The marked activities (#) are not covered by ENAC accreditation

Certificate of analysis

Certified reference material code: CRM-02-AZA2

Name: **Azaspiracid-2 in methanol.** Batch: **19-001**

Description: This Certified Reference Material (CRM) is a solution of Azaspiracid-2 (CAS Number 265996-92-7) 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 Bruker AVANCE NEO 750 equipment (USC).

Concentration of Azaspiracid-2 and 37-epi-Azaspiracid-2 (95% Confidence Interval)	**(1.50 ± 0.12) µmol / kg**
	(1.29 ± 0.10) 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 for additional information.

The starting material is measured by ^{1}H-qNMR against a Benzoic acid TraceCERT certified reference material (Sigma-Aldrich) 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}, 0.52%. 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}, 2.74%. 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, ISO35.

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-AZA2, 19-001 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.

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

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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-AZA2 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 withdrawn at 15 °C to 25 °C 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 few 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-2 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 AZA2 solution were dispensed into 2-mL amber argon-filled glass ampoules, which were then flame sealed.

#Non-certified and non-accredited data:

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-2 and 37-epi-Azaspiracid-2* (95% Confidence Interval)	(1.19 ± 0.09) µM
	(1.02 ± 0.08) µg / mL

* Concentration of AZA2 and impurities are neglected in order to apply solution density value.

Certified concentration of Azaspiracid-2 presents a low level (0.015 ± 0.007) µg/g (1.2%) of tentative 37-epi-Azaspiracid-2. In order to get a certified concentration, quantification for these isomers have been conducted jointly, therefore certified concentration is the sum of epimeric pair. *Percentage value not certified.*

Purity analysis was performed by qNMR and UPLC MS/MS. 37-epi-Azasparicid-2 amount is within the principal component quantification. This material presents 0.7 % of tentative Azaspiracid-2 isomer as major impurity and a low level of Azaspiracid-1 (0.4%) and Azaspiracid-3 (<0.1%). Traces (<0.1%) of tentative isomers of Azaspiracid-1, -3 and -6 are found. Material molar ratio purity is ≥ 98 %. *Percentage values not certified*.

Additional lectures:

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

2. ISO Guide 35 *Reference materials - Guidance for characterization and assessment of homogeneity and stability*.

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Figures

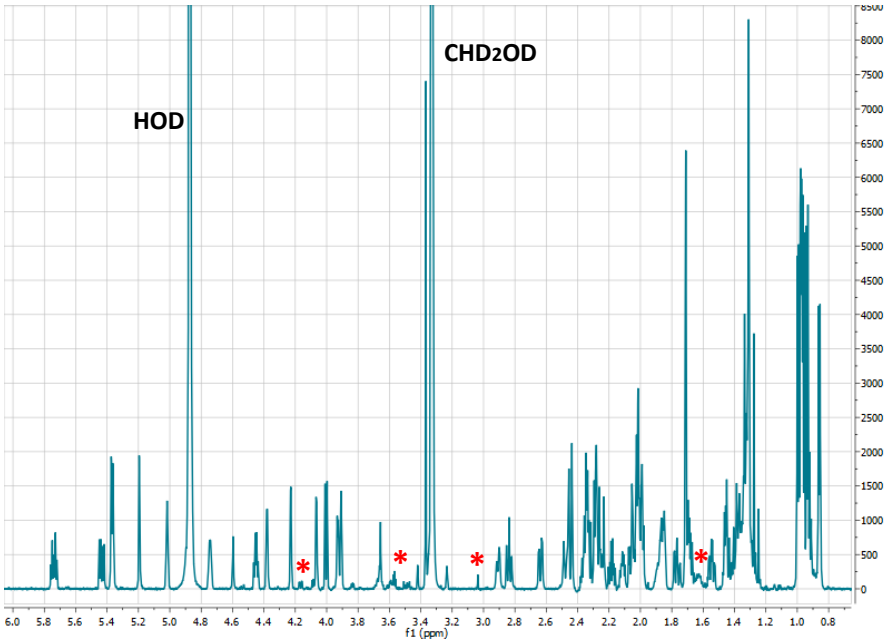

Figure 1. ^{1}H-NMR spectrum of AZA-2 solution, Bruker AVANCE NEO 750 MHz, 1 mM solution of AZA-2 in CD_3OD used in preparation of CRM-02-AZA2 batch 19-001. No identified impurities are highlighted. As the structure of these impurities is not known, their exact amount cannot be calculated. Because the 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.

