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The Preparation of Certified Calibration Solutions for Azaspiracid 1, -2 and -3, Potent Marine Biotoxins found in Shellfish

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Abstract

The production and certification of a series of azaspiracid (AZA) calibration solution reference materials is described. Azaspiracids were isolated from contaminated mussels, purified by preparative liquid chromatography and dried under vacuum to the anhydrous form. Purity was assessed by liquid chromatography–mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Final concentration of each AZA in a CD₃OH stock solution was determined accurately by quantitative NMR spectroscopy. This solution was then diluted very accurately in degassed, high purity methanol to a concentration of $1.47 \pm 0.08 \mu\text{mol/L}$ for AZA1, $1.52 \pm 0.05 \mu\text{mol/L}$ for AZA2, and $1.37 \pm 0.13 \mu\text{mol/L}$ for AZA3. Aliquots were dispensed into argon-filled glass ampoules, which were immediately flame-sealed. The calibration solutions are suitable for method development, method validation, calibration of liquid chromatography or mass spectrometry instrumentation and quality control of shellfish monitoring programs.

Introduction

Azspiracids (AZAs) are a class of lipophilic polyether marine biotoxins that were first detected in harvested mussels (*Mytilus edulis*) from Killary Harbour on the west coast of Ireland in 1995. Symptoms resembling those of diarrhetic shellfish poisoning (DSP) were reported by those affected, including nausea, vomiting, stomach cramps, and severe diarrhea. A relationship between these incidents and a specific toxin could not be immediately determined because DSP and PSP toxins were only present in low levels and known toxin producing phytoplankton species were absent in the associated water samples [1, 2]. A new toxic compound was soon identified as the causative agent and provisionally named Killary toxin-3 (KT3) in recognition of the location where the mussels originated [3]. Following elucidation of the structure, it was renamed azaspiracid-1 (AZA1) [4]. AZAs possess a unique spiral ring assembly, a cyclic amine and a carboxylic acid group (Fig. 1). Shortly after the initial discovery of AZA1, two further analogs, 22-desmethylazaspiracid (AZA3) and 8-methylazaspiracid (AZA2) were discovered [5]. Subsequently, further hydroxylated analogues were discovered by the use of mass spectrometry [6]. To date, some 20 analogues are known, including dihydroxy, carboxy and carboxy-hydroxy-derivatives [7]. *Azadinium spinosum* has recently been identified as the producer [8, 9], while AZA3 is likely a product of metabolism in shellfish [10]. AZAs have since been found throughout the North and West of Europe [11-15] and have most recently been detected in mussels from Morocco [16].

These compounds have a high oral toxicity to humans and have been responsible for incidents of shellfish poisoning [17]. AZAs can be found in various species of filter-

feeding bivalve mollusks such as oysters, mussels, scallops, and clams. Toxicological properties of this group of toxins are being studied to determine the maximum allowable levels in shellfish for human consumption. Due to the limited data available from many of the AZP events, nearly all information regarding AZA toxicology has been obtained from controlled *in vitro* and *in vivo* experiments [18-21]. Many of these efforts have been directed towards assessing the risk of AZA consumption in contaminated shellfish and in turn, identifying the molecular target(s) of AZA, which is currently unknown [22]. All symptoms observed in humans following consumption of shellfish contaminated with AZAs appear within hours of ingestion, and include nausea, vomiting, severe diarrhea and stomach cramps. The illness persists for 2-3 days and full recovery has been established in all cases during the incident in Arranmore Island, 1997 [23]. As of yet, no long term effects or illness have been reported. Azaspiracid poisoning (AZP) remains a rare illness, as only few intoxication events have been reported to date [17, 24]. Its similarity to ‘food poisoning’ or DSP and because the symptoms of the illness disappear rapidly (i.e., days) and are not fatal [14], more cases of AZP are thought to occur than are reported. Within the European Union, the maximum allowable level of AZAs in shellfish is 160 µg/Kg AZA equivalents [25]. This regulation specifically includes AZA1, -2, -3, as preliminary risk assessment suggested that low concentrations and low toxicities of other analogues do not necessitate a particular need to monitor these compounds [26].

Certified reference materials (CRMs) play a vital role in shellfish toxin monitoring programs as they facilitate method validation, ensure accuracy of results, and maintain consistency between laboratories. CRMs are also essential for laboratories maintaining a quality assurance system such as ISO-17025 [27], as they ensure accuracy

and traceability to international standards. However, shellfish toxin CRM availability is limited, generally due to insufficient quantities of the toxin of interest available for CRM production. The complex structures of marine toxins do not allow for efficient synthetic production, thus naturally occurring sources are generally required for production. The isolation of shellfish toxins from natural sources is not only labour-intensive, but generally yields relatively low quantities of purified toxin. Working with such small quantities of material for CRM production presents a significant analytical challenge and specialized methodologies and advanced analytical instrumentation are essential. These factors drastically increase the cost of producing a CRM, which has likely limited the widespread availability of shellfish toxin reference materials.

In this report, the production and certification of a series of certified calibration solutions for azaspiracids-1, -2, and -3 will be described. The workflow and methodologies employed make use of advanced analytical techniques, such as liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy for characterization and quantification. Major steps in the production and certification will be described [28-30] and parameters such as structural confirmation, purity assessment, homogeneity, stability, and final certified value assignments will be discussed.

Experimental

Chemicals

High purity methanol (MeOH) and ethanol (EtOH) from Burdick and Jackson (B&J) was used. Acetonitrile (ACN), ethyl acetate (EtOAc) and hexane were purchased

from Caledon and were of high purity (distilled in glass). Formic acid (99.9% purity) and caffeine (USP grade) were acquired from Sigma Aldrich. Reagent quality glacial acetic acid was purchased from Caledon. Ammonium formate was obtained from Merck. D₃ Methanol (D₃, 99.5%) was purchased from Cambridge Isotope Laboratories (CIL).

