

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

## Certificate of analysis

### Certified reference material code: CRM-01-DTX2

Name: Dinophysistoxin-2 sodium salt in methanol.

Batch: 12-001

**Description:** This Certified Reference Material (CRM) is a solution of Dinophysistoxin-2 sodium salt (CAS Number 139933-46-3 for acid form) in methanolic solution.

**Intended use:** Calibration of analytical instruments. Spiking of control samples for recovery experiments. 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 <sup>1</sup>H-qNMR in Varian Inova 750 MHz equipment (USC).

<b>Concentration of Dinophysistoxin-2 sodium salt (95% Confidence Interval)</b>	<b>(3.07 ± 0.17) μmol / kg</b>
	<b>(2.54 ± 0.14) μg / g</b>

NOTE: In order to perform the analysis, it should be noted that the certificate value corresponds to the sum of Dinophysistoxin-2 and minor analogues concentration and therefore the sum of the areas of the chromatographic signals should be considered. See #Non-certified and non-accredited data section of this certificate.

The starting material is measured by <sup>1</sup>H-qNMR against a Benzoic acid NIST certified reference material (SRM 350b) 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  $u_{bb}$ , 0.65%. 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  $u_{lts}$ , 0.79%. 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-01-DTX2, 12-001 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.

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**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-01-DTX2 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 °C (freezer) until use. Unopened ampoules should be stored upright under normal laboratory conditions inside the original container supplied.

**Source and Preparation of Material:** Dinophysistoxin-2 sodium salt was isolated from harmful algae bloom located on the Galician coast (Spain) and happened in November 2005. After purification, identity of the material was confirmed by 1H-NMR and LC-MS/MS, Figures 1 and 2. Finally, 0.5 mL aliquots of DTX2 solution were dispensed into 2-mL amber argon-filled glass ampoules, which were then flame sealed.

**#Non-certified and non-accredited data:**

The certified concentration of Dinophysistoxin-2 sodium salt presents a low level ( $0.020 \pm 0.004$ )  $\mu\text{g/g}$  (**0.7%**) of okadaic acid sodium salt and tentative Dinophysistoxin-2 sodium salt isomer ( $0.020 \pm 0.004$ )  $\mu\text{g/g}$  (**0.7%**). In order to get a certified concentration, quantification for these isomers have been conducted jointly, therefore certified concentration is the sum of all these compounds. Percentage values 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 Dinophysistoxin-2 sodium salt (95% Confidence Interval)	( $2.43 \pm 0.13$ ) $\mu\text{M}$
	( $2.01 \pm 0.11$ ) $\mu\text{g} / \text{mL}$

Concentration of DTX2 and impurities are neglected in order to apply solution density value.

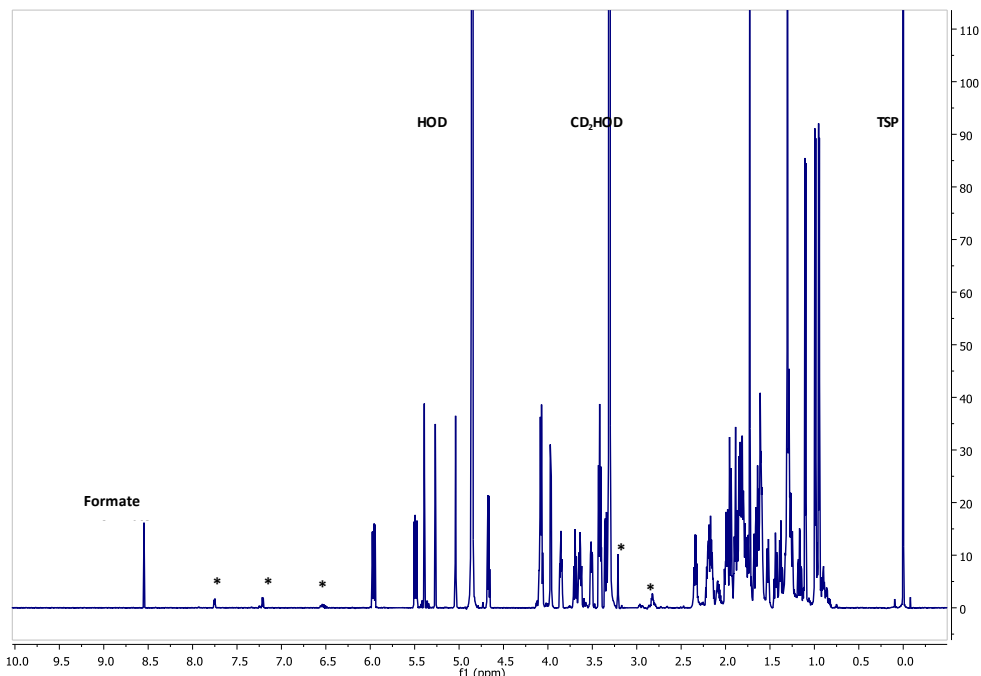
Purity analysis was performed by qNMR and UPLC MS/MS. The percentage purity of principal signal of DTX-2 was measured by the difference qNMR method and the material molar ratio purity is  $\geq 98$  %. Percentage value not certified.

