National Research Council Canada | Conseil national de recherches Canada

Certificate of Analysis

Certified Reference Material

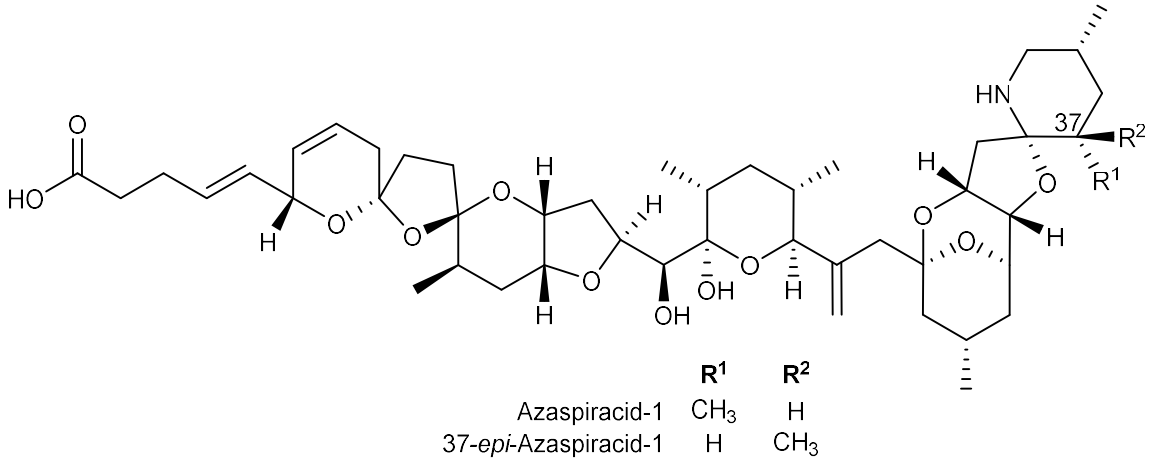
CRM-AZA1-c (Lot# 20220525)

Certified Calibration Solution for Azaspiracid-1

Azaspiracids (AZAs) are a potent class of algal toxins produced by the dinoflagellate *Azadinium spinosum* [1] that possess unique spiro assemblies with both carboxyl and amine moieties [2,3]. CRM-AZA1-c is a certified calibration solution of azaspiracid-1 (AZA1) in methanol and is a replacement for the CRM-AZA1-b.

Table 1: Certified concentration and uncertainty for CRM-AZA1-c.

Compound	µg/g	µg/mL (15 - 30 °C)	µmol/L (15 - 30 °C)
Azaspiracid-1 (AZA1 + 37-*epi*-AZA1 sum)	1.54 ± 0.08	1.22 ± 0.06	1.44 ± 0.07



Azaspiracid-1
CAS registry No.: 214899-21-5
InChIKey: BVZWTQCTAVYACS-KVGHCGRPSA-N
Molecular formula: $C_{47}H_{71}NO_{12}$
Molecular weight: 842.1 g/mol
$[M+H]^+$: *m/z* 842.5049

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

Canada

Intended Use

CRM-AZA1-c is a certified calibration solution for analytical method development and accurate quantitation of AZA1. The concentration is suitable for preparing a dilution series for calibration of instruments such as liquid chromatography with detection by mass spectrometry (LC–MS), as well as for spiking control samples for recovery experiments.

Instructions for Storage and Use

To ensure the stability of CRM-AZA1-c, ampoules should be stored at −12 °C or below.

It is important to note that volume of the solution is not certified. Only the concentration is certified. Therefore, the ampoule contents should not simply be transferred to a volumetric container and diluted to volume.
Prior to opening, each ampoule should be allowed to equilibrate to room temperature and the contents thoroughly mixed. The CRM solution should be transferred using calibrated equipment for accuracy. Repeated sub-sampling and storage of the CRM solution after initial opening may impact concentration values. However, users may take responsibility for demonstrating that their sub-sampling and storage procedures do not impact concentrations.

Preparation of CRM-AZA1-c

Azaspiracid-1 was obtained from Marine Institute (Rinville, Oranmore, Co. Galway, Ireland). The structure and purity of AZA1 was confirmed by LC–MS (Figures 1 and 2), LC with charged aerosol detection and nuclear magnetic resonance (NMR) spectroscopy. A measured accurate *m*/*z* of 842.5041 (Δ = -0.9 ppm for $C_{47}H_{72}NO_{12}^{+}$) was obtained for the $[M+H]^{+}$ ion of AZA1 using LC–high-resolution MS (LC–HRMS).

