Certificate of Analysis

NRC·CNRC Certified Reference Material

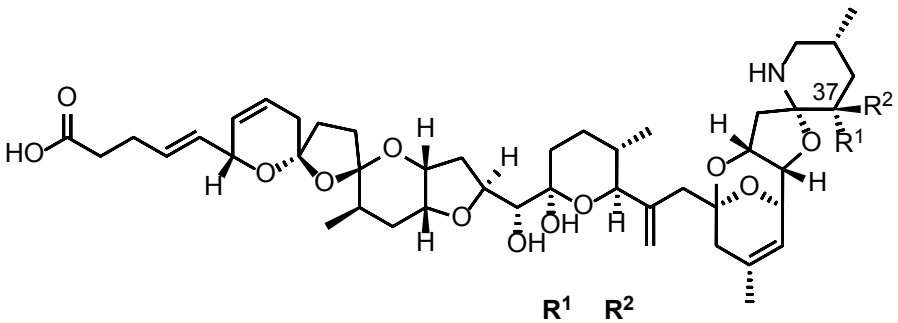
CRM-AZA3-b (Lot# 20150527)

Certified Calibration Solution for Azaspiracid-3

Azaspiracids (AZAs) are a class of shellfish toxins first discovered in Ireland in 1995 after a human intoxication event linked to the consumption of shellfish [1,2]. AZAs possess unique spiro assemblies with both carboxyl and amine moieties [3,4]. The origin of AZAs has been identified as the dinoflagellate *Azadinium spinosum* [5]. Intoxication symptoms can include nausea, stomach cramps, headache, vomiting and diarrhea. The maximum permissible total level of AZAs (AZA1, AZA2, and AZA3) in shellfish is currently 0.16 mg/kg in whole tissue [6]. CRM-AZA3-b is a certified calibration solution of AZA3 in methanol and is a replacement for CRM-AZA3, which was released in 2011.

Table 1: Certified concentration values for CRM-AZA3-b

Compound	µmol/L (at +20 °C)	µg/mL (at +20 °C)	µg/g
Azaspiracid-3 (AZA3 + 37-*epi*-AZA3 sum)	1.43 ± 0.06	1.18 ± 0.05	1.51 ± 0.06



	R^1	R^2
Azaspiracid-3	CH_3	H
37-*epi*-Azaspiracid-3	H	CH_3

Azaspiracid-3
CAS registry No.: 265996-93-8
Molecular formula: $C_{46}H_{69}NO_{12}$
Formula molecular weight: 828.05 g/mol
$[M+H]^+$: monoisotopic mass: 828.4893 Da

Period of validity: 1 year from date of sale
Storage conditions: -12 °C or below

Intended Use

CRM-AZA3-b is designed for use as an instrument calibration solution to aid the analyst in the determination of AZA3 in plankton and shellfish sample extracts. The most widely used method of analysis is liquid chromatography-mass spectrometry (LC-MS) and the concentration of AZA3 in CRM-AZA3-b is suitable for the calibration of most LC-MS instruments. CRM-AZA3-b can also be used for spiking shellfish control samples for recovery experiments.

Instructions for Storage and Use

To ensure the stability of CRM-AZA3-b, ampoules should be stored at -12 °C or below. Likewise, any aliquots or dilutions of this material should be stored in low-headspace vials at -12 °C.

Prior to opening, each ampoule should be allowed to warm to room temperature and the contents thoroughly mixed. The ampoules should be opened at the pre-scored mark. Once an ampoule has been opened, accurate aliquots should be removed with calibrated volumetric equipment and transferred to volumetric flasks or vials. An increase in concentration due to evaporation of methanol will occur if the solution is left opened for more than a few minutes. It is recommended that solutions of CRM-AZA3-b should not be evaporated to dryness, due to potential decomposition on glass surfaces. *Note:* The volume of the solution is not certified; only the concentration is certified. Therefore, the entire contents of the ampoule should not simply be transferred to a volumetric flask and diluted to volume.

Preparation of CRM-AZA3-b

AZA3 was isolated from shellfish tissue collected in 2005 at Bruckless, Co. Donegal, Ireland. The preparative extraction and purification of AZA3 was carried out at the Marine Institute, Ireland, with final clean-up and purity checks performed at the National Research Council, Halifax. The structure and purity of the AZA3 was confirmed by NMR and LC-MS/MS. No significant impurities were detected. A measured accurate mass of 828.4877 +/- 0.0002 (Δ = -1.8 ppm for $C_{46}H_{70}NO_{12}$) was obtained for the $[M+H]^+$ of AZA3 using high-resolution MS. Figure 1 shows the high-resolution MS product ion spectrum.

