

In 2008, Nzoughet *et al.* reported that AZAs bind to proteins within the mussel tissue matrices [42]. The presence of protein bound AZAs was further suggested in later work examining the stability of AZAs [43]. However, in studies conducted as part of this work there

was no evidence of significant amounts of protein bound toxins in CRM-AZA-Mus, therefore there is no perceived risk of changes to the certified concentrations (see Electronic Supplementary Material).

3.3 Certification measurements

Matrix CRMs are valuable in assessing the performance of entire analytical methods and it is essential that values assigned to the CRM are as accurate as possible. For CRM-AZA-Mus there were several challenges to be considered in the value assignment process. As the analyte was present in a complex matrix, the first challenge was to ensure complete (exhaustive) recovery during the extraction procedure. For CRM-AZA-Mus, two extraction methods were optimized and validated in-house to provide complete recovery. A procedure based on the traditional liquid solid extraction (LSE) approach (Method B) was optimized for CRM-AZA-Mus through a number of experiments examining the number of extraction steps (n=4), the type of solvent (MeOH), and the sample-to-solvent ratio (1:12.5) (see Electronic Supplementary Material). The second extraction procedure (Method C) was based on matrix solid phase dispersion (MSPD). MSPD involves disruption of the sample on a solid support phase, which also maximizes surface area, with subsequent exhaustive elution of the analytes of interest from the sample/silica mixture packed in an SPE tube [44]. The main considerations in development of the MSPD method were the support phase (Bakerbond C8), the sample to support ratio (1:4), solvent (100% MeOH), and elution volume (10 mL) (see Electronic Supplementary Material). The optimized LSE and MSPD methods both gave recoveries >99% for AZA1-3 from CRM-AZA-Mus.

The second challenge was accurate quantitation by LC-MS/MS. The complex nature of the mussel matrix made the analysis susceptible to matrix effects, which can enhance or suppress

electrospray ionization of analytes [45,46]. Various approaches to deal with matrix effects in the analysis of shellfish toxins were discussed previously [35]. The methods most applicable to compensate for matrix effects in CRM certification work are standard addition [47,46], matrix-matched calibration [48] and dilution. These methods deal with the matrix effect issues, without the risk of losing analyte during the process, which can be a problem with clean-up procedures such as solid phase extraction. Standard addition and matrix-matched calibration procedures were optimized and validated in-house to ensure that the most accurate results were achieved.

In a previous study, it was demonstrated that AZA1-3 showed linearity of response when spiked into mussel tissue extracts [35]. Therefore, the standard addition experiments on CRM-AZA-Mus were performed using a single toxin spike (spiked at a concentration equal to that of the analyte in the extract) and a zero spike (with 20% dilution to compensate for spike solution volume) level for both LSE and MSPD extracts. This greatly reduced the number of samples for analysis and ensured that the experiment could be run under repeatable conditions. The standard addition results (Table 2) were in good agreement for the LSE and MSPD extracts. Comparing the standard addition results with those from analysis of the neat extracts with external calibration shows that levels of suppression ranged from 16 – 24% for LSE extracts and from 11 – 21 % for MSPD extracts. The reduced matrix effects associated with the MSPD extracts are due to the higher sample-to-solvent ratio and possibly some clean-up of the extract provided by the MSPD process.

CRM-Zero-Mus extract was used for preparation of matrix-matched calibration standards. Through standard addition experiments, it was shown that AZAs were suppressed to the same extent in CRM-Zero-Mus as they were in CRM-AZA-Mus (see Electronic Supplementary Material). LSE and MSPD extracts of CRM-Zero-Mus were spiked with a mixed

dilution series of AZA1-3. These matrix-matched calibration curves were then used to quantitate AZA1-3 levels in CRM-AZA-Mus (Table 2). The matrix-matched calibration results on the LSE and MSPD extracts were in very good agreement. The extent of matrix effects was assessed through comparison with calibrants spiked in pure MeOH, and show levels of suppression very similar to those determined by standard addition. Again, matrix suppression was slightly reduced for MSPD extracts.