The stock solution was prepared by dissolving the purified AZA1 in CD_3OD for quantitation using ^{1}H NMR (qNMR) [4]. The CRM-AZA1-c solution was prepared by accurately diluting the stock solution in degassed high purity methanol. Aliquots were dispensed into clean argon-filled amber glass ampoules and immediately flame-sealed. Each ampoule contains approximately 0.5 mL.

Analytical Methods and Value Assignment

The certified value for CRM-AZA1-c (Table 1) is based on results obtained at the NRC with qNMR using benzoic acid for calibration and LC-MS/MS using CRM-AZA1-b as the calibrant.
CRM-AZA1-c contains low levels of AZA analogues, including AZA2 and AZA3, which combined are less than 0.3% of the concentration of AZA1. The proportion of 37-*epi*-AZA1 in CRM-AZA1-c is approximately 8% of the total concentration of AZA1 and 37-*epi*-AZA1.

Homogeneity

A representative number of CRM-AZA1-c ampoules were selected from across the fill series and AZA1 response was measured by LC–MS/MS. No heterogeneity was observed.

Stability

Studies with AZA1 in methanol have demonstrated good stability stored in sealed ampoules at temperatures of −12 °C and below.

Uncertainty

All reasonable sources of uncertainty related to the characterization of CRM-AZA1-c were considered and measured. The overall uncertainty estimate (U_{CRM}) includes uncertainties associated with batch characterization (u_{char}) and instability during storage (u_{stab}) [5]. These components are listed in Table 2, 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 2: Uncertainty components for the certified value of CRM-AZA1-c.

Uncertainties	Relative*
u_{char}	0.023
u_{hom}	negligible
u_{stab}	0.009

*Relative to concentration shown in Table 1.

Safety Instructions

Only qualified personnel should handle the solution and appropriate disposal methods should be used. Suitable personal protective equipment should be used when opening the ampoule in the event glass shatters. A safety data sheet (SDS) is available for CRM-AZA1-c.

Period of Validity

If stored unopened at the recommended storage condition of −12 °C or below, the certified concentration of CRM-AZA1-c is valid for 1 year from the date of sale.

Metrological Traceability

Results presented in this certificate are traceable to the SI (*Système international d'unités*) through a gravimetrically prepared standard of NIST benzoic acid certified reference material (PS1), and NRC CRM-AZA1-b (lot # 20131120).

Quality Management System (ISO 17034, ISO/IEC 17025)

This material was produced in compliance with the National Research Council of Canada (NRC) Metrology Quality Management System, which conforms to the requirements of ISO 17034 and ISO/IEC 17025.

The Metrology Quality Management System supporting the 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 approval is available upon request.

References

1. Tillmann U, Elbrachter M, Krock B, John U, Cembella A (2009). *Azadinium spinosum* gen. et sp. nov. (*Dinophyceae*) identified as a primary producer of azaspiracid toxins. Eur J Phycol 44 (1):63-79. doi:10.1080/09670260802578534

2. Satake M, Ofuji K, Naoki H, James KJ, A. F, McMahon T, Silke J, Yasumoto T (1998). Azaspiracide, a new marine toxin having unique sprio ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J Am Chem Soc 120:9967-9998. doi:10.1021/ja981413r

3. Kenton NT, Adu-Ampratwum D, Okumu AA, McCarron P, Kilcoyne J, Rise F, Wilkins AL, Miles CO, Forsyth CJ (2018). Stereochemical definition of the natural product (6R,10R,13R, 14R,16R,17R,19S,20S,21R,24S,25S,28S,30S,32R,33R,34R,36S,37S,39R)-azaspiracid-3 by total synthesis and comparative analyses. Angew Chem Int Edit 57 (3):810-813. doi:10.1002/anie.201711008

4. Burton IW, Quilliam MA, Walter JA (2005). Quantitative ^{1}H NMR with external standards: Use in preparation of calibration solutions for algal toxins and other natural products. Anal Chem 77:3123-3131. doi:10.1021/ac048385h