Source of the material and isolation of AZAs

AZAs were isolated from the hepatopancreas (HP) tissue of contaminated blue mussels (*Mytilus edulis*) originating from Bruckless, Co. Donegal, Ireland, which were harvested in August 2005. Portions of HP tissue (0.5 kg) were extracted 3 times with 300 mL of 95% EtOH (Fig. 2). The mixture was centrifuged (2000 g's for 20 min) and the ethanolic supernatant was evaporated to dryness using rotary evaporator (Büchi) equipped with a dry-ice/acetone-cooled coldfinger. The dry residue was partitioned between EtOAc (300 mL) and 1M NaCl (100 mL). The aqueous phase was re-extracted two additional times with 300 mL of EtOAc. The organic layers were combined and taken to dryness using a rotary evaporator equipped with a dry-ice/acetone-cooled coldfinger.

The dried EtOAc extract was taken up in equal volumes (200 mL) of hexane and 80/20 v/v MeOH/water. The aqueous phase was collected and the hexane phase re-extracted with a further 200 mL of 80/20 v/v MeOH/water. The aqueous MeOH portions were combined, evaporated to dryness, pre-absorbed onto silica gel (Sigma, 10-40 µm, Type H, No Binder) using EtOAc and then applied onto a silica gel (Sigma, 10-40 µm, Type H, No Binder) column (5 cm i.d. x 6.5 cm). The column was eluted using a stepwise gradient with equal amounts (300 mL) of hexane, EtOAc, 90/10 v/v

EtOAc/MeOH 70/30 v/v EtOAc/MeOH, 50/50 v/v EtOAc/MeOH and MeOH using vacuum (Fraction 1-6 respectively). All eluents apart from hexane contained 0.1% acetic acid. Fraction 4 (70% EtOAc) was dried and applied to a open glass column (1.6 cm x 81 cm) packed with Sephadex LH-20 (Amersham Biosciences) and eluted with MeOH with gravity flow. A total of 70 fractions (1.5 mL) were collected. Fractions 52-59 were combined, evaporated to dryness, dissolved in MeOH (1.5 mL) and applied onto a flash column (1 cm x 21 cm) packed with C8-silica (LiChroPrep, C₈, Merck, 25-40 μm). A step gradient elution was performed with 50/50 v/v ACN/water, 60/40 v/v ACN/water, 70/30 v/v ACN/water and 100% ACN, all of which contained 0.1% acetic acid. Again a total of 70 fractions were collected (70 drops per fraction). Fractions 14-22 were combined, the solvent evaporated and the sample dissolved in 200 μL of MeOH.

Final purification of AZAs was carried out on C₈-silica HPLC column (Phenomenex Luna, 10 mm i.d. x 250 mm) using 50/50 v/v ACN/water with 1 mM ammonium formate and 0.05% formic acid at a flow rate of 5 mL/min. Mass-directed fraction collection was used to collect the eluting toxin on an Agilent 1100 HPLC system coupled to an Agilent MSD single quadrupole mass spectrometer (1946 series) with an active splitter device (Agilent). The active splitting ratio was set to 500:1. The organic solvent was evaporated and the remaining aqueous solution was partitioned with high purity EtOAc to remove formic acid and ammonium formate from the purified AZAs. The organic phase was evaporated to dryness, re-dissolved in 700 μL CD₃OH and then transferred into a Wilmad 535-PP NMR tube.

Recovery quantitation

Recovery of toxin from the individual isolation steps was measured by LC-MS, using an API 165 single quadrupole mass spectrometer (MDS Sciex). Separation of the toxins was achieved on a Hypersil BDS C₈ column (2.1 mm i.d. x 5 cm, 3 μm) using isocratic flow of 70% B where A was H₂O and B was 95% ACN, both containing 2 mM ammonium formate and 40 mM formic acid. The flow rate was 200 μL/min.

Structural confirmation

The molecular structures of AZA-1,-2 and -3 were confirmed by NMR spectroscopy and quadrupole/time-of-flight (Q-ToF) mass spectrometry using a Waters QToF Premier (Milford, MA, USA). For accurate mass determinations, appropriate dilutions of each toxin were carried out using MeOH and were then infused at a rate of 5-10 μL/min into the QToF mass spectrometer.

Purity assessment by LC-MS

Purity analysis by LC-MS was carried out on API 165 MS coupled to an Agilent 1100 series HPLC. A gradient elution was performed using H₂O (A) and 95% ACN (B), both containing 2 mM ammonium formate and 40 mM formic acid, running from 10% B to 100% B in 20 min and holding at 100% B for 20 min. Chromatography was performed on a Hypersil BDS C₈ column (2 mm i.d. x 150 mm, 3 μm, Keystone) at a flow rate of 200 μL/min. The column temperature was set to 25°C. The MS scan range was set from m/z 600 to m/z 1000.

Contamination from other analogs of the toxin was assessed using an isocratic run on the same system. Mobile phase was 70% B and 30% A, at 200 $\mu\text{L}/\text{min}$ on the same column. The MS was set to monitor for the $[\text{M}+\text{H}]^+$ ions of the three main AZAs, m/z 828.5 (AZA3), m/z 842.5 (AZA1) and m/z 856.5 (AZA2) as well as m/z 810.5 (AZA25) [7].

Purity assessment by NMR

One dimensional ^1H NMR spectroscopy was employed to assess the purity of the AZAs. The sample was dissolved in 700 μL of CD_3OH and transferred to a high precision 5 mm NMR tube (Wilmad 535-PP). The ^1H spectrum was acquired with single frequency presaturation on resonance with the $-\text{OH}$ resonance from the MeOH solvent set to the center of the spectrum. The presaturation power was set to 55 dB attenuation down from 50 W (approx. 100 mw) and was applied during the relaxation delay of 2 s. The spectrum at 500.13 MHz was acquired with a Bruker DRX-500 spectrometer using a 90° pulse of approximately 6.4 μs over a sweep width of 7507 Hz (15 ppm at 500 MHz) into 32K points for an acquisition time of 2.18 s. A total of 128 scans were acquired to ensure adequate signal-to-noise of any impurity peaks. The spectrum was Fourier Transformed after zero filling to 64K points and applying an exponential window function with a line broadening parameter of 0.3 Hz. The spectrum was then phased to pure absorption mode.