The occurrence of isomeric forms of the major AZA analogs was previously reported [35], and these have recently been characterised as C37 epimers [49]. The presence of these epimers in CRM-AZA-Mus is illustrated in Fig. 4A and is significant from the point of view of assigning accurate certified values. While not generally separated under acidic conditions (Fig. 4B), it has been established that these epimers can be separated from their parent analogs when using neutral mobile phase conditions [35]. Molar response studies have shown that when monitoring using the LC-MS/MS conditions described for certification in this work, there is no significant difference between the response of AZA1 and its isomer [49]. For assignment of certified values the equivalence of molar response under the conditions used facilitated reporting certified values as a sum of the parent AZA analogs and their respective epimers. A combined concentration value is appropriate as acidic pH mobile phase systems are frequently used for the analysis of shellfish toxins, including AZAs, and under these conditions the AZA epimers are not resolved. In CRM-AZA-Mus the epimer peak areas correspond to approximately 10% of the total AZA1 and AZA2 peak areas, and approximately 7% for AZA3. Only the combined concentrations of AZA1-3 and their isomers are certified in CRM-AZA-Mus.

3.4 Assignment of certified values and uncertainties

For certification of RMs a number of different approaches can be taken [50]. At the NRCC the typical procedure is to assign values based on analyses using two or more independent methods. The methods available for use in a certification exercise vary significantly between analytes and the type of RM being produced. Quantitative ^{1}H nuclear magnetic resonance is an important method for the certification of toxin calibration solution CRMs [51] but this technique is not applicable to matrix materials. The analytical methods available for AZA determination are somewhat limited due to the absence of a suitable chromophore for direct analysis by optical detection methods such as UV and fluorescence. A method based on derivatisation of AZAs with 9-anthryldiazomethane (ADAM) has been developed to enable analysis by LC with fluoresence detection (LC-FLD) [28]. The method has produced accurate results for AZAs in CRM-AZA-Mus [28]. However, the precision of the ADAM results for CRM-AZA-Mus was not sufficient for certification purposes. The derivatisation step and clean-up procedure involved in the process requires several steps over two days, and it can suffer from matrix effects on the reaction yields. . Therefore, while the ADAM LC-FLD method provided good supporting data for the CRM-AZA-Mus, it was not used for certification. The methods for AZA quantitation in the certification exercise were therefore based on LC-MS/MS. The separate LSE and MSPD extractions procedures were optimized for CRM-AZA-Mus to provide complete recovery (>99%) and to provide independent approaches for value assignment. Two separate approaches were also taken to compensate for the matrix effects associated with LC-MS analysis of AZA-Mus extracts (Table 2). The independence of the separate extraction procedures was best complemented by selecting data acquired using separate calibration approaches to deal with matrix effects. Therefore, the final certified values for CRM-AZA-Mus were obtained by using

data from standard addition on the LSE extracts (1.184, 0.284 and 0.225 mg/kg for AZA1-3, respectively) and matrix-matched calibration on the MSPD extracts (1.132, 0.261 and 0.197 mg/kg for AZA1-3, respectively). Following ISO guidelines [52] and in-house protocols the two sets of values were combined by taking an average of the mean values of each method, to assign certified values for AZA1-3 in CRM-AZA-Mus: 1.16 mg/kg for AZA1; 0.27 mg/kg for AZA2; and 0.21 mg/kg for AZA3. The uncertainty associated with characterization (μ_{char}) was calculated as in Equation 3 from two components: the method dependent uncertainty (μ_i) and the uncertainty of the average of the method means (μ_R). The method dependent uncertainty (μ_i) is the combined uncertainty of the two analytical approaches (standard addition with LSE and matrix-matched calibration with MSPD) that were used to obtain the certified values. Method uncertainties for each of the two approaches were determined first and each included uncertainties associated with extraction, LC-MS and the uncertainty from the CRM calibrant used. These uncertainties for both approaches were then combined to obtain the method dependent uncertainty (μ_i). The uncertainty of the average of the method means (μ_R) is the uncertainty associated with assigning a value based on the average of a number of values. In the certification of CRM-AZA-Mus data from two different analytical approaches were used. The μ_{char} ranged from 0.008 to 0.029 for AZA1-3 (Table 3).

