



# Certificate of Analysis

## Certified Reference Material

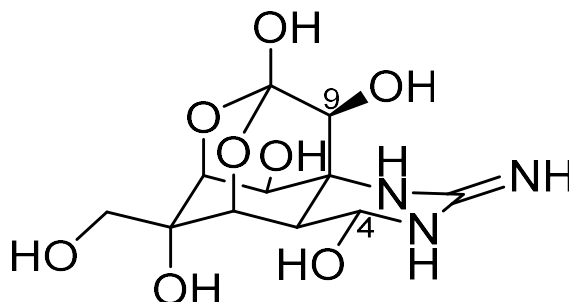
### CRM-TTX (Lot # 20170328)

#### Certified Calibration Solution for Tetrodotoxin

Tetrodotoxin (TTX) is a naturally occurring toxin associated with poisoning incidents due to the consumption of contaminated marine species, most notably puffer fish [1,2]. NRC CRM-TTX is a certified calibration solution of TTX in aqueous 1 mM acetic acid. It is designed as an instrument calibration solution to aid in the identification and quantitation of TTX.

**Table 1:** Certified mass fraction and concentration values for CRM-TTX.

Compound	$\mu\text{g/g}$	$\mu\text{g/mL}$ (15-30 °C)	$\mu\text{mol/L}$ (15-30 °C)
Tetrodotoxin (TTX)	$6.75 \pm 0.24$	$6.74 \pm 0.24$	$21.10 \pm 0.76$



#### Tetrodotoxin

CAS registry no.: 4368-28-9

InChIKey: CFMYXEVWODSLAX-QOZOJJKESA-N

Molecular formula: C<sub>11</sub>H<sub>17</sub>N<sub>3</sub>O<sub>8</sub>

Molecular weight: 319.27 g/mol

Period of validity: 1 year from date of sale

Storage conditions: -12 °C or below

## Intended Use

CRM-TTX is a certified calibration solution designed for analytical method development and accurate quantitation of TTX. The concentration is suitable for preparing a dilution series for calibration of analytical instruments such as liquid chromatography-mass spectrometry (LC-MS), as well as for spiking control samples for recovery experiments.

## Instructions for Storage and Use

To ensure the stability of CRM-TTX, ampoules should be stored at -12 °C or below.

Prior to opening, each ampoule should be allowed to warm to room temperature and the contents mixed thoroughly. The ampoule should be opened at the pre-scored mark. Once an ampoule has been opened, accurate aliquots should be removed with calibrated volumetric equipment and transferred to volumetric flasks or vials. An increase in concentration due to evaporation of solvent will occur if the solution is left opened for more than a few minutes. It is recommended that the CRM should not be evaporated to dryness because of the potential for losses or decomposition. *Note:* The volume of the solution is not certified. Therefore, the entire contents of the ampoule should not simply be transferred to a volumetric flask and diluted to volume.

## Preparation of CRM-TTX

Purified TTX was obtained from Latoxan (Portes-lès-Valence, France). A measured accurate  $m/z$  of 320.1088 matching the theoretical mass of  $C_{11}H_{18}N_3O_8^+$  ( $\Delta = 0$  ppm) and characteristic TTX fragment ions [2] were obtained for the  $[M+H]^+$  ion of TTX using high-resolution MS (Figure 1). Impurities were assessed by proton nuclear magnetic resonance ( $^1H$ -NMR), liquid chromatography with chemiluminescence nitrogen detection (LC-CLND) [3] and LC-MS. Analyses revealed small amounts of the TTX equilibrium products 4,9-anhydro-TTX and 4-*epi*-TTX at below 1% of TTX (Figure 2). The approximate concentrations of individual analogues are provided in Table 2 as non-certified information values.

The CRM-TTX solution was prepared by making an accurate dilution of the qNMR stock solution in degassed 1 mM acetic acid. Aliquots were dispensed into clean argon-filled amber glass ampoules and immediately flame-sealed. Each ampoule contains approximately 0.5 mL of solution.

**Table 2:** Information values for equilibrium products of TTX in CRM-TTX as measured by  $^1H$ -NMR.

Compound	Molecular Weight (g/mol)	Concentration ( $\mu g/g$ )*
4,9-anhydro-TTX	301.26	0.04
4- <i>epi</i> -TTX	319.27	0.06

\* These concentrations are not certified

## Analytical Methods and Value Assignment

The certified value for CRM-TTX (Table 1) is based on results obtained at the NRC using qNMR [4]. A NIST potassium hydrogen phthalate certified reference material (NIST SRM 84L) was used for calibration.

### Homogeneity

A representative number of CRM-TTX ampoules were selected from across the fill series and relative TTX concentrations measured by LC-MS. No heterogeneity was observed.

### Stability

One year (long-term) and one month (transport) studies have demonstrated good stability of CRM-TTX stored in sealed ampoules at temperatures of +4 °C or lower, with no detectable decomposition observed within the limits of uncertainty of the LC-MS method. Based on these results, a period of validity of one year from the date of sale has been applied for CRMs stored at -12 °C or below.