Quantification by NMR

Quantification of AZAs was performed on three replicate aliquots of the stock solution dissolved in CD_3OH using previously published QNMR methodology [31]. The

aliquots were run against three external standards of caffeine dissolved in H₂O at a concentration of 4.10 mM. All samples were run in 5 mm high-precision NMR tubes (Wilmad 535-PP) at 20°C using 90° pulses and 15 s relaxation delays with continuous wave presaturation of the protonated solvent resonance at 55 dB attenuation down from 50W during the relaxation delay. The receiver gain was kept constant over all of the samples and standards. All samples were tuned and matched to 50 Ω resistive impedance before calibrating the 90° pulse to ¼ of the 360° pulse length determined from the null signal. The 360° pulse value was used during the calculations to correct the measurements for probe damping [31]. All other conditions for the NMR spectra were the same as for the spectra acquired for purity assessment. The spectra were processed in the same way as for the purity spectrum except that the integration was done with constant scaling between the spectra.

Preparation of NRC CRM AZA1, -2 and -3

Quantitative results from QNMR analysis were used to determine the amount of stock solution required for the production of NRC CRM-AZA1, -2 and -3. Stock solutions containing 1.47 μM of AZA1 (MW= 842.0845), 1.52 μM of AZA2 (MW = 856.1072 g/mole) and 1.37 μM of AZA3 (MW = 828.0534 g/mol) were prepared, based on the density of CD₃OH of 0.866 g/mL (at 23°C). Solutions were weighed in screw-cap vials and then transferred quantitatively into pre-weighed calibrated volumetric flasks. The flask for each AZA was filled to the mark with degassed high purity MeOH (Burdick and Jackson, CA, high purity solvent). Care was taken to avoid turbulent mixing of the solvent with oxygen from the air. The final solution was weighed and sealed to prevent

evaporation. The solution was kept cool with ice during ampouling operations. All ampoules were washed with MeOH and dried prior to use. Ampoules were purged with Argon and then filled with 510 μ L of the prepared CRM solution, and flame-sealed using an ampouling machine (Cozzoli Model FPS1-SS-428). The ampoules were removed from the machine, inspected and labeled in sequential order.

LC-MS quantification for homogeneity and stability studies

All analytical LC-MS experiments were performed on an Agilent 1100 system coupled to an API 4000 triple-quadrupole system (Applied Biosystems/MDS Sciex) equipped with an electro spray ionization interface (ESI). The API 4000 instrument was used with an ion spray voltage (IS) of 5500, a declustering potential (DP) of 60 V, a collision energy (CE) of 55 eV and a source temperature of 275 °C. Multiple reaction monitoring (MRM) experiments were carried out in positive ion mode by selecting the following transitions (precursor ion > fragment ion): (i) AZA1: m/z 842.5 > 672.5; (ii) AZA2: m/z 856.5 > 672.5 and (iii) AZA3: m/z 828.5 > 658.5. A binary mobile phase was used, with (A) water and (B) 95% aqueous ACN, each containing 2 mM ammonium formate and 50 mM formic acid. A Hypersil BDS C₈ column (50 mm \times 2.1 mm i.d., 3 μ m) (Thermo Fisher Scientific, Loughborough, U.K.) was eluted isocratically with 70% B for 15 min. Retention times for AZA3, -1, and -2 were ca. 6.9, 9.5, and 11.5 min, respectively.

Results and discussion

Isolation of AZAs

Azaspiracids were isolated from contaminated blue mussels (*Mytilus edulis*) according to the extraction and isolation procedure summarized in Figure 2. The recovery of individual components at each step in the isolation procedure are reported in Figure 3. Briefly, extraction of approximately 500 g of mussel HP with EtOH in triplicate yielded an extraction efficiency of 90%. Although the extraction efficiency could have been increased by additional extraction replicates, this would have introduced undesired matrix compounds that would have to be removed at a later stage. It was therefore decided that an extraction efficiency of 90% was sufficient for purification work.

Liquid-liquid-partitioning steps did not result in any significant toxin losses with recoveries nearing 95% (Fig. 3). Partitioning with EtOAc readily formed emulsions when used with pure deionised water, likely due to the high lipid content of the mussel tissue. The use of a 1 M NaCl solution instead of pure water prevented the formation of an emulsion and increased the effectiveness of this step. The hexane partitioning typically did not form any emulsions and separation was achieved rapidly.

While liquid-liquid partitioning typically did not result in any significant toxin losses, chromatography on silica gel led to a noticeable loss (Fig. 3). Previous trials using gravity flow chromatography on slightly more coarse silica material (silica 60, Merck) with 100% acetone followed by 100% MeOH yielded a toxin recovery of only 35%. This loss could have been due to irreversible absorption of the toxin to the stationary phase. This step however, significantly improved purity so measures to minimize the loss were

preferable to replacing or omitting it from the procedure. Use of a vacuum setup rather than gravity flow reduced the time the compounds spent on the column. The use of a smaller particle size silica gel and a multiple step gradient increased toxin recovery to 75%. Chromatography on silica gel with the described parameters removed over 90% of the loaded sample weight. This efficient clean-up justified the loss of 25% of AZAs during the procedure.

Chromatography on Sephadex LH-20 also yielded high clean-up efficiency (removal of 75% of sample mass) and resulted in high recoveries of ca. 95%. Chromatography on C₈ material by flash-chromatography did result in a 25-30% loss of toxin, although most of this was due to dissolution problems during the loading of the column. The use of a large amount of solvent to load the column in order to achieve complete dissolution resulted in peak broadening, and reduced the clean-up effect of the column. It was therefore decided to dissolve as much as possible with the maximum amount of solvent allowing for good chromatography and to store the undissolved remainder of the sample at -20 °C to be used in future purification work.