Equation 3: $$\mu_{char} = \sqrt{\mu_i^2 + \mu_R^2}$$

The assignment of final combined uncertainties to CRM-AZA-Mus was in accordance with ISO guidelines [52] and the guide to expression of uncertainty in measurement (GUM) [53]. The overall uncertainty estimates (U_{CRM}) include uncertainties associated with batch

characterization (μ_{char}), between-bottle variation (μ_{hom}) and instability during long-term (μ_{stab}), as expressed in Equation 4.

Equation 4:
$$U_{CRM} = k\sqrt{\mu_{char}^2 + \mu_{hom}^2 + \mu_{stab}^2}$$

The uncertainty components relating to homogeneity, stability, and characterization for AZA1-3 are shown in Table 3. A coverage factor (k) of 2 for a 95% confidence level was applied to obtain the final expanded uncertainties, and these corresponded to relative uncertainty values of 8.2, 8.2 and 11% for AZA1-3, respectively.

3.5 Characterization of additional toxins in CRM-AZA-Mus

As CRM-AZA-Mus was prepared from naturally contaminated tissues the presence of toxins in additional to those certified was a consideration. CRM-AZA-Mus was analyzed for a range of AZA analogues (Fig. 5) and Table 4 shows indicative concentrations of AZA4-10 present. A range of additional minor AZA analogues [7] were also detected (data not shown).

The CRM was screened for a broad suite of lipophilic toxins including OA, DTXs, YTXs and PTXs using a previously reported method [35]. Indicative concentrations of OA and DTX2, total OA concentrations including esters, and YTX are reported in Table 5. Finally, a number of cyclic imines group toxins were detected in the CRM, which included several spirolides (SPX) and pinnatoxin-G (PnTX-G) (see Electronic Supplementary Material). The majority of these cyclic imines were present at trace levels and were not quantitated.

4 Conclusions

Based on prior experience and feasibility studies on RMs for AZAs, CRM-AZA-Mus was prepared from naturally contaminated mussels and packaged as a wet homogenate. Anti-oxidants and antibiotics were added as stabilizers and gamma-irradiation was used as post-bottling stabilization process. AZA levels are appropriate for laboratories that perform testing for AZAs on a routine basis in a regulatory setting. The homogeneity and stability of the CRM were comprehensively assessed and the CRM was found to be fit for purpose. Fully exhaustive extraction procedures were developed and a number of different approaches were used to account for the matrix effects associated with analysis by LC-MS. Certified values and associated uncertainties were assigned following internationally accepted procedures for the production of CRMs. As the CRM was prepared from naturally contaminated tissues, a range of additional AZA analogues were identified in the matrix, along with toxins from the DSP and YTX groups, although certified values were not assigned for these analytes. CRM-AZA-Mus will be a valuable tool for analytical labs in the development, validation and quality control of methods for monitoring AZAs.

