Certificate of analysis

Certified reference material code: CRM-02-AZA1

Name: Certified Reference Material of Azaspiracid-1 Batch: 23-001

Description: This Certified Reference Material (CRM) is a solution of Azaspiracid-1 (CAS Number 214899-21-5) in methanolic solution.

Intended use: Calibration of equipment or a measurement procedure. Establishing metrological traceability. Method validation. Quality control of a measurement or measurement procedure. For laboratory use only.

Certified values and uncertainties: The certified concentration given below is based on results obtained from the gravimetric preparation of this solution previous quantity determination by ^{1}H-qNMR in Varian WB 500 MHz equipment (Universidad de Santiago de Compostela).

Concentration of Azaspiracid-1 and 37-epi-Azaspiracid-1 (95% Confidence Interval)	**(1.67 ± 0.16) µmol / kg**
	(1.40 ± 0.14) µg / g

NOTE: In order to perform the analysis, it should be noted that the certificate value corresponds to the sum of two epimers concentration and therefore the sum of the areas of the two chromatographic signals should be considered. See #Non-certified and non-accredited data section of this certificate.

The starting material is measured by ^{1}H-qNMR against a Benzoic acid certified reference material followed by gravimetric preparation using high precision balances calibrated with SI-traceable weights. Consequently, the quantified values are traceable to the International System of Units (SI) via an unbroken chain of comparisons through validated methodology.

Homogeneity was evaluated by analysis of variance on 2% of stratified randomly selected ampoules over the entire batch (6 replicate analyses per ampoule). A possible heterogeneity is hidden by the method repeatability. Therefore, a maximum possible heterogeneity was calculated as u_{bb}, 1.03%. The Stability studies after storage of selected units were performed at -20°C for a storage time of 12 months. Using the data from the long-term study, the uncertainty due to possible degradation was calculated as u_{lts}, 2.16%. Both studies were verified against an independently prepared calibration solution.

The components of Standard Uncertainty include related uncertainties due to characterization, heterogeneity, long term instability, short term instability (dispatch), and bulk assay, Table 1.

The results are expressed as the certified value ± the expanded uncertainty (calculated by combination of the squared contribution values). Estimated expanded uncertainty with a coverage factor k = 2, corresponding to a level of confidence of about 95 %, as defined in the Guide to the expression of uncertainty in measurement, ISO33405.

The certificate is a summary of an extensive program of work involving selection and purification of the material, assessing its suitability and measuring the properties to be certified.

Expiration of Certification: *This certificate of CRM-02-AZA1, 23-001 batch is valid 12 months after purchase date within the measurement uncertainties specified*, provided the CRM is handled and stored in accordance with the instructions given in this certificate. This validity may be extended if further evidence of stability becomes available.

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Maintenance of Certification: The material will be subjected to regular Laboratorio CIFGA stability monitoring programme to control its further stability over the period of its certification. If substantive changes occur that affect the certification before the expiration date, Laboratorio CIFGA will notify the purchaser.

Instructions for proper handling use: This material contains toxic material, and should be handled with care. Read MSDS before using. Material Safety Data sheet for information regarding CRM-02-AZA1 is enclosed in the package. Use proper disposal methods.

Before opening an ampoule, it must be guaranteed that the content is a liquid and properly mixed to ensure homogeneity. Sample aliquots for analysis should be handled at 15ºC to 25ºC immediately after opening the ampoules and should be processed without delay for the certified value to be valid within the stated uncertainty. Ampoule should not remain open longer than five minutes due to solvent volatility and air contamination.

Storage conditions: The original sealed ampoules should be stored at temperatures below/at -20 ºC (freezer) until use. Unopened ampoules should be stored upright under normal laboratory conditions inside the original container supplied.

Source and Preparation of Material: Azaspiracid-1 was isolated from samples of mussels collected during outbreak in the coast of Ireland and purified via preparative HPLC-MS/MS. After purification, identity of the material was confirmed by ^{1}H-NMR and UPLC-MS/MS, Figures 1, 2 and 3. Finally, 0.5 mL aliquots of AZA1 solution were dispensed into 2-mL amber argon-filled glass ampoules, which were then flame sealed.