The final purification step was carried out on a preparative LC-MS system, which facilitated automatic mass-directed fraction collection. This technique proved to be very helpful in correct identification of collected fractions during collection and also significantly increased sensitivity of detection in comparison to a UV or PDA detector. The low absorbance of AZAs due to the lack of a chromophore within the molecule requires monitoring of the substances at a wavelength near 200 nm when using UV detection. This can result in misidentification of peaks in the spectrum and can lead to contamination of the collected fractions. Use of a buffer during this step was necessary

for separation and better peak shape. However, it was observed that strong buffer concentrations and/or longer periods of exposure of AZAs to acidic buffer can lead to rearrangement reactions, resulting in the production of different toxin diastereomers. The concentrations of formic acid and ammonium formate were therefore kept to a minimum and the buffer components were removed from the isolated toxin within the shortest possible time period by EtOAc partitioning. The low concentration of buffer resulted in some tailing of peaks and in order to prevent overlap, only 20-30 μg of AZAs were injected per run. This part of the procedure proved to be the most labour intensive. While all other steps took between 3 and 10 hours, many replicate injections (>150) were needed for the final purification. The use of the Agilent preparative LC-MS system facilitated automation of this step and enabled up to 30 injections per day. Collection of only the center of peaks (heart-cut) to ensure high purity of the target compound resulted in some loss of toxin during this step although the recovery of the AZAs using this column material was established at 95%. During this stage 3.3 mg of AZA1, 0.67 mg of AZA2 and 1.59 mg of AZA3 were obtained in high purity using the isolation procedure shown in Figure 2.

Structural confirmation and purity assessment

NMR spectroscopy was used to confirm the identity and purity of the AZAs by comparing spectra to literature chemical shift values. Tandem mass spectrometry was also employed to obtain product ion scans to confirm the AZA structures. Accurate masses were obtained on a quadrupole/time-of-flight mass spectrometer, and all

measured masses were found to be within 5 ppm (0.002 Da) of the theoretical monoisotopic masses.

LC-MS was employed to estimate the purity of the AZA stock solutions. Firstly, full spectrum data was acquired to identify potential impurities, and then single ion monitoring was employed to obtain maximum sensitivity to estimate the abundance of low-level impurities. Several low-abundance impurities were identified, many of which appeared to be structural isomers of the AZAs (Fig 4). These impurities are generally well resolved chromatographically from AZA1,-2, and 3, and are present at less than 1 % relative abundance to the AZAs.

Characterization

Accurate quantification of the AZAs in a CD₃OH stock solution at the mM-level was performed by quantitative ¹H-NMR [31], using external calibration with USP-grade caffeine. ¹H-NMR is inherently a quantitative technique and yields equal response (area of resonances *vs* concentration) for all protons in a molecule. These precise measurements require an in-depth knowledge of the structural assignments of chemical shifts, as the number of protons assigned to each group of resonances in the spectra must be known. The procedures have been validated by cross-comparison of gravimetrically prepared solutions of USP-certified standard compounds (caffeine, theophylline, arginine and sucrose) [31] and have been successfully applied to the quantification of other phycotoxins such as domoic acid and okadaic acid.

Although typical characterization of CRMs employs at least two orthogonal methods for quantification, only one method was used in this case as no other definitive

methods were available. Gravimetric analysis is generally not accurate for marine toxin quantification as it cannot allow for impurities such as salts, which is unavoidable for the extremely small quantities of toxin material isolated from contaminated shellfish. Liquid chromatography coupled to chemiluminescence nitrogen detection (LC-CLND) was investigated as a potential definitive method for AZAs, but sensitivity was insufficient for accurate quantification. Although CLND has been implemented successfully for paralytic shellfish poisoning (PSP) toxins and domoic acid [32], the lone nitrogen atom of the AZAs coupled with their larger structure relative to PSPs or domoic acid, results in a significantly reduced mole fraction of nitrogen for the AZAs. In addition to CLND, fluorescence derivatization methods were also attempted but did not generate quantitative results. Therefore, given the high demand for AZA calibration solutions due to the potential risk AZAs pose to food safety and increasing use of LC-MS for regulatory programs, only one method was employed for their characterization.

Homogeneity Study

The within- and between-bottle homogeneity was studied following the production of each material. The homogeneity testing protocol was developed from the recommendations of Ellison et al. [33]. For each of the AZA CRMs, approximately $3n^{1/3}$ (n ; total number of ampoules) of all ampoules produced were selected from over the entire ampoule range. The ampoules were randomly selected and AZA1, -2 and -3 were measured by LC-MS in MRM mode. The between-bottle variation was measured to be less than 2.5%, no greater than the variation for replicate analyses of one solution, thus demonstrating acceptable homogeneity over the entire ampoule range.

Stability Study

To evaluate the stability of the calibration solutions during potential transport and storage, individual short-term and long-term stability studies were carried out. In both cases, the isochronous stability study approach was employed, whereby samples are subjected to the test condition of interest at the appropriate interval to allow for simultaneous analysis of all samples in order to minimize analytical variability [34]. Temperatures studied were -12°C, 4°C, 20°C, and 37°C, and data at these temperatures were compared to a reference temperature of -80°C.