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6 Tables

Table 1. Relative concentrations of AZA1-3 monitored in the stability study on CRM–AZA-Mus. Values are expressed relative to day 0 samples that had been stored at the reference condition (−80°C)

	Time point (days)	-12°C	+4°C	+20°C	+37°C
AZA1	14	1.03 (0.05)	1.05 (0.03)	1.01 (0.02)	0.98 (0.07)
	67	0.98 (0.09)	1.06 (0.07)	0.97 (0.09)	0.92 (0.13)
	144	1.02 (0.05)	0.97 (0.10)	1.02 (0.01)	0.86 (0.01)
	267	1.00 (0.02)	1.01 (0.02)	1.00 (0.07)	0.87 (0.09)
AZA2	14	1.03 (0.05)	1.05 (0.07)	1.03 (0.01)	0.99 (0.08)
	67	0.98 (0.11)	1.06 (0.04)	0.98 (0.10)	0.92 (0.08)
	144	1.05 (0.06)	1.00 (0.13)	1.03 (0.03)	0.87 (0.06)
	267	0.97 (0.08)	1.01 (0.01)	1.04 (0.09)	0.93 (0.11)
AZA3	14	1.01 (0.05)	1.04 (0.03)	1.00 (0.01)	0.78 (0.09)
	67	0.99 (0.04)	1.03 (0.03)	0.96 (0.03)	0.26 (0.01)
	144	0.99 (0.03)	1.03 (0.01)	0.90 (0.01)	0.09 (0.01)
	267	1.01 (0.02)	1.05 (0.08)	0.71 (0.04)	0.07 (0.03)

Numbers in parentheses are standard deviations (SD, n=3)

Table 2. Concentrations (mg/kg) of AZAs in CRM-AZA-Mus determined in certification exercises. Concentrations are a sum of the main AZA compound and its C37 epimer.

Method	Calibration	AZA1	AZA2	AZA3
LSE extraction & Standard Addition	Standard addition	1.184	0.284	0.225
	External calibration	0.895	0.233	0.188
	% difference	24.4	18.1	16.3
MSPD extraction & Matrix-Matched Calibration	Matrix calibrants	1.132	0.261	0.197
	MeOH calibrants	0.92	0.219	0.181
	% difference	18.7	16.2	8.1
** MSPD extraction & Standard Addition*	*Standard addition*	*1.101*	*0.28*	*0.202*
	External calibration	*0.867*	*0.228*	*0.179*
	% difference	*21.3*	*18.4*	*11.5*
** LSE extraction & Matrix-Matched Calibration*	*Matrix calibrants*	*1.129*	*0.284*	*0.212*
	MeOH calibrants	*0.878*	*0.229*	*0.195*
	% difference	*22.3*	*19.4*	*8.08*

*****data not used in assignment of certified values for CRM-AZA-Mus in italics

Table 3. The certified values, estimated standard uncertainties for the individual components, and combined uncertainty estimates for AZA1, AZA2 and AZA3. Certified concentrations (mg/kg) taken from LSE standard addition data and MSPD matrix-matched calibration data are a sum of the main AZA compound and its C37 epimer.

	AZA1	**AZA2**	**AZA3**
Certified values (mg/kg)	1.16	0.27	0.21
Combined uncertainty (k=2)	0.09	0.02	0.02
μ_{char}	0.029	0.008	0.009
μ_{homo}	0.04	0.006	0.007
μ_{stab}	0.014	0.006	0.001

Table 4. Information values for additional AZA analogues present in CRM-AZA-Mus and relative retention times

Analog	$[M+H]^+$	RRT*	mg/kg**
AZA1	842.5	1.00	-
AZA2	856.5	1.04	-
AZA3	828.5	0.93	-
AZA4	844.5	0.77	0.16
AZA5	844.5	0.83	0.04
AZA6	842.5	0.97	0.08
AZA7	858.5	0.83	0.02
AZA8	858.5	0.86	0.03
AZA9	858.5	0.81	0.04
AZA10	858.5	0.87	0.02

* RRTs relating to AZA1 using LC-MS method 3.
** Information values only: these concentrations are not certified

Table 5. Information values for other lipophilic toxins in CRM-AZA-Mus.

