

Certificate of analysis

Certified reference material code: CRM-02-AZA3

Name: Certified Reference Material of Azaspiracid-3

Batch: 20-001

Description: This Certified Reference Material (CRM) is a solution of Azaspiracid-3 (CAS Number 265996-93-8) 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 ¹H-qNMR in Bruker NEO 750 equipment (Universidad de Santiago de Compostela).

Concentration of Azaspiracid-3 and 37-epi-Azaspiracid-3 (95% Confidence Interval)	(1.59 ± 0.15) µmol / kg
	(1.32 ± 0.12) 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 ¹H-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} , 1.45%. 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} , 2.00%. 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-AZA3, 20-001 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.*



CRM-02-AZA3, 20-001,
rev.2, 12/01/2026

F-01-08 Edición 6 Página 1 de 5

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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-AZA3 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 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 five minutes due to solvent volatility and air contamination.

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

Source and Preparation of Material: Azaspiracid-3 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 ¹H-NMR and UPLC-MS/MS, Figures 1 and 2. Finally, 0.5 mL aliquots of AZA3 solution were dispensed into 2-mL amber argon-filled glass ampoules, which were then flame sealed.

#Non-certified and non-accredited data:

Certified concentration of Azaspiracid-3 presents a low level (0.056 ± 0.010) µg/g (4.2%) of 37-epi-Azaspiracid-3. In order to get a certified concentration, quantification for these isomers have been conducted jointly, therefore certified concentration is the sum epimeric pair. Percentage value not certified.

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

Concentration of Azaspiracid-3 and 37-epi-Azaspiracid-3 (95% Confidence Interval)	(1.26 ± 0.12) µM
	(1.04 ± 0.10) µg / mL

Concentration of Azaspiracid-3 and impurities are neglected in order to apply solution density value.

Purity analysis was performed by qNMR and UPLC MS/MS. 37-epi-Azaspiracid-3 amount is within the principal component quantification. This material presents 0.9 % of Azaspiracid-4 isomer as major impurity, low levels: 0.2 % at $t_r = 2.64$ min and 0.1% at $t_r = 3.93$ min of Azaspiracid-3 isomers and traces (<0.1%) of another Azaspiracid analogues. 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 33405 *Reference materials - Approaches for characterization and assessment of homogeneity and stability.*



CRM-02-AZA3, 20-001,
rev.2, 12/01/2026

F-01-08 Edición 6 Página 2 de 5

Figures

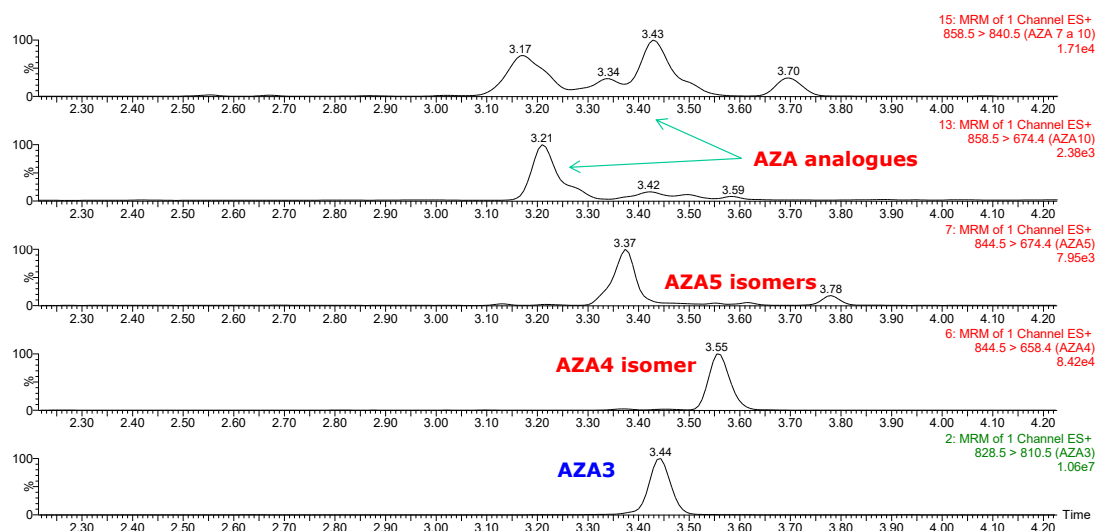


Figure 1. Analysis of the CRM-02-AZA3 (20-001) by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7 μ m, 100x2.1 mm, UPLC column. Elution conditions, gradient mode with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 2mM ammonium formate and 50mM formic acid; Gradient: 0.00 min 30% 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. Azaspiracid-4 isomer is checked as major impurity and moreover, traces of Azaspiracids are highlighted.