For the short-term stability study, the calibration solutions were stored for 7, 10, 15 and 30 days and then analyzed in the same batch over a 24-h period by LC-MS. As shown in Figure 5a, results for AZA1 and AZA2 showed that no significant variation of concentration occurred over the range of temperatures during the 30-day period. However, AZA3 proved to be least stable of the three compounds, with significant degradation of 20% occurring at 37°C after 10 days and roughly 50% after 30 days. For a statistical assessment of the stability, the slope of the regression lines were tested for significance using the Student t-test, whereby $t_{\text{exp}} = b/u_b$ (where b is the slope of the regression line and u_b is the standard error of the slope) obtained from analysis of variance (ANOVA) is compared to the t-statistic (t_{tab}), with a 95% confidence level and $n-2$ degrees of freedom [35]. Using this approach, the slope is considered insignificant and a material is deemed stable when t_{exp} is lower than t_{tab} [35]. With the exception of AZA3 at 37 °C, all slopes of the stability plots for the short-term study were insignificant. As the AZA calibration solutions will be shipped with refrigerants that have been shown to keep the contents of the insulated shipping container near 0°C for up to 96 h, 37 °C would

likely represent the worst case scenario for a lengthy delay in shipping. Therefore, these results indicate that the AZA calibration solutions demonstrate reasonable short-term stability, and are suitable for transport under the appropriate conditions.

The calibration solutions were stored for 30, 90, 180, and 360 days for the long-term stability study before analysis by LC-MS. As shown in 5b, AZA1 and AZA2 were over 90% degraded after 360 days at 37°C, and less than 20% loss was observed at room temperature over this period. AZA3 exhibited the greatest amount of degradation, with roughly 40% loss occurring after 360 days at room temperature and nearly 90% after 180 days at 37°C. This was in accordance with previous findings by McCarron et al. [10, 36]. Unfortunately, due to ampoules being misplaced over the course of this study, no stability data was obtained beyond 180 days at 4°C. Regardless, -12°C was chosen as the recommended storage temperature as conventional laboratory freezers are generally available in most laboratories. No significant degradation was observed for any of the AZAs at -12°C during this period of study and this was confirmed by testing the slopes for significance as described above [35]. Therefore, these data show that the AZAs are sufficiently stable for use as certified calibration solutions if stored at the recommended storage temperature of -12°C or lower.

Estimation of uncertainty

The overall uncertainty estimate of the CRM (U_{CRM}) is composed of individual uncertainties associated with the batch characterisation (u_{char}), uncertainty related to possible between-bottle variation (u_{bb}), as well as uncertainty related to potential

instability due to long-term storage (u_{lts}) [35]. Using a coverage factor, K , these components can be combined according to Equation 1.:

$$U_{CRM} = K \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2} \quad (1)$$

Values for the individual contributions to the overall uncertainty are listed in Table 1. For u_{har} , standard deviations of the quantification data was used to estimate of the uncertainty. The primary source of error in this case was the variability of the quantitative NMR measurements, with error associated with glassware and pipetting contributing to a lesser extent.

To estimate u_{bb} , ANOVA results from the homogeneity data were used to calculate the variation between bottles, S_{bb} , according to Equation 2:

$$S_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \quad (2)$$

with $MS_{between}$ being the mean squares between groups, MS_{within} the mean squares within groups and n the number of replicates per ampoule. The maximum between-ampoule variability u^*_{bb} was calculated using Equation 3:

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{\nu_{MS_{within}}}} \quad (3)$$

with $\nu_{MS_{within}}$ being the degrees of freedom of MS_{within} [37]. The uncertainty of between-unit homogeneity (u_{bb}) is given by the larger value obtained from either u^*_{bb} or S_{bb} . The estimated value for u^*_{bb} was found smaller than S_{bb} for AZA1, -2 and -3,

therefore S_{bb} was subsequently used as an estimate for u_{bb} . The resulting values for u_{bb} are listed in Table 1.

Although both short-term and long-term stability studies were carried for the AZA calibration solution, typically only the uncertainty of long-term stability associated with storage is included in the certified uncertainty [35]. As described above, although the knowledge of the stability during transport is crucial, shipping conditions must be chosen such that the uncertainty due to instability is negligible [35]. The uncertainty due to long-term storage (u_{lts}) can be estimated by $u_{lts} = u_b x_{shelf\ life}$, where u_b is the standard error of the slope of the stability curve at the recommended storage temperature (-12°C) and $x_{shelf\ life}$ is the shelf-life of the reference material [35]. In our case, u_b was obtained directly from the ANOVA results used to assess stability (see above) and the shelf-life employed was 365 days. The uncertainties associated with long-term storage for the AZA calibration solutions are summarized in Table 1.

Certified values

Using a coverage factor of 2, the certified values of AZA1, -2 and -3 were determined to be $1.47 \pm 0.08 \mu\text{mol/L}$, $1.52 \pm 0.05 \mu\text{mol/L}$, and $1.37 \pm 0.13 \mu\text{mol/L}$, respectively, as summarized in Table 2. These concentration levels are suitable for their intended use, primarily the calibration of LC-MS instrumentation. Although significantly lower in concentration than most other marine toxin certified reference materials, AZAs generate stronger response than most toxins in MS by electrospray ionization (ESI) in positive mode, due to the presence of the amine group in the AZAs' structure.

Conclusions

AZA1,-2 and -3 were purified in sufficient amounts and purity to produce CRMs. Approximately 3600 ampoules containing 0.5 mL of a 1.47 μM solution of AZA1, 988 ampoules of a 1.52 μM solution of AZA2 and 1960 ampoules of 1.37 μM solution of AZA3 were prepared. A purification procedure was developed that yielded sufficient AZA recoveries for effective isolation of AZAs from contaminated mussel tissue. Quantitative NMR techniques were used to accurately certify the concentration of the calibrations solutions. The solutions were shown to be homogeneous and sufficiently stable for use as certified calibration solutions if stored at the recommended conditions of -12°C . In addition to these calibration solutions, the NRC Certified Reference Materials Program (CRMP) is currently developing a mussel tissue CRM with certified values for AZA1, -2, and -3.

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Figure Captions

Figure 1. Structures of the three predominant azaspiracids. AZA1 (R1 = H, R2 = H, R3=CH3); AZA2 (R1 =H, R2= CH3, R3= CH3); AZA3 (R1= H, R2 =H, R3 =H).

Figure 2. Schematic of the isolation procedure used for the production of CRM-AZA1, AZA2 and AZA3.

Figure 3. Comparison of the % recovery of individual isolation steps for the reported extraction and isolation procedure.