A stock solution was prepared by dissolving the AZA3 in CD_3OH for quantitative NMR (qNMR). The CRM solution was prepared by making an accurate dilution of the qNMR stock solution in degassed, high purity methanol. This solution was thoroughly mixed with a Teflon stir bar and magnetic mixer while being cooled in an ice bath under an argon atmosphere. Aliquots were dispensed into clean argon-filled amber glass ampoules, which were then flame-sealed immediately.

Analytical Methods and Value Assignment

The certified value for CRM-AZA3-b is based on the results obtained at NRC using two independent methods: qNMR [7] on the stock AZA3 solution using high purity benzoic acid standards for external calibration and LC-MS/MS with a neutral pH mobile phase using CRM-AZA3 (Lot# 20081210) for calibration. It is important to note that the certified value of CRM-AZA3-b was calculated as a sum of the concentrations of AZA3 and an epimer, 37-*epi*-AZA3 [8]. The epimer is resolved from AZA3 with a neutral mobile phase-column system (Figure 2A). With some more commonly used mobile phase-column systems (Figure 2B), the compounds may be unresolved. Equimolar response factors were assumed when combining the peak area of AZA3 and 37-*epi*-AZA3 on the basis of previous studies [8]. The 37-*epi*-AZA3 peak area corresponds to approximately 6% of the total peak area of AZA3 and 37-*epi*-AZA3. Trace amounts of AZA6 and other AZA isomers are present in CRM-AZA3-b (Table 2).

Table 2: AZA impurities present in CRM-AZA3-b (values are not certified)

Compound	Relative Retention Time*	$[M+H]^+$, *m/z*	Relative %*
AZA6	1.05	842.5	0.25
AZA isomer	1.08	842.5	0.45
AZA isomer	1.11	842.5	0.07

* Relative to AZA3 using conditions described for Figure 2A

Homogeneity

A representative number of CRM-AZA3-b ampoules (n = 28) were selected from across the entire fill series and AZA3 concentrations measured by LC-MS/MS. Results were evaluated using ANOVA and no heterogeneity was detected across the entire fill series.

Stability Study

Short and long-term stability studies were conducted for CRM-AZA3. The production process and concentrations are equivalent for CRM-AZA3-b, therefore comparable stability is reasonably assumed. Stability studies showed no significant degradation of AZA3 in methanol at -12°C or lower over a period of one year. AZA3 exhibited significant instability with 20% degradation occurring at +37ºC after 10 days, degrading approximately 50% after 30 days. In the long-term stability study, greater than 40% degradation was observed after 360 days at +20°C and nearly 90% after 180 days at +37ºC.

Uncertainty

All reasonable sources of error related to the characterization of CRM-AZA3-b were considered and quantitated. A combined uncertainty component relating to the two analytical methods used is included (u_{char}). The overall uncertainty estimate (U_{CRM}) includes uncertainties associated with batch characterization (u_{char}), between-bottle variation (u_{hom}), and instability during long-term storage (u_{stab}) [9] . These components are listed in Table 3, and are combined and expanded as follows:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{hom}^2 + u_{stab}^2}$$

where *k* is the coverage factor for a 95% confidence level (= 2).

Table 3: Uncertainty components for the certified value of CRM-AZA3-b

Uncertainties	[µmol/L]
u_{char}	0.03
u_{hom}	negligible
u_{stab}	0.006
U_{CRM} (k = 2)	0.06

Safety Instructions

AZA3 is toxic when swallowed or inhaled. If ingested, AZA3 can cause nausea, vomiting, diarrhea and stomach cramps. Inhalation and ingestion of methanol are harmful; ingestion may cause blindness or death, while prolonged skin contact may result in dermatitis and/or kidney damage. Only qualified personnel should handle the solution and appropriate disposal methods should be used. Gloves and eye protection should be used when opening the ampoule. An MSDS is available for CRM-AZA3-b.

Period of Validity

If stored unopened at the recommended conditions, the certified concentration of CRM-AZA3-b is valid for 1 year from the date of sale. The label on the original packaging includes the period of validity.

Metrological Traceability

Results presented in this certificate are traceable to the SI (*Système international d'unités*) through gravimetrically prepared standards of established purity.

Quality Management (ISO/IEC 17025, ISO Guide 34)

This material was produced in compliance with the documented National Research Council of Canada (NRC) Measurement Science and Standards (MSS) Quality System, which conforms with the requirements of ISO/IEC 17025 and ISO Guide 34.