[#]Non-certified and non-accredited data:

Certified concentration is the sum of epimeric pair: Azaspiracid-1 and 37-epi-Azaspiracid-1. Concentration of 37-epi-Azaspiracid-1 is (0.06 ± 0.01) µg/g (4.7%). *Percentage value not certified.*

Taking into account the density of methanol at 20 ºC ($\rho^{20} = 0.7915$ g/mL), *non-certified volumetric concentration* values are:

Concentration of Azaspiracid-1 and 37-epi-Azaspiracid-1 (95% Confidence Interval)	**(1.32 ± 0.13) µM**
	(1.11 ± 0.11) µg / mL

Concentration of Azaspiracid-1 and impurities are neglected in order to apply solution density value.

Purity analysis was performed by ^{1}H-qNMR and UPLC MS/MS. This material presents a low level of Azaspiracid-2 isobaric analogue (0.2 %), Azaspiracid-2 (0.1%). Tentative Azaspiracid-1 methyl ester, Azaspiracid-3 and other azaspiracid analogues at trace level (<0.1%). Material molar ratio purity is ≥ 99 %. *Percentage values not certified*.

Additional lectures:

1. UNE-EN ISO 17034 *General Requirements for the competence of reference material producers*.

2. ISO 33405 *Reference materials - Approaches for characterization and assessment of homogeneity and stability*.

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Figures

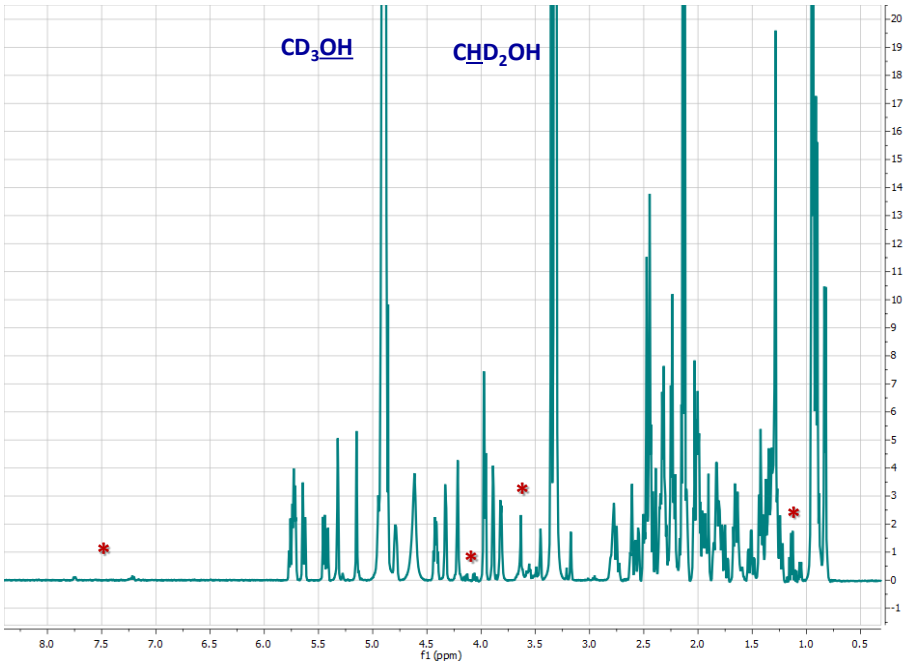

Figure 1. ^{1}H-NMR spectrum of AZA-1 solution, Varian 500 MHz WB, 2 mM of AZA-1 in CD_3OH used in preparation of CRM-02-AZA1 batch 23-001. (*) No identified impurities are highlighted. As the structure of these impurities is not known, their exact amount cannot be calculated. Because the impurities usually are smaller molecules (otherwise, more NMR signals would arise), it can be estimated from the corresponding integrals, but this purity is not relative to close-related impurities to Azaspiracid analogues.