Figure 4. LC/MS analysis of NRC CRM AZA1, AZA2 and AZA3 using MRM at m/z 842.5>672.5, 856.5>672.5 and 828.5>658.5 respectively.

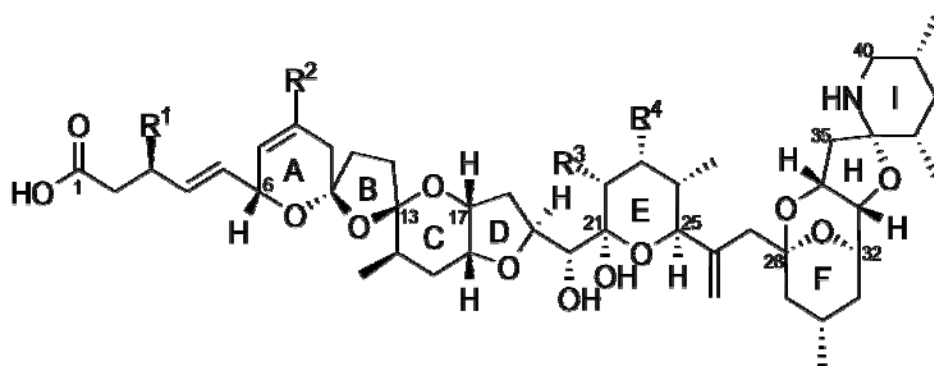
Figure 5. Short-term (a) and long-term (b) stability study data for AZA1, AZA2 and AZA3 at -12°C, 4°C, room temperature (RT; ~ 20°C), and 37°C.

Table 1. Uncertainty components for AZA1, AZA2 and AZA3

Uncertainty component	AZA1 ($\mu\text{mol/L}$)	AZA2 ($\mu\text{mol/L}$)	AZA3 ($\mu\text{mol/L}$)
u_{char}	0.02	0.02	0.05
u_{bb}	0.03	0.01	0.01
u_{lts}	0.02	0.01	0.04
U_{CRM} (k=2)	0.08	0.05	0.13

Table 2. Certified concentrations for AZA1, AZA2 and AZA3

Analyte	Certified concentration ($\mu\text{mol/L}$)
AZA1	1.47 \pm 0.08
AZA2	1.52 \pm 0.05
AZA3	1.37 \pm 0.13



	R^1	R^2	R^3	R^4
Azaspiracid-1 (AZA1)	H	H	CH ₃	H
Azaspiracid-2 (AZA2)	H	CH ₃	CH ₃	H
Azaspiracid-3 (AZA3)	H	H	H	H

Figure 1.

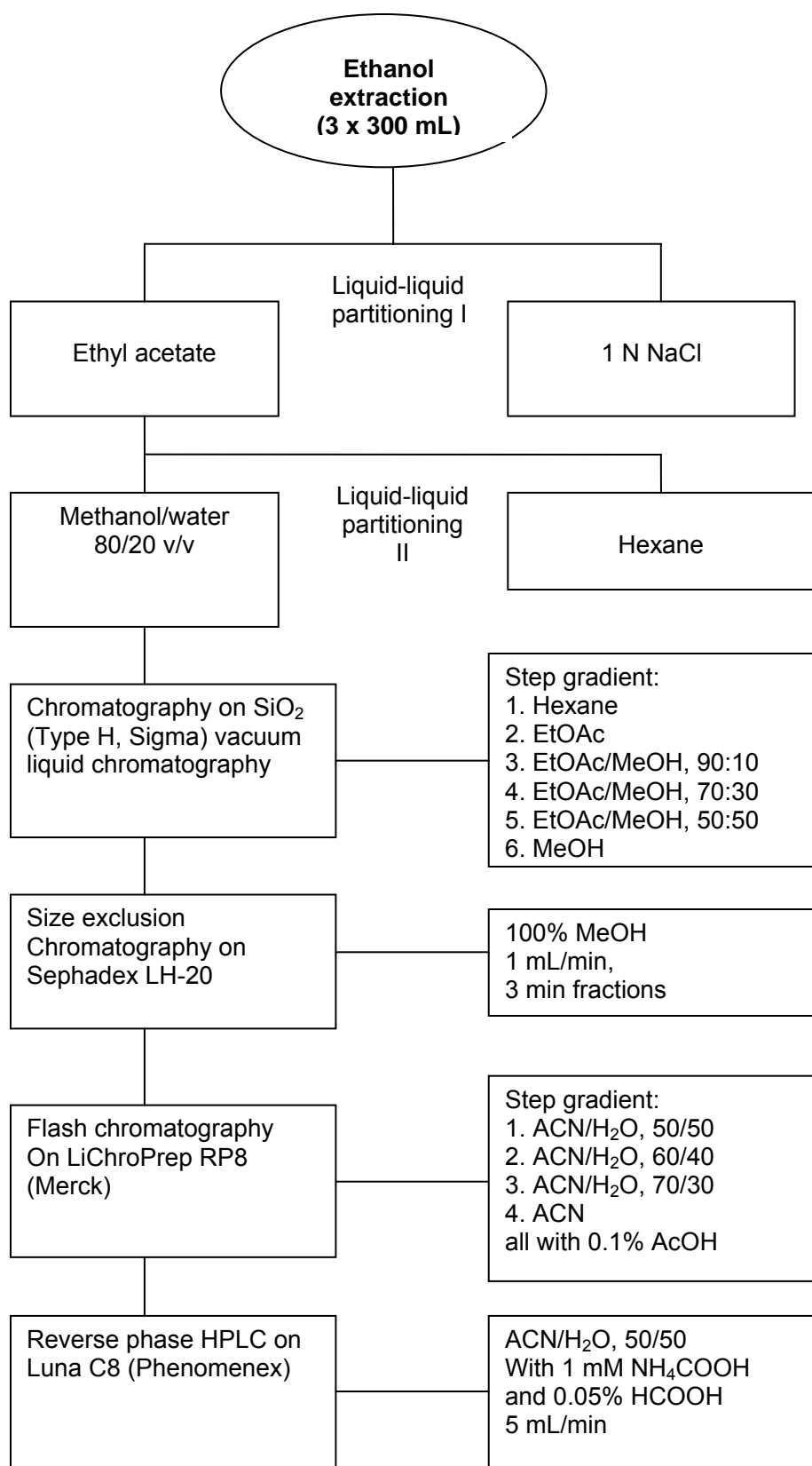


Figure 2.

