

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

Certificate of analysis

Certified reference material code: CRM-00-YTX

Name: Yessotoxin di-sodium salt in methanol.

Batch: 19-001

Description: This Certified Reference Material (CRM) is a solution of Yessotoxin di-sodium salt (CAS Number 112574-50-2) 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 Varian 500 MHz WB equipment (USC).

Concentration of Yessotoxin di-sodium salt and analogues (95% Confidence Interval)	(6.60 ± 0.36) μmol / kg
	(7.84 ± 0.43) μg / g

The starting material is measured by ¹H-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} , 0.44%. 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} , 1.58%. 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-00-YTX, 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-00-YTX 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: Yessotoxin di-sodium salt was isolated from large scale culture of *Protoceratium reticulatum* and purified via preparative HPLC chromatography system. 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 YTX 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 °C ($\rho^{20} = 0.7915 \text{ g/mL}$), non-certified volumetric concentration values are:

Concentration of Yessotoxin di-sodium salt (95% Confidence Interval)	(5.22 ± 0.28) µM
	(6.20 ± 0.34) µg / mL

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

Purity analysis was performed by qNMR and UPLC MS/MS. Additional low amounts of YTX tentative analogues quantified out from principal component are presented, see #Table 2. The percentage purity of principal signal of YTX was measured by the difference ¹H-qNMR method and the 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

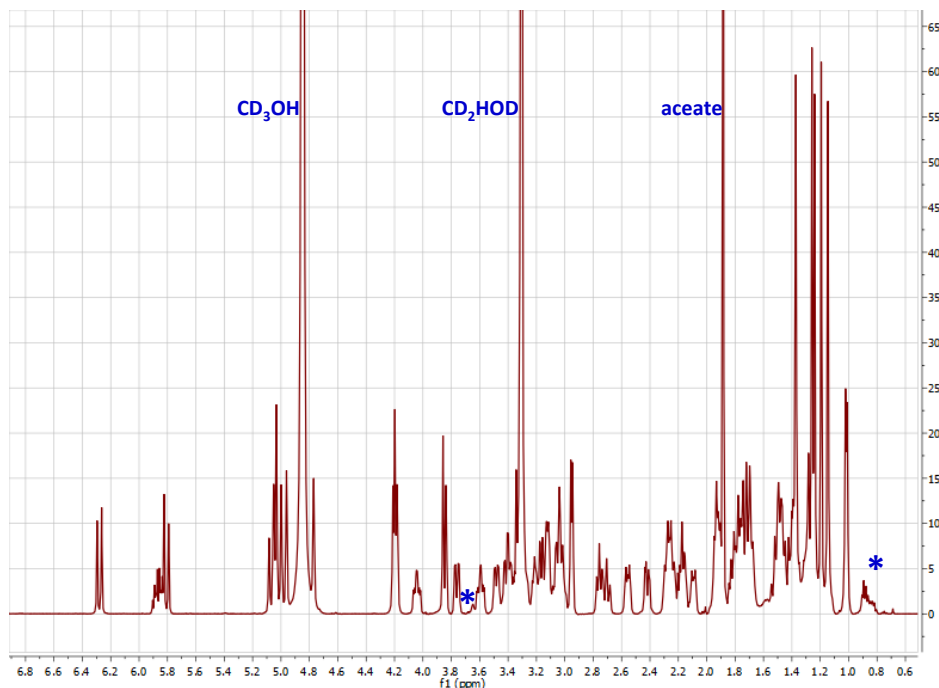


Figure 1. ¹H-NMR spectrum of YTX solution, Varian 500 MHz WB, 7 mM solution of YTX in CD₃OD used in preparation of CRM-00-YTX batch 19-001. No identified impurities are highlighted (*).