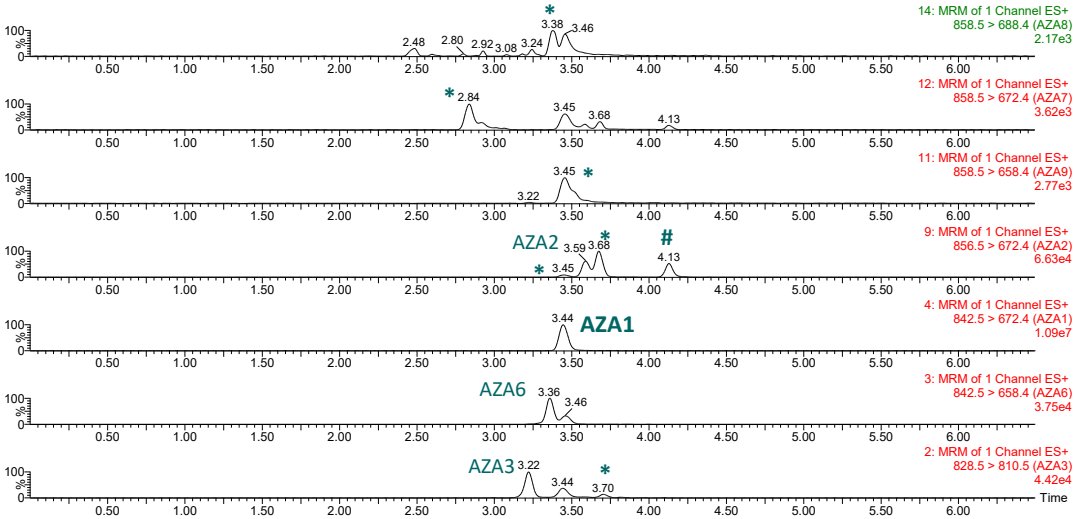

Figure 2. Analysis of the **CRM-02-AZA1** (23-001) by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7µm, 100x2.1 mm, UPLC column. Elution conditions, gradient mode with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 2mM ammonium formate and 50mM formic acid; Gradient: 0.00 min 30% B; 3.00 min 90% B; 4.50 min 90% B; 4.51 min 30% B; 6.50 min 30% B; flow rate: 0.40 mL/min. Temperature: 30 °C. MS detector: Waters XEVO TQ-MS using the typical MRM transitions of Azaspiracid family. Traces of minor and non-identified (*) analogues are highlighted. (#) identified as tentative AZA1 methyl ester analogue

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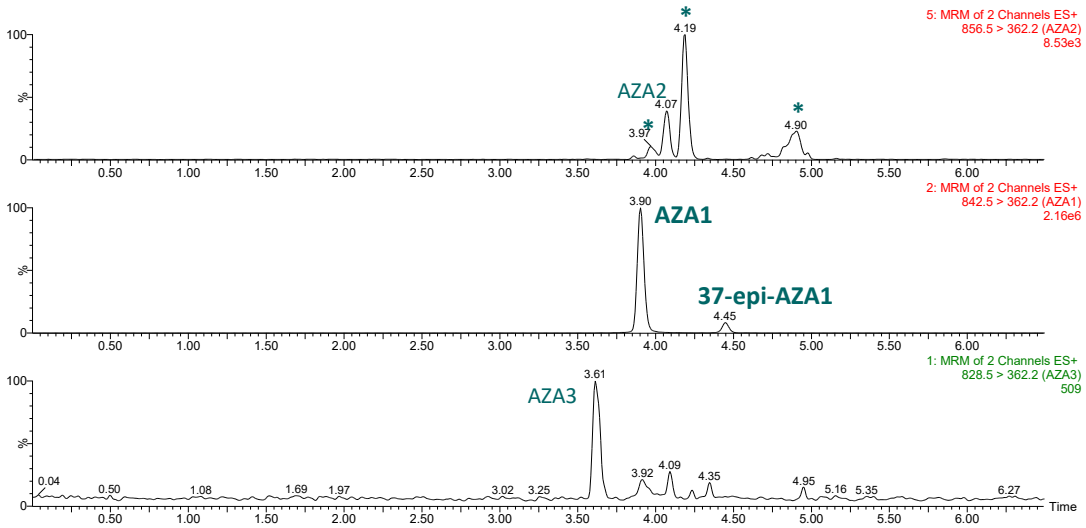