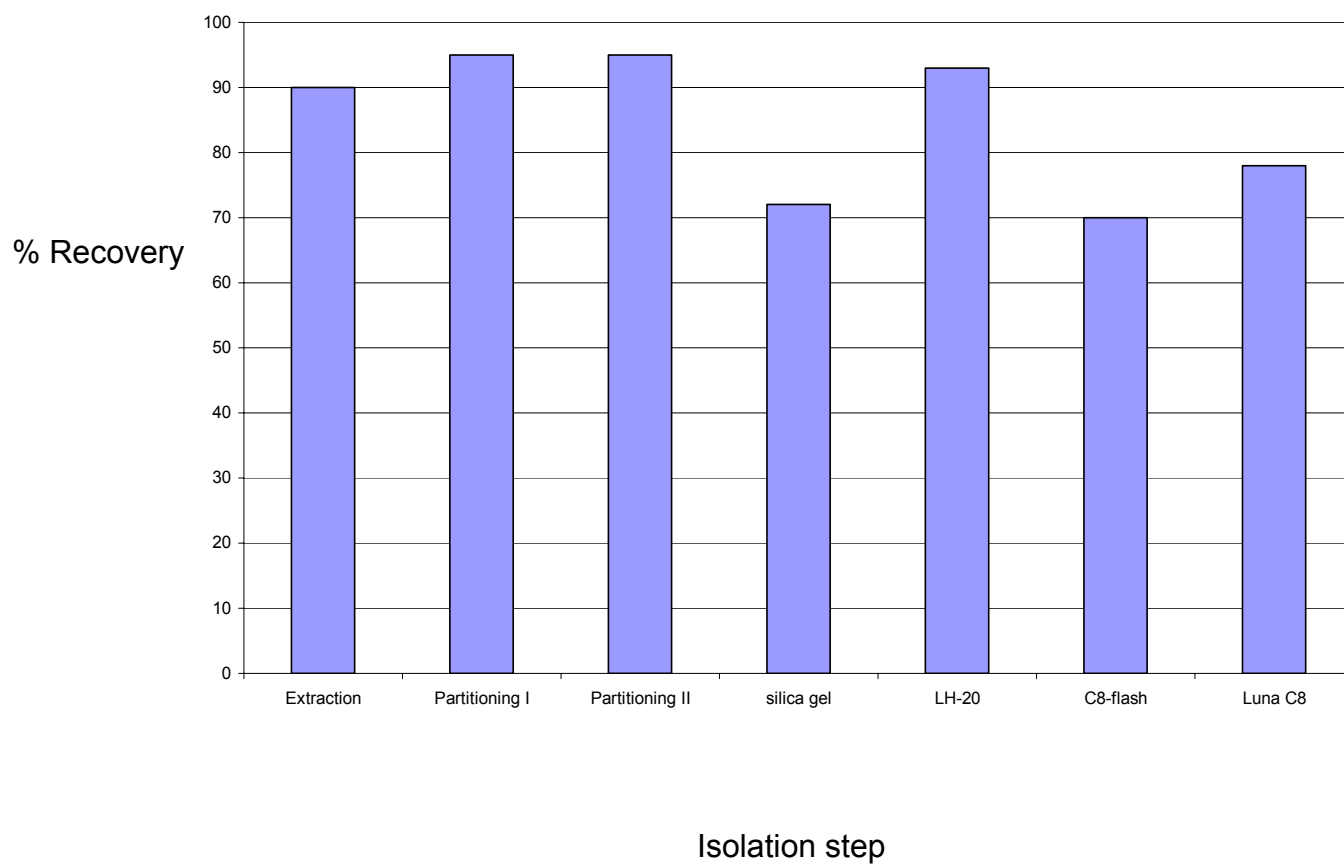


Figure 3.