### Uncertainty

All reasonable sources of error related to the characterization of CRM-TTX were considered and measured. The overall uncertainty estimate ( $U_{CRM}$ ) includes uncertainties associated with batch characterization ( $u_{char}$ ), instability during storage ( $u_{stab}$ ) and between-bottle variation ( $u_{hom}$ ) [5-8]. These components are listed in Table 3, and are combined and expanded as follows:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{hom}^2 + u_{stab}^2}$$

where  $k$  is the coverage factor for a 95% confidence level (= 2).

Uncertainties for solution concentrations shown in Table 1 ( $\mu\text{mol/L}$  and  $\mu\text{g/mL}$ ) include an additional component for density correction covering the temperature range 15-30 °C.

**Table 3:** Uncertainty components for the certified value of CRM-TTX.

Uncertainty	Relative*
$u_{char}$	0.011
$u_{hom}$	negligible
$u_{stab}$	0.014

\* Relative to mass fraction and concentrations shown in Table 1

## Safety Instructions

Ingestion of sufficient quantities of TTX can cause paralysis and even death [9,10], therefore only qualified personnel should handle the solution and appropriate disposal methods should be used. Suitable personal protective equipment should be used when opening the ampoule in the event the glass shatters. A safety data sheet (SDS) is available for CRM-TTX.

**Period of Validity**

If stored unopened at the recommended storage condition of -12 °C (or below), the certified concentration of CRM-TTX is valid for 1 year from the date of sale.

**Metrological Traceability**

Results presented in this certificate are traceable to the SI (*Système international d'unités*) through gravimetrically prepared standards of a NIST potassium hydrogen phthalate certified reference material (NIST SRM 84L).

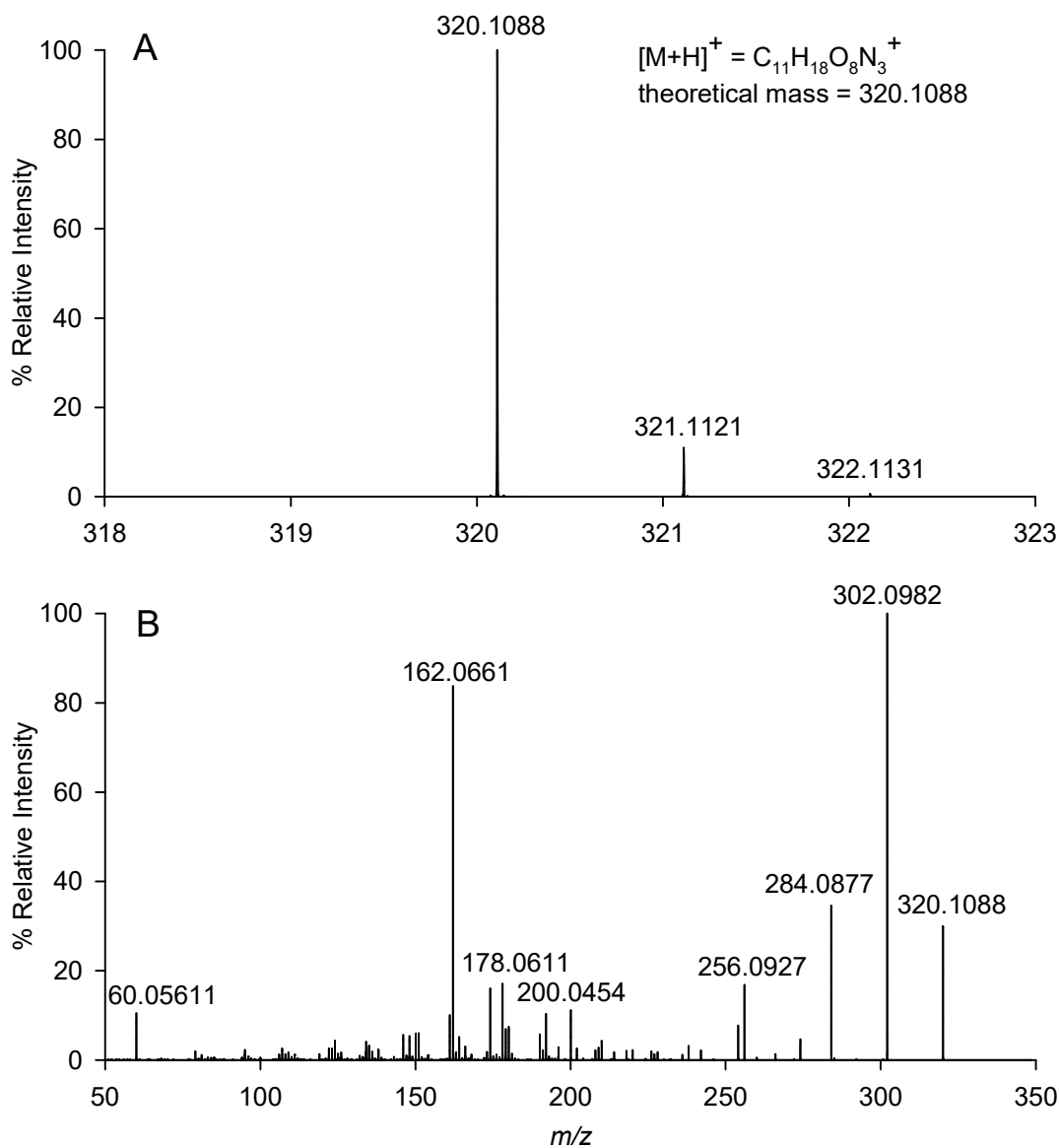
**Quality Management System (ISO 17034, ISO/IEC 17025)**

This material was produced in compliance with the National Research Council of Canada (NRC) Metrology Quality Management System, which conforms to the requirements of ISO 17034 and ISO/IEC 17025.