Toxin	$[M+H]^+$	mg/kg*
OA	805.5	0.08 (0.13)**
DTX2	805.5	0.01 (0.01)**
SPX C	706.5	0.01
YTX	1143.5	0.01
13-desMe-SPX C	692.5	trace

* These concentrations are not certified

** Values in parentheses are total amounts of OA and DTX2 determined following base hydrolysis

7 Figure Captions

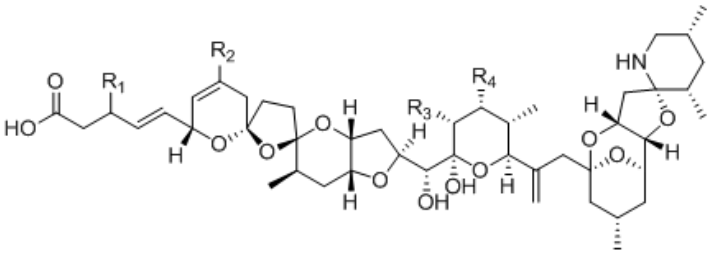
Fig. 1. Structure and *m/z* values for the $[M+H]^+$ ions of selected AZA analogues

Fig. 2. Schematic of CRM-AZA-Mus preparation, bottling and stabilization process.

Fig. 3. Concentrations of AZA1-3 measured in aliquots (*n=3*) of uncooked mussel tissue after heating at different temperatures. Error bars shown represent ±1SD.

Fig. 4: LC-MS/MS chromatogram for AZA1-3 and AZA6 in CRM-AZA-Mus using neutral pH (A) and acidic pH (B) mobile phases, showing resolution and co-elution of 37-*epi*-AZAs, respectively.

Fig. 5. LC-MS/MS analysis for selected additional AZA analogs present in CRM-AZA-Mus.



	R^1	R^2	R^3	R^4	$[M+H]^+$ m/z
AZA1	**H**	**H**	**CH_3**	**H**	**842.5**
AZA2	**H**	**CH_3**	**CH_3**	**H**	**856.5**
AZA3	**H**	**H**	**H**	**H**	**828.5**
AZA4	OH	H	H	H	844.5
AZA5	H	H	H	OH	844.5
AZA6	H	CH_3	H	H	842.5
AZA7	OH	H	CH_3	H	858.5
AZA8	H	H	CH_3	OH	858.5
AZA9	OH	CH_3	H	H	858.5
AZA10	H	CH_3	H	OH	858.5

Fig. 1.