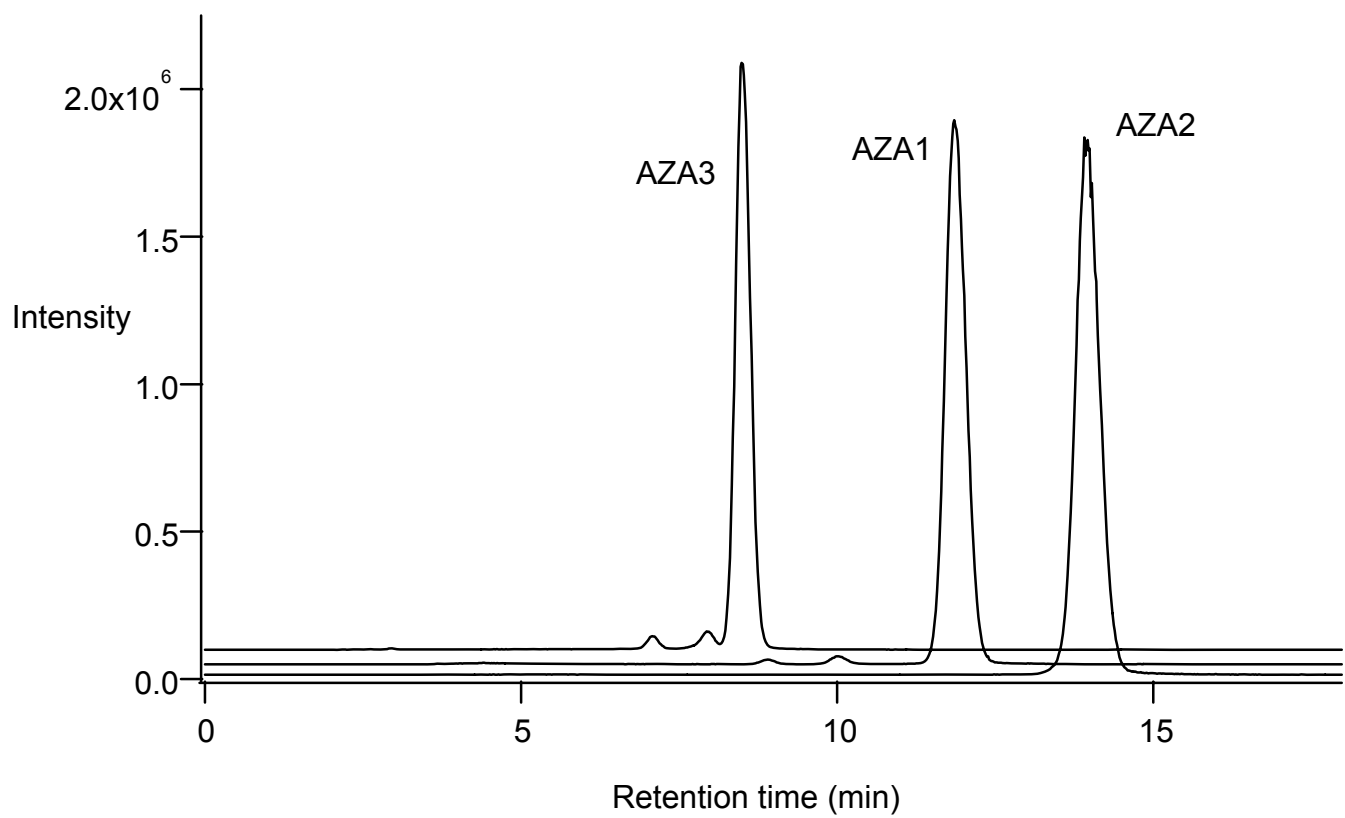


Figure 4.

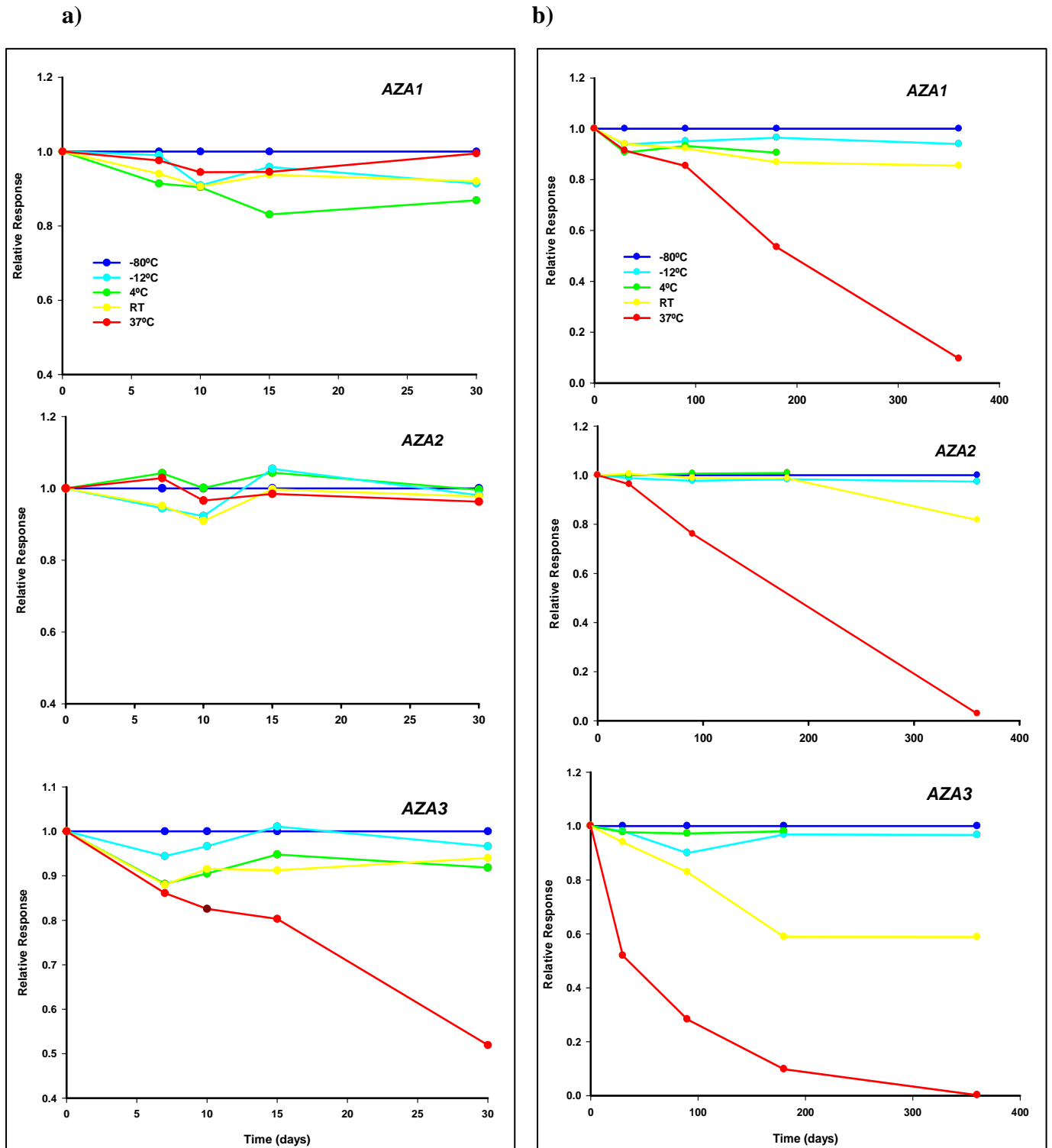


Figure 5.