The MSS Quality System supporting NRC calibration and measurement capabilities, as listed in the Bureau international des poids et mesures (BIPM) key comparison database (http://kcdb.bipm.org/), has been reviewed and approved under the authority of the Inter-American Metrology System (SIM) and found to be in compliance with the expectations of the Comité international des poids et mesures (CIPM) Mutual Recognition Arrangement. The SIM certificate of approval is available upon request.

References

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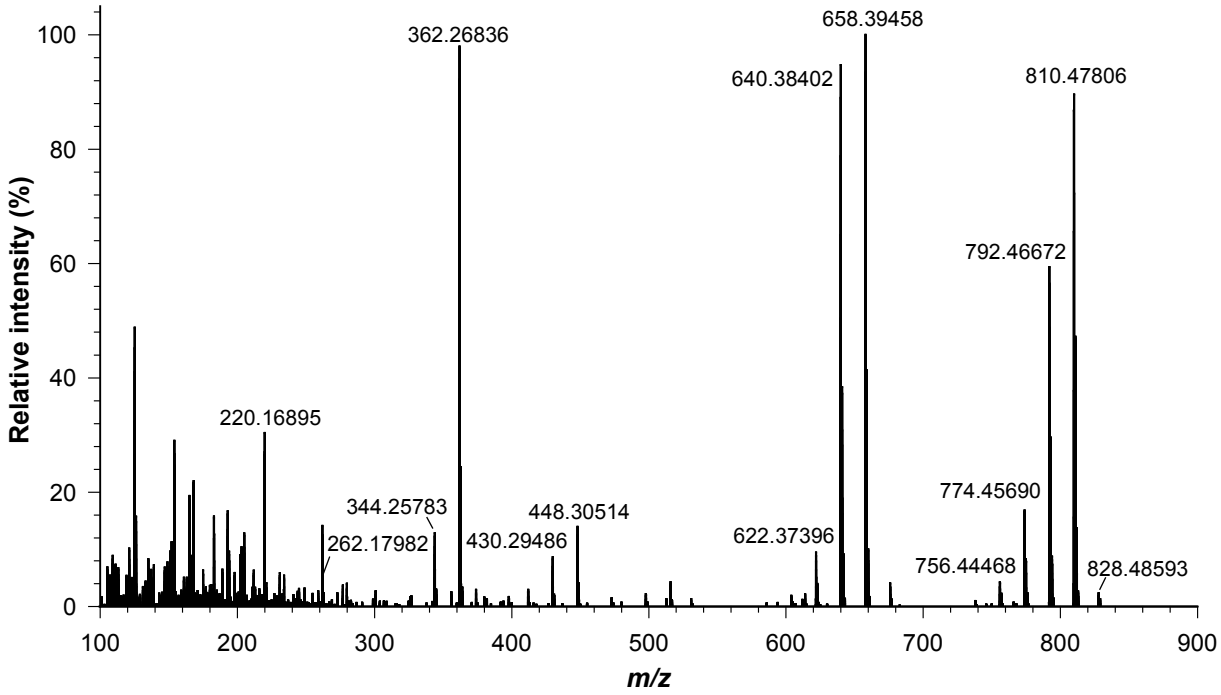


Figure 1: High-resolution MS product ion spectrum of the $[M+H]^+$ ion, *m/z* 828.5, of AZA3 in CRM-AZA3-b measured on a Thermo Exactive Orbitrap mass spectrometer equipped with a heated electrospray ionization probe. Data acquired in positive ion mode with a 3.0 kV spray voltage, +250 °C capillary temperature, and +300 °C heater temperature. This higher energy collisional dissociation scan (70 eV HCD) was obtained at the 50,000 resolution instrument setting (2 Hz scan rate).