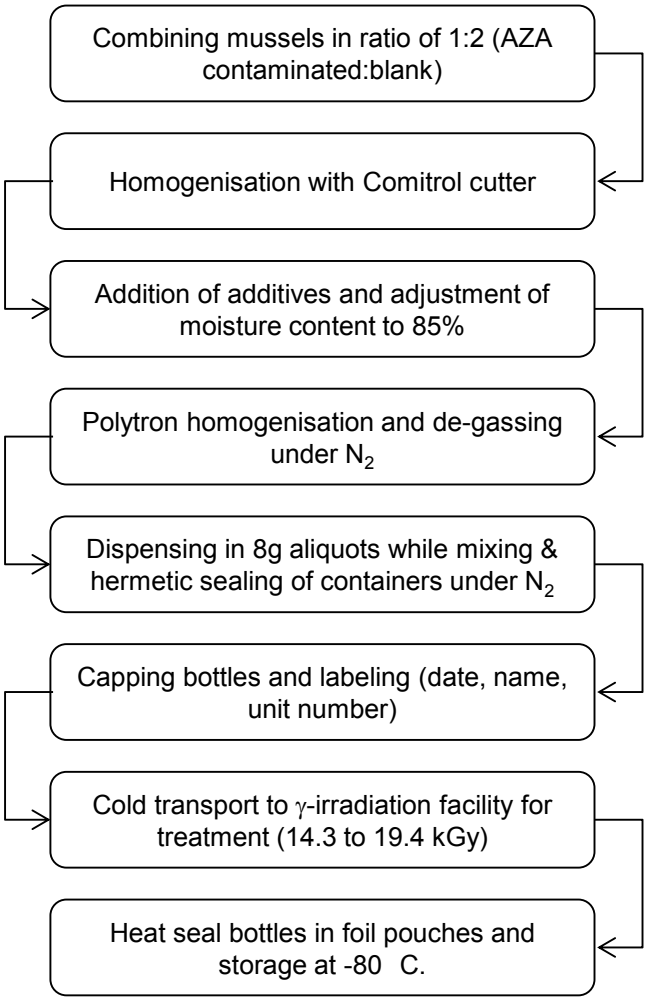

Combining mussels in ratio of 1:2 (AZA contaminated:blank)
Homogenisation with Comitrol cutter
Addition of additives and adjustment of moisture content to 85%
Polytron homogenisation and de-gassing under N_2
Dispensing in 8g aliquots while mixing & hermetic sealing of containers under N_2
Capping bottles and labeling (date, name, unit number)
Cold transport to γ-irradiation facility for treatment (14.3 to 19.4 kGy)
Heat seal bottles in foil pouches and storage at -80 C.

Fig. 2.

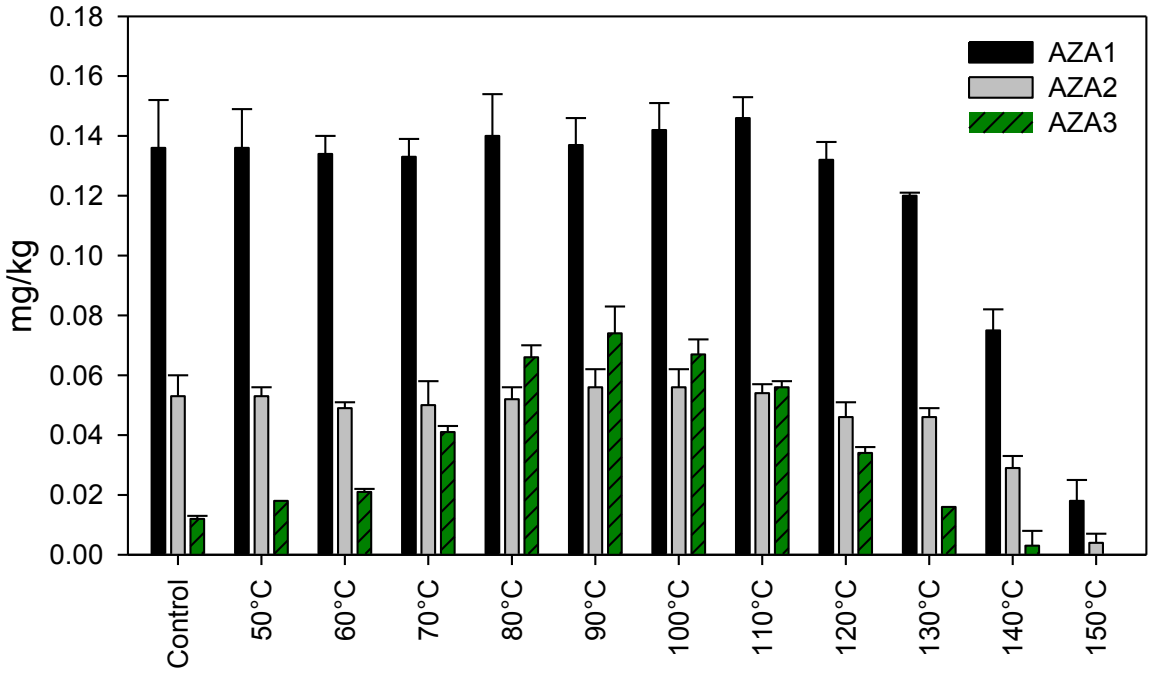

0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
mg/kg
AZA1
AZA2
AZA3
Control
50°C
60°C
70°C
80°C
90°C
100°C
110°C
120°C
130°C
140°C
150°C

Fig. 3.