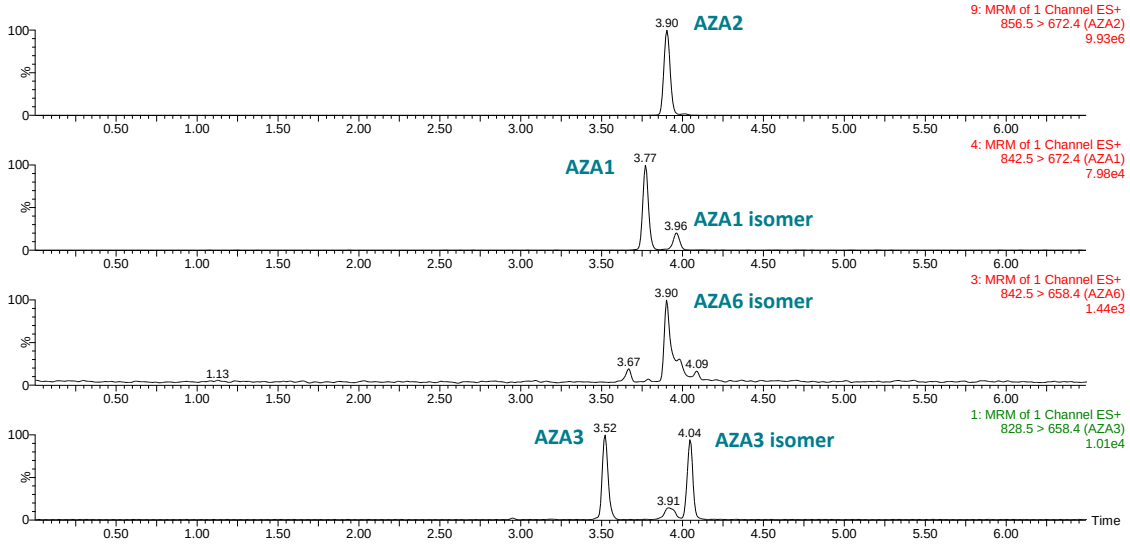

Figure 2. Analysis of the **CRM-02-AZA2** solution by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7µm, 100x2.1 mm, UPLC column. Elution conditions, gradient mode with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 2mM ammonium formate and 50mM formic acid; Gradient: 0.00 min 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 Quattro Premier XEVO using the typical group-2 MRM transitions of Azaspiracid family. Tentative isomers of principal Azaspiracids are highlighted.

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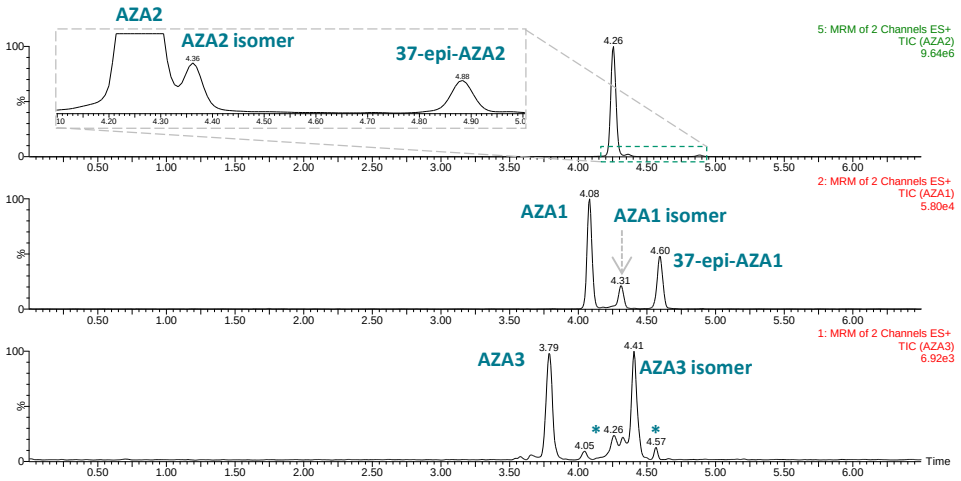

Figure 3. Analysis of the **CRM-02-AZA2** solution by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7µm, 100x2.1 mm, UPLC column. Elution conditions, gradient mode with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 5 mM ammonium acetate, pH = 6.85; Gradient: 0.00 min 20% B; 1.00 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 Quattro Premier XEVO using the typical group-2 and -4 MRM transitions of Azaspiracid family.

Table 1. Uncertainties contribution for the certification of CRM-02-AZA2. ***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-2 + 37-epi-Azaspiracid-2
u_{char}/%	2.73
u_{ba}/%	0.60
u_{bb}/%	0.52
u_{lts}/%	2.74
Expanded uncertainty (*k*=2) / %	7.89

Short-term stability study, test for slope significance, has been carried out to simulate the conditions of transport. Packaged vials were exposed to 40ºC for 6 days, 4 days, 48 hr and 0 hr (-80 ºC, control), with an isochronous design being used to enable subsequent LC-MS/MS analysis of CRMs to be conducted under repeatability conditions. After 6 days, results indicated continued stability of total epimeric pair. With these conditions, no change is expected in the property values of the CRM. The short-term stability uncertainty (u_{sts}) was not considered relevant as an uncertainty component and it was not included in the u_{CRM} uncertainty calculation.

For the short-term study two more criteria were evaluated: the concentration of Azaspiracid-2 and 37-epi-azaspiracid-2 exposed at the study temperature against concentration of each epimer exposed at the reference temperature. This data suggest epimerization equilibrium is undergone under thermal kinetic dependence. These minor individual changes are detected for a lengthy delay in shipping (6 days) while no evidence of statistical epimerization is detected for dispatch within less than four days indicating a satisfactory concordance of data and evidencing the stability of the material.

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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, 25th 2019* (Original certificate issue date, revision 1).