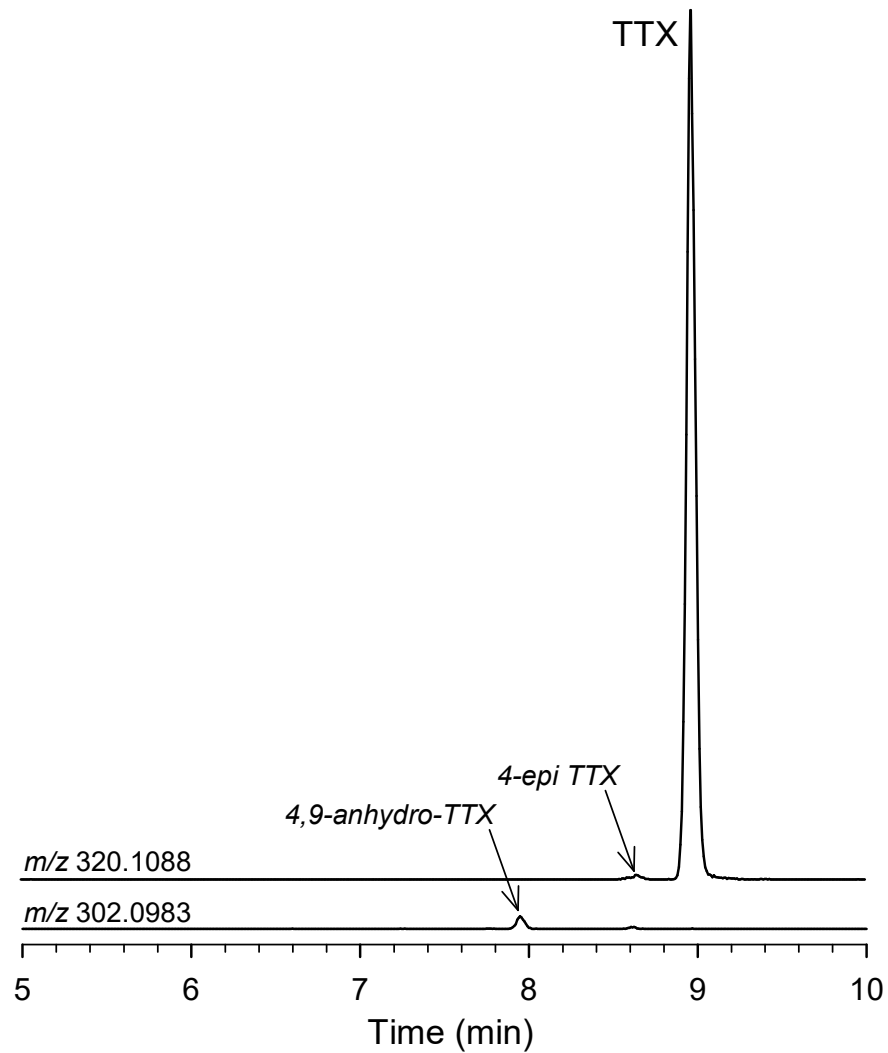
The Metrology Quality Management System supporting the NRC Calibration and Measurement Capabilities, as listed in the *Bureau international des poids et mesures* (BIPM) Key Comparison Database (<http://kcdb.bipm.org/>), has been reviewed and approved under the authority of the Inter-American Metrology System (SIM) and found to be in compliance with the expectations of the *Comité international des poids et mesures* (CIPM) Mutual Recognition Arrangement. The SIM approval is available upon request.

## References

1. U.S. Department of Health and Human Services. Tetrodotoxin Poisoning Associated With Eating Puffer Fish Transported from Japan - California, 1996. Volume 45: 389-391. Centers for Disease Control and Prevention; Atlanta, GA, USA. *Morbidity and Mortality Weekly Report*.
2. Bane V, Lehane M, Dikshit M, O'Riordan A, Furey A (2014). Tetrodotoxin: Chemistry, Toxicity, Source, Distribution. *Toxins* 6: 693-755.
3. Thomas K, Wechsler D, Chen YM, Crain S, Quilliam MA (2016). Analysis of natural toxins by liquid chromatography-chemiluminescence nitrogen detection and application to the preparation of certified reference materials. *J AOAC Int* 99: 1173-1184.
4. Burton IW, Quilliam MA, Walter JA (2005). Quantitative  $^1\text{H}$  NMR with external standards: Use in preparation of calibration solutions for algal toxins and other natural products. *Anal Chem* 77: 3123-3131