5. Pauwels J, Lamberty A, Schimmel H (2000). Evaluation of uncertainty of reference materials. Accredit Qual Assur 5:95-99. doi:10.1007/s007690050020

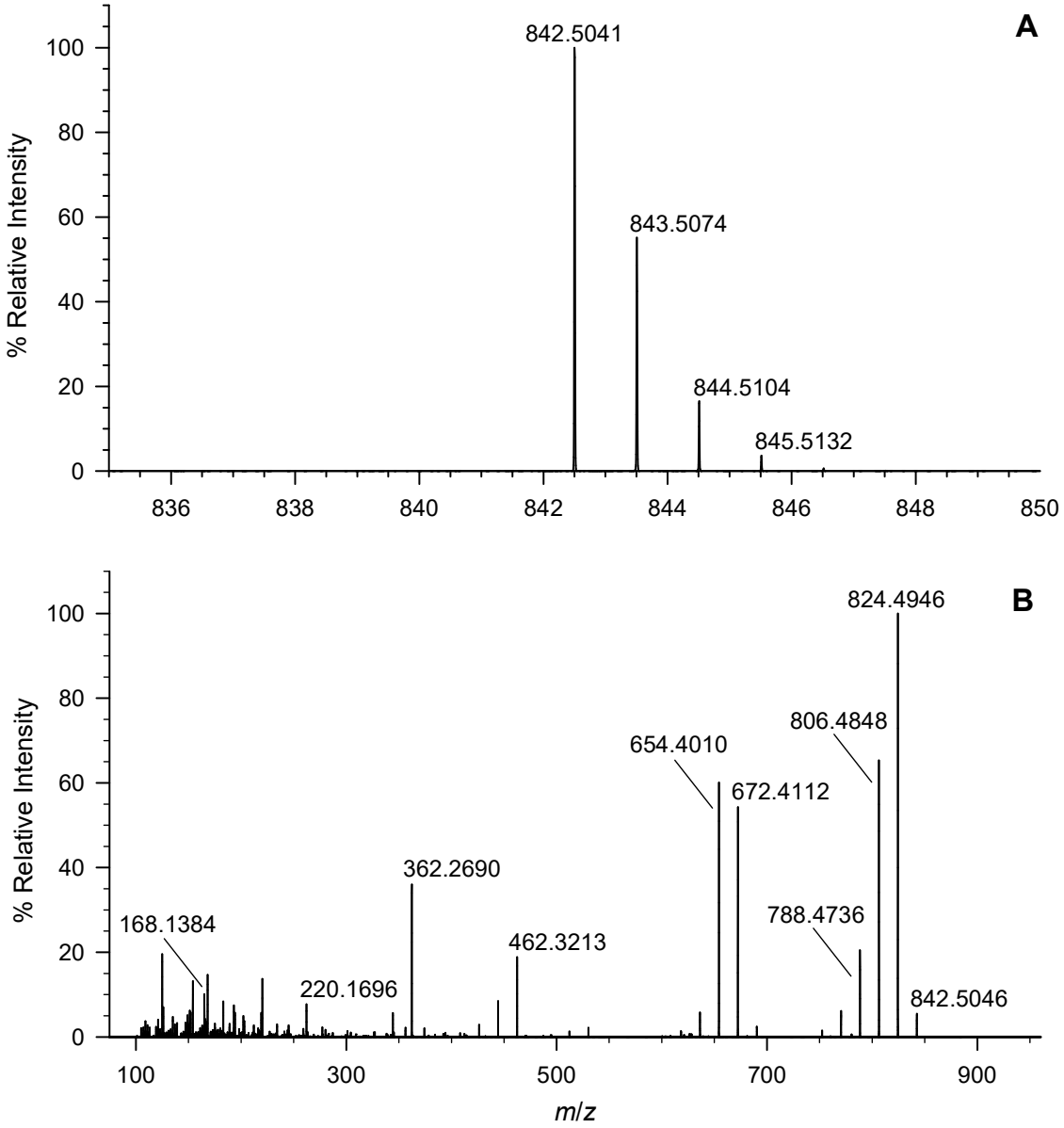


Figure 1: Full-scan (A) and collision-induced dissociation (MS/MS) (B) LC–HRMS spectra of AZA1 used for preparation of CRM-AZA1-c, acquired using a Q Exactive-HF mass spectrometer in positive mode. Full-scan data was acquired with a resolution setting of 120 000. MS/MS data was acquired in parallel reaction monitoring scan mode with the same resolution setting using a stepped collision energy of 60, 65, 70 V.