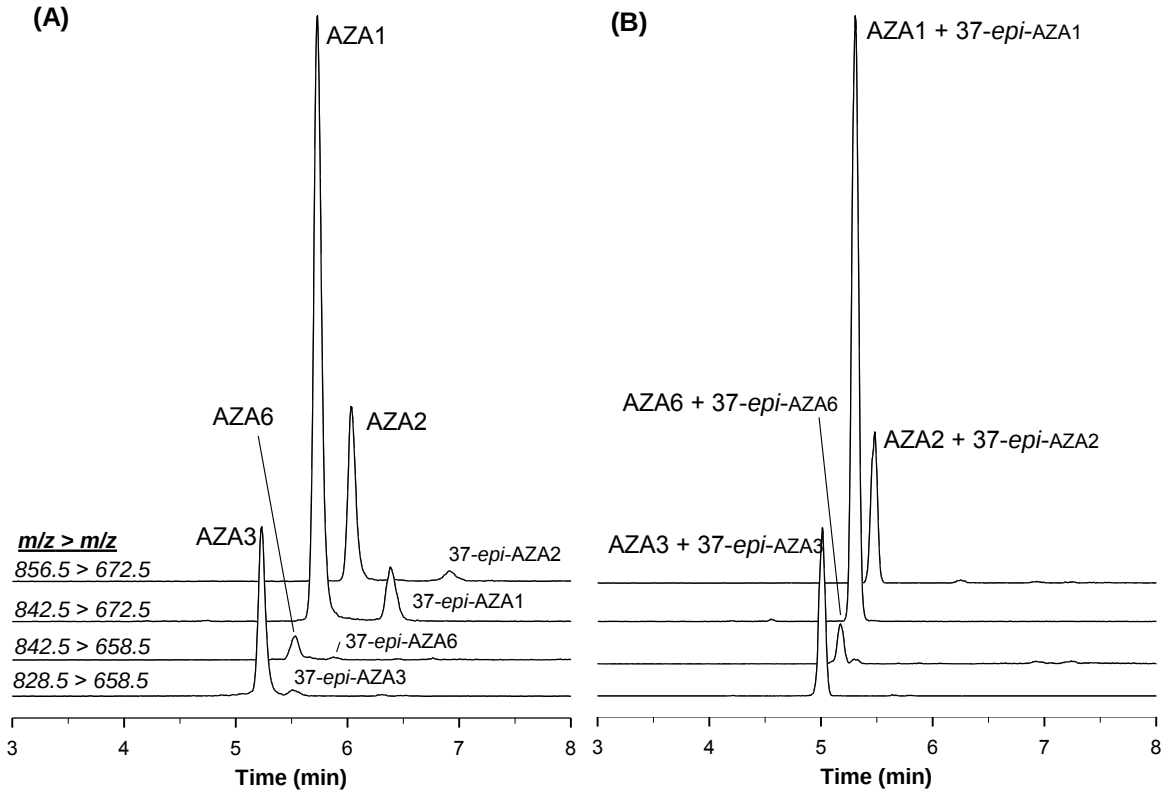

(A)
AZA1
AZA6
AZA2
AZA3
m/z > m/z
856.5 > 672.5
842.5 > 672.5
842.5 > 658.5
828.5 > 658.5
37-epi-AZA2
37-epi-AZA1
37-epi-AZA6
37-epi-AZA3
3
4
5
6
7
8
Time (min)
(B)
AZA1 + 37-epi-AZA1
AZA6 + 37-epi-AZA6
AZA2 + 37-epi-AZA2
AZA3 + 37-epi-AZA3
3
4
5
6
7
8
Time (min)