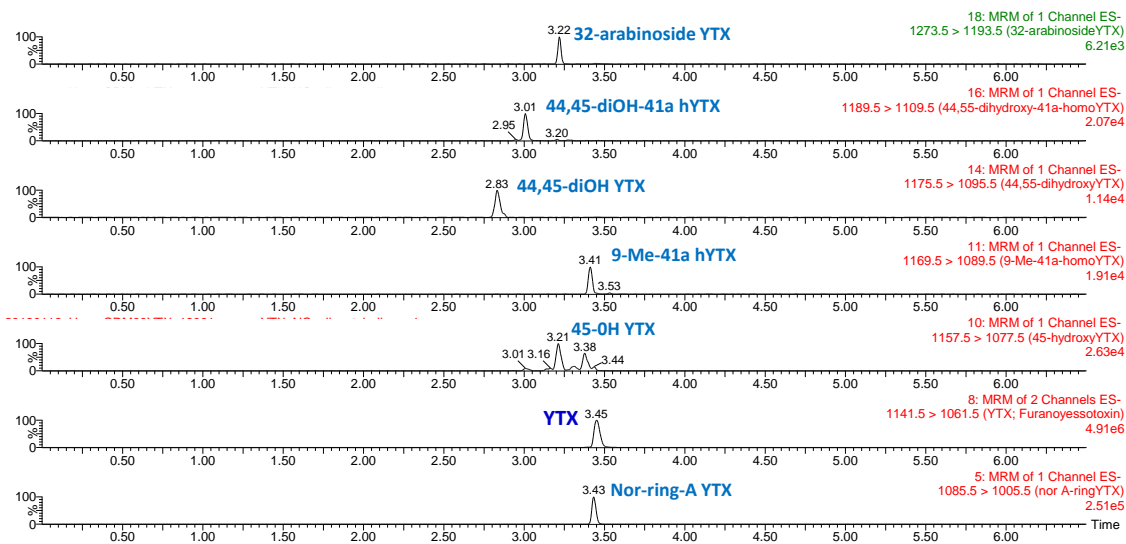


Figure 2. Analysis of the CRM-00-YTX solution by UPLC-MS/MS. Conditions: ACQUITY UPLC BEH C18 1.7µm, 100x2.1 mm, UPLC column. Elution conditions, gradient method with mobile phases: (A) Water and (B) Aqueous 95% acetonitrile, both with 5 mM ammonium acetate, pH = 6.85; Gradient: 0.00 min 20% B; 1.00 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 Yessotoxin tentative analogues.

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Table 1. Uncertainties contribution for the certification of CRM-00-YTX. *Char*: qNMR quantification; *ba*: bulk assay; *bb*: homogeneity test; *lts*: long term stability test; *sts*: short term stability test.

Uncertainty contribution parameter	Uncertainty values	
	Yessotoxin	
$u_{char}/\%$	2.08	
$u_{ba}/\%$	0.60	
$u_{bb}/\%$	0.44	
$u_{lts}/\%$	1.58	
Expanded uncertainty ($k=2$) / %	5.44	

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.

#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.

Toxin	Concentration / μM	Percentage
YTX	5.23 ± 0.26	Principal component
32-arabinoside-YTX	-	< 0.05 %
Nor-ring-A-YTX	0.07 ± 0.01	1.3 %
9-Me-41a-homoYTX	-	< 0.1 %
45-hydroxy-YTX	0.010 ± 0.005	0.2 %
44,45-dihydroxy-41a-homoYTX	-	< 0.1 %

Table 3. Molecular information.

Name	Yessotoxin di-sodium salt
CAS NUMBER	112514-54-2 (for component yessotoxin di acid form)
Molecular formula	$\text{C}_{95}\text{H}_{80}\text{O}_{21}\text{S}_2\text{Na}_2$
Molecular Weight	$1187.3 \text{ g}\cdot\text{mol}^{-1}$
Monoisotopic molecular weight	$1186.4 \text{ g}\cdot\text{mol}^{-1}$

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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, 11th 2019* (Original certificate issue date, revision 1).

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