**Additional lectures:**

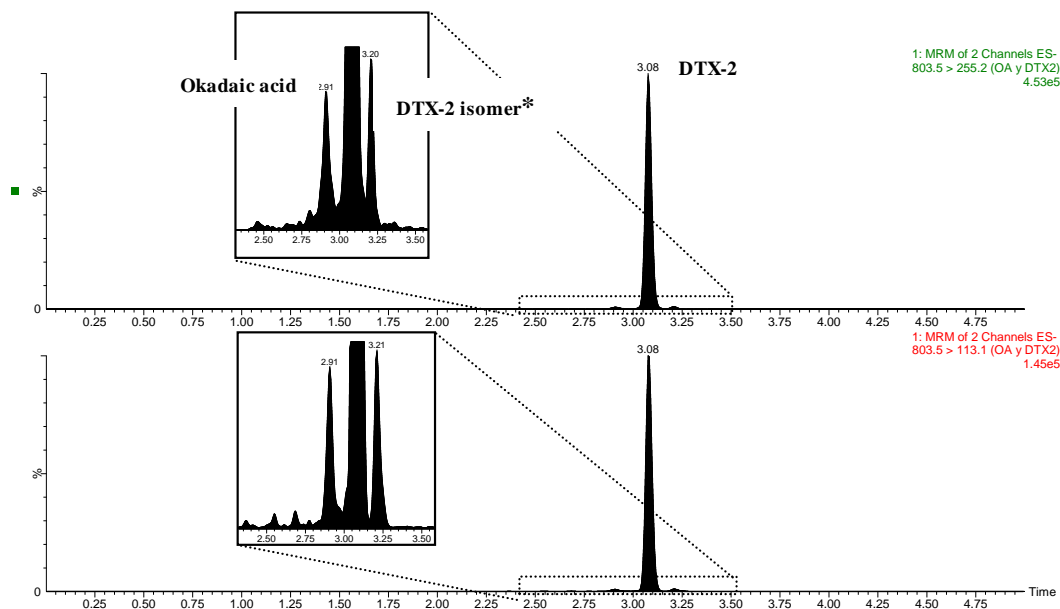
1. ISO Guide 34:2009 *General Requirements for the competence of reference material producers*. International Organisation for Standardization (ISO).
2. ISO Guide 35:2006 *Reference materials - General and statistical principles for certification*. International Organisation for Standardization (ISO).

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**Figures**



**Figure 1.** <sup>1</sup>H-NMR spectrum of DTX2 solution, Varian Innova 750 MHz, 3mM solution of DTX2 in CD<sub>3</sub>OD used in preparation of CRM-01-DTX2 batch 12-001. 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 okadaic acid analogues.



**Figure 2.** Analysis of the CRM-01-DTX2 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 min 30% B; 3.0 min 90% B; 4.5 min 90% B; 4.51 min 30% B; 6.5 min 30% B; flow rate: 0.4 mL/min. Temperature: 30°C. MS detector: Waters Quattro Premier XEVO using MRM transitions of Okadaic acid and Dinophysistoxin-1. Two most important transitions for Okadaic acid and DTX2 are shown. \* Okadaic acid isomer is probably the most appropriate name.

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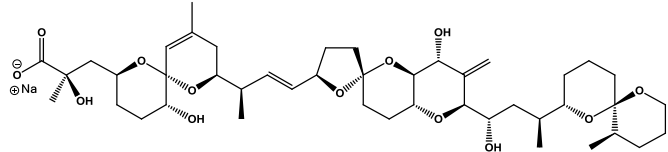
**Table 1.** Uncertainties contribution for the certification of CRM-01-DTX2. *Char*: qNMR quantification; *ba*: bulk assay; *bb*: homogeneity test; *lts*: long term stability test; *sts*: short term stability test.

Uncertainty contribution parameter	Uncertainty values	
	Dinophysistoxin-2 sodium salt + minor analogues*	
$U_{char}/\%$	2.59	
$U_{ba}/\%$	0.15	
$U_{bb}/\%$	0.65	
$U_{lts}/\%$	0.79	
Expanded uncertainty ( $k=2$ ) / %	5.57	

**Short-term stability study**, test for slope significance, has been carried out to simulate the conditions of transport. Packaged vials were exposed for 9 days, 6 days, 3 days and 0 hr (-80 °C, control), with an isochronous design being used to enable subsequent LC-MS/MS analysis of CRMs to be conducted under repeatability conditions. After 9 days at 37 °C - 40°C temperature results indicated continued stability. 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.

\* Dinophysistoxin-2 sodium salt, Okadaic acid sodium salt and Dinophysistoxin-2 sodium salt isomer have been quantified jointly.

**Table 2.** Molecular information.

Name	Dinophysistoxin-2 sodium salt
<b>CAS NUMBER</b>	139933-46-3 (acid form)
<b>Molecular formula</b>	$C_{44}H_{67}O_{13}Na$
<b>Molecular Weight</b>	$827.0 \text{ g}\cdot\text{mol}^{-1}$
<b>Monoisotopic molecular weight</b>	$826.4 \text{ g}\cdot\text{mol}^{-1}$
	

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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:** *April, 14<sup>th</sup> 2013* (Original certificate issue date, revision 1).  
*September, 1<sup>st</sup> 2013* (*certificate rev. 2*).  
*April, 8<sup>th</sup> 2014*. This certificate has undergone revision to change the uncertainty following stability testing, extension of certification period (*certificate rev. 3*).  
*October, 24<sup>th</sup> 2015*. This certificate has undergone revision to change the uncertainty following stability and robustness testing, extension of certification period, editorial changes (*certificate rev. 4*).  
*March, 07<sup>th</sup> 2017*. Introduction of the accreditation mark, stability uncertainty changes and editorial changes. Format F-01-08, revision 1 (*certificate rev. 5*).  
*May, 30<sup>th</sup> 2017*. Minor editorial changes. No attempt was made to re-evaluate the certificate values or any technical data presented on this certificate (*certificate rev. 6*).

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