Figure 3. Analysis of the **CRM-02-AZA1** (23-001) by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7µm, 100x2.1 mm, UPLC column. Elution conditions, gradient mode with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 5 mM ammonium acetate, pH = 6.85; Gradient: 0.00 min 20% B; 1.00 min 20% B; 4.00 min 90% B; 5.00 min 90% B; 5.10 min 20% B;6.50 min 20% B; flow rate: 0.50 mL/min. Temperature: 35 °C. MS detector: Waters XEVO TQ-MS using the typical MRM transitions of Azaspiracid family. Traces of minor and non-identified (*) analogues are highlighted.

Table 1. Uncertainties contribution for the certification of **CRM-02-AZA1 (23-001)**. ***Char**: qNMR quantification; **ba**: bulk assay; **bb**: homogeneity test; **lts**: long term stability test; **sts**: short term stability test.*

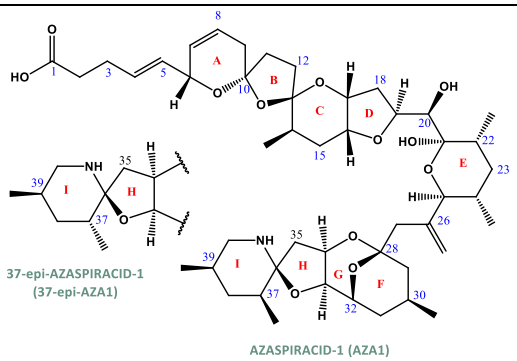
Uncertainty contribution parameter	Uncertainty values Azaspiracid-1 + 37-epi-Azaspiracid-1*
u_{char}/%	2.28
u_{ba}/%	0.60
u_{bb}/%	1.03
u_{lts}/%	2.16
u_{sts}/%	3.48
Expanded uncertainty (*k*=2) / %	9.68

Short-term stability study. The uncertainty due to transport instability was estimated from the test for slope significance at 40°C. The results indicated continued stability of total epimeric pair, no change is expected in the property values of the CRM. The combined uncertainty budget of the assigned value was upgraded to incorporate short-term stability at 40°C.

Time-efficient dispatch for CRM-02-AZA1 is settled in 3 or 6 days based on a structural stability assessment for target boxes under cooled conditions.

***** Azaspiracid-1 + 37-epi-Azaspiracid-1 have been quantified jointly.

Table 2. Molecular information.

Name	AZASPIRACID-1
CAS NUMBER	214899-21-5
Molecular formula	$C_{47}H_{71}NO_{12}$
Molecular Weight	842.08 g·mol^{-1}
Monoisotopic molecular weight	841.497 g·mol^{-1}
2D structure	

Inquiries concerning this Reference Material should be directed to:

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RM PRODUCER'S APPROVING OFFICER:

Certification Manager: Álvaro Antelo

This Certificate is only valid if the product was obtained directly from Laboratorio CIFGA S.A. or one of our authorized distributors.

Certificate Revision History:

February, 24th 2023 (Original certificate issue date, revision 1).

January, 12th 2026. Correction of the originally AZA1 reported concentration due to a typographical error, the correction does not affect the metrological traceability of the material. Address update. Revised and added associated uncertainties following additional testing. *(certificate rev. 2).*

NOTE: This certificate of analysis shall not be reproduced, except in full, without written approval of Laboratorio CIFGA S.A.

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