Fig. 4

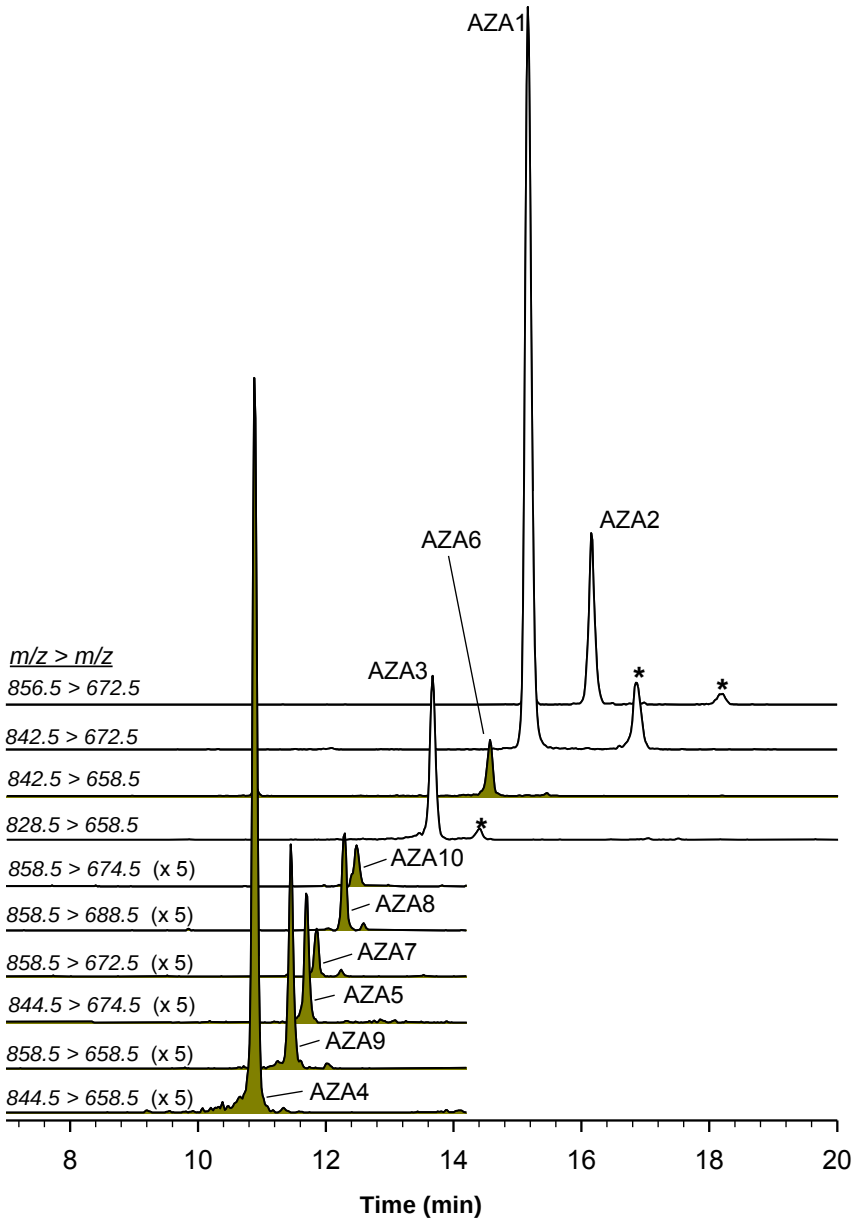

AZA1
AZA2
AZA6
AZA3
m/z > m/z
856.5 > 672.5
842.5 > 672.5
842.5 > 658.5
828.5 > 658.5
858.5 > 674.5 (x 5)
858.5 > 688.5 (x 5)
858.5 > 672.5 (x 5)
844.5 > 674.5 (x 5)
858.5 > 658.5 (x 5)
844.5 > 658.5 (x 5)
AZA10
AZA8
AZA7
AZA5
AZA9
AZA4
8
10
12
14
16
18
20
Time (min)

Fig. 5