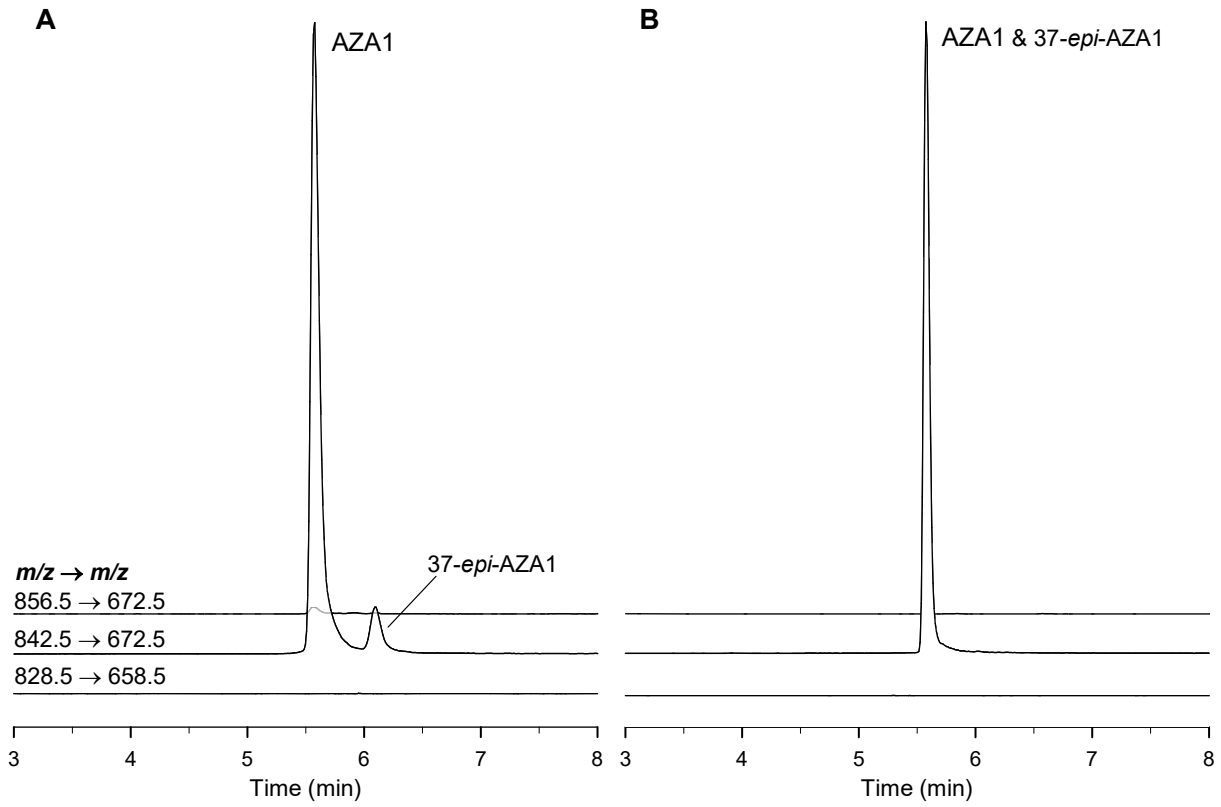


Figure 2: LC–MS/MS analysis of CRM-AZA1-c using neutral pH (A) and acidic pH (B) mobile phases, showing the resolution and co-elution of AZA1 and 37-*epi*-AZA1, respectively. Conditions: both methods used a Luna C18(2) column (50 × 2.1 mm i.d., 2.5 µm) with 1 µL injection volumes. Neutral pH method: mobile phase: 5 mM ammonium acetate (pH 6.8) in both deionized water (a) and 95% acetonitrile (b); gradient 25-100% b over 5 min, 350 µL/min at +20°C. Acidic pH method: mobile phase: 2 mM ammonium formate and 50 mM formic acid (pH 2.3) in both deionized water (a) and 95% acetonitrile (b); gradient 25-100% b over 5 min, 350 µL/min at +20°C.

Acknowledgements

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This Certificate is only valid if the corresponding material was obtained directly from the NRC or an Authorized Reseller.

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