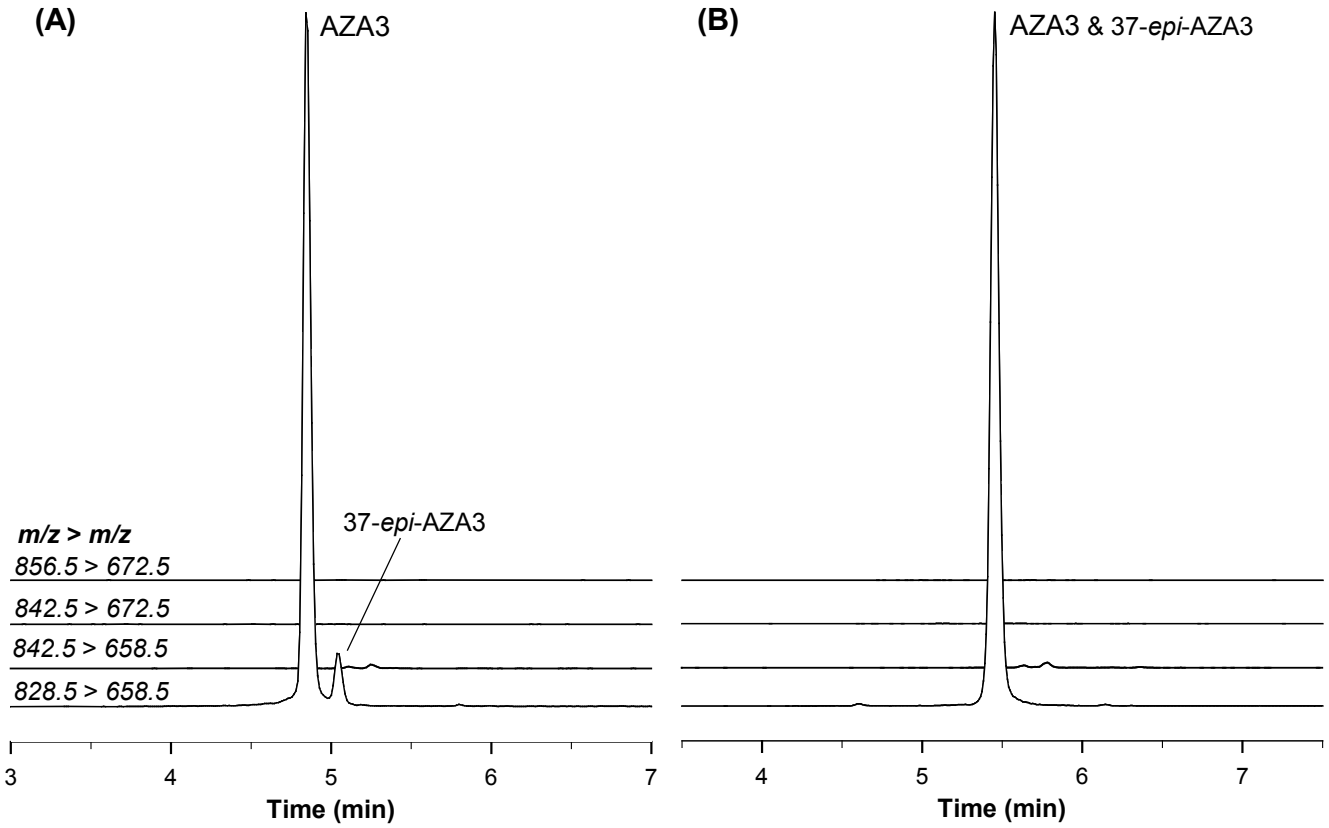


Figure 2: LC-MS/MS chromatograms of CRM-AZA3-b using neutral pH (A) and acidic pH (B) mobile phases, showing resolution and co-elution of AZA3 and 37-*epi*-AZA3, respectively. Neutral conditions: Luna C18(2) column (2.0 × 50 mm, 2.5 µm); mobile phase: 5 mM ammonium acetate (pH 6.8) in both deionised water (A) and 95% acetonitrile (B); gradient: 25 -100% B over 5 min, 350 µL/min at +15 °C; injection volume: 1 µL. Acidic conditions: Luna C18(2) column (2.0 × 50 mm, 2.5 µm); mobile phase: 2 mM ammonium formate and 50 mM formic acid (pH 2.3) in both deionised water (A) and 95% acetonitrile (B); gradient: 25 -100% B over 5 min, 300 µL/min at +20 °C; injection volume: 5 µL.

Acknowledgements

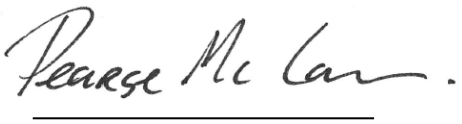
The following staff members of Measurement Science and Standards at NRC contributed to the production and certification of CRM-AZA3-b: S. Crain, D. Beach, S.D. Giddings, W. Hardstaff, P. LeBlanc, N. Lewis, P. McCarron, R.A. Perez, K. Thomas.

Jane Kilcoyne, Conor Duffy and Joe Silke of the Marine Institute (Galway, Ireland) are acknowledged for supply of purified AZA3.

This document should be cited as:

R.A. Perez, S. Crain, P. LeBlanc, M.A. Quilliam, P. McCarron, "CRM-AZA3-b, certified calibration solution for azaspiracid-3" Biotoxin Metrology Technical Report CRM-AZA3-b-20150527, National Research Council Canada, Halifax.

Date of issue: December 2016
Document version: 20161209

Approved by: 

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