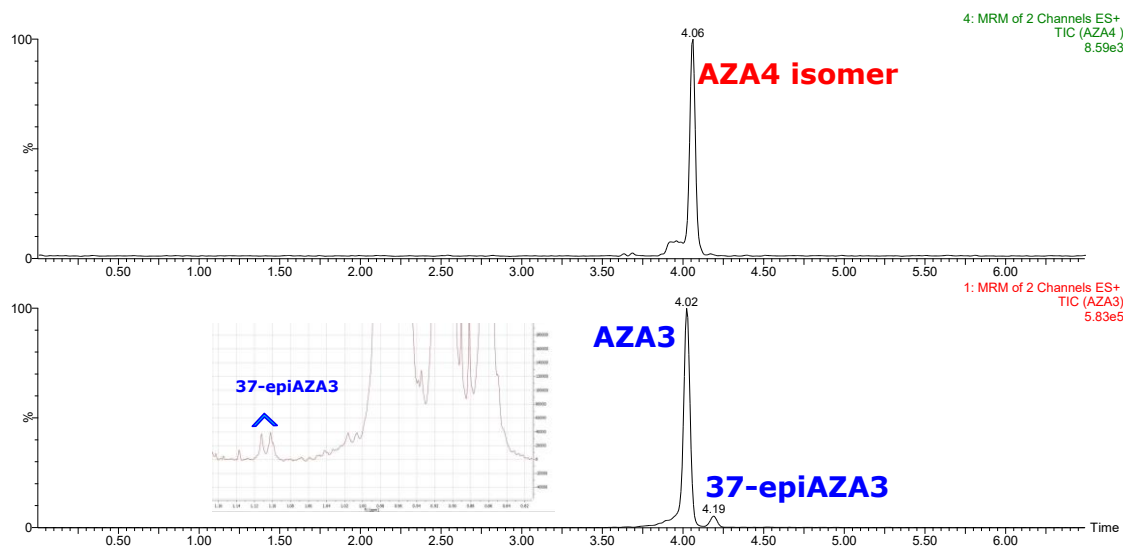


Figure 2. Analysis of the CRM-02-AZA3 (20-001) by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7 μ m, 100x2.1 mm, UPLC column. Elution conditions, gradient mode with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 5 mM ammonium acetate, pH = 6.85; Gradient: 0.00 min 20% B; 1.00 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 MRM transitions of Azaspiracid family. ¹H-NMR spectrum of methyl region is overlapped.



CRM-02-AZA3, 20-001,
rev.2, 12/01/2026

F-01-08 Edición 6 Página 3 de 5

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Table 1. Uncertainties contribution for the certification of CRM-02-AZA3 (20-001). *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-3 + 37-epi-Azaspiracid-3*	
$u_{char}/\%$	2.34	
$u_{ba}/\%$	0.60	
$u_{bb}/\%$	1.45	
$u_{lts}/\%$	2.00	
$u_{sts}/\%$	3.00	
Expanded uncertainty ($k=2$) / %	9.16	

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-AZA3 is settled in 3 or 6 days based on a structural stability assessment for target boxes under cooled conditions.

* Azaspiracid-3 + 37-epi-Azaspiracid-3 have been quantified jointly.

Table 2. Molecular information.

Name	AZASPIRACID-3
CAS NUMBER	265996-93-8
Molecular formula	C ₄₆ H ₆₉ NO ₁₂
Molecular Weight	828.053 g·mol ⁻¹
Monoisotopic molecular weight	827.482 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, 25th 2021 (Original certificate issue date, revision 1).

January, 12th 2026. Correction of typographical errors. 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.



CRM-02-AZA3, 20-001,
rev.2, 12/01/2026

F-01-08 Edición 6 Página 5 de 5

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