

Certificate of analysis

Certified reference material code: CRM-03-TTXs

Name: Certified Reference Material of Tetrodotoxin, 4,9-anhydro Tetrodotoxin and 4-epi Tetrodotoxin.

Batch: 20-001

Description: This Certified Reference Material (CRM) is a solution of Tetrodotoxin (CAS Number 4368-28-9), 4,9-anhydro Tetrodotoxin (CAS Number 13072-89-4) and 4-epi Tetrodotoxin (CAS Number 98242-82-1) in aqueous [AcOH] = 1 mM, pH 3.91.

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 Tetrodotoxin (TTX) (95% Confidence Interval)	$(65.8 \pm 4.3) \mu\text{mol} / \text{kg}$
	$(21.0 \pm 1.4) \mu\text{g} / \text{g}$
Concentration of 4,9-anhydro Tetrodotoxin (4,9-anhTTX) (95% Confidence Interval)	$(18.0 \pm 1.5) \mu\text{mol} / \text{kg}$
	$(5.44 \pm 0.45) \mu\text{g} / \text{g}$
Concentration of 4-epi Tetrodotoxin (4-epiTTX) (95% Confidence Interval)	$(5.24 \pm 0.56) \mu\text{mol} / \text{kg}$
	$(1.67 \pm 0.18) \mu\text{g} / \text{g}$

The starting material is measured by ^1H -qNMR against a Sucrose 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} , 0.18% for TTX, 1.24% for 4,9-anhTTX and 1.15% for 4-epiTTX. 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} , 0.97% for TTX, 2.18% for 4,9-anhTTX and 2.33% for 4-epiTTX. 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 $\pm$ 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.



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Expiration of Certification: This certificate of CRM-03-TTXs, 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.

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 has been classified as non-hazardous, but it should still be handled with care. Read MSDS before using. Material Safety Data sheet for information regarding CRM-03-TTXs 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: The raw material used in the preparation of this CRM was obtained from commercial source. Identity of the material was confirmed by ¹H-NMR and LC-MS/MS. Figures 1 and 2. Finally, 0.5 mL aliquots of toxin solution were dispensed into 1.5 mL amber argon-filled glass ampoules, which were then flame sealed.

Non-certified and non-accredited data:

Taking into account the density of water at 20 °C ($\rho^{20} = 0.9982 \text{ g/mL}$), non-certified volumetric concentration values are:

Concentration of Tetrodotoxin (TTX) (95% Confidence Interval)	(65.7 ± 4.3) µM
	(21.0 ± 1.4) µg / mL
Concentration of 4,9-anhydro Tetrodotoxin (4,9-anhTTX) (95% Confidence Interval)	(18.0 ± 1.5) µM
	(5.43 ± 0.45) µg / mL
Concentration of 4-epi Tetrodotoxin (4-epiTTX) (95% Confidence Interval)	(5.24 ± 0.56) µg / mL
	(1.67 ± 0.18) µg / mL

NOTE: Concentration of TTX, 4,9-anhTTX, 4-epiTTX and impurities are neglected in order to apply solution density value.

Purity analysis was performed by qNMR and UPLC MS/MS. In order to perform the analysis, it should be noted impurity Tetrodotoxin analogues content. Small amounts of tentative 4,4a-anhydro Tetrodotoxin (4,4a-anhTTX), monoformyl Tetrodotoxin (monoformylTTX) and 11-deoxy Tetrodotoxin (11-deoxyTTX) are revealed (#Table 2). *Concentration values are presented as non-certified.*



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Results of nuclear magnetic resonance (NMR) analysis were consistent with structure and a molar ratio purity of principal components > 97 %.

No purity correction over certified concentration should be done.

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.*

Figures

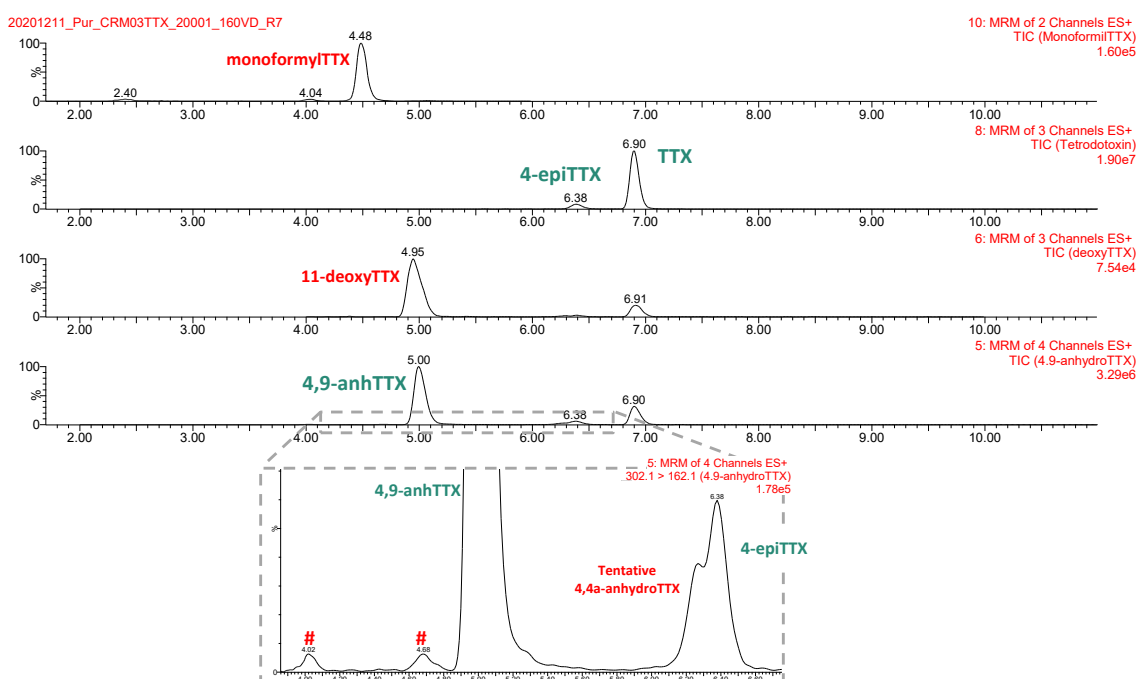


Figure 1. Analysis of the CRM-03-TTXs (20-001) by ACQUITY UPLC system, BEH AMIDE 1.7µm, (150 x 2.1) mm column. Elution conditions, gradient mode with mobile phases: (A) Water, [FmONH₄] = 1.0 mM and (B) Acetonitrile 95% (V/V), 0.01% FmOH; Gradient: 0 min [73% B]; 5 min [73% B]; 7.5 min [40% B]; 7.6 min [20% B]; 9.5 min [20% B]; 9.6 min [73% B]; 11.0 min [73% B]; flow rate: 0.40 mL/min. Temperature: 55 °C. MS detector: Waters XEVO TQ-MS using the typical MRM transitions for TTX and analogues. MonoformylTTX (0.3%) and 11-deoxyTTX (0.1%) are highlighted and other minor (#) no identified impurities with fragmentation pattern similar to 4,9-anhTTX including tentative 4,4a-anhydroTTX (0.8%).



Table 1. Uncertainties contribution for the certification of CRM-03-TTXs (20-001). *Char*: qNMR quantification; *ba*: bulk assay; *bb*: homogeneity test; *lts*: long term stability test; *sts*: short term stability test.

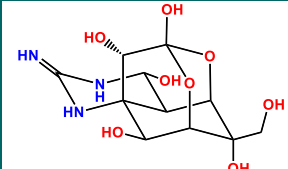
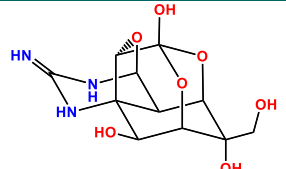
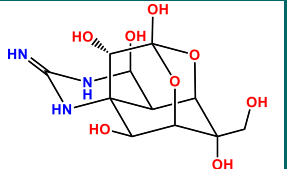
Uncertainty contribution parameter	Uncertainty values		
	TTX	4,9-anh TTX	4-epiTTX
$u_{char}/\%$	1.91	1.98	1.83
$u_{ba}/\%$	0.32	0.32	0.32
$u_{bb}/\%$	0.18	1.24	1.15
$u_{lts}/\%$	0.97	2.18	2.33
$u_{sts}/\%$	2.40	2.65	4.26
Expanded uncertainty ($k=2$) / %	6.48	8.32	10.65

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, 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 for 6 days.

Table 2. Non-certified reference values of impurities concentration (µM) determined by UPLC MS/MS. Assigned analogues entries for compounds must be regarded as tentative according to MRM transitions agree. Assumption of equimolar response. Estimated uncertainty corresponds to a level of confidence in terms of one standard deviation.

Toxin	Concentration / µM	%
Monoformyl TTX	0.24 ± 0.02	0.3
11-deoxyTTX	0.05 ± 0.01	0.1
Tentative 4,4a-anhydroTTX	0.72 ± 0.06	0.8

Table 3. Molecular information.

Name	TTX	4,9-anh TTX	4-epiTTX
CAS NUMBER	4368-28-9	13072-89-4	98242-82-1
Molecular formula	C ₁₁ H ₁₇ N ₃ O ₈	C ₁₁ H ₁₅ N ₃ O ₇	C ₁₁ H ₁₇ N ₃ O ₈
Molecular Weight	319.27 g·mol ⁻¹	301.25 g·mol ⁻¹	319.27 g·mol ⁻¹
Monoisotopic molecular weight	319.10 g·mol ⁻¹	301.09 g·mol ⁻¹	319.10 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:

July, 07th 2021 (Original certificate issue date, revision 1).

January, 12th 2025. Editorial changes. Revised and added associated uncertainties following additional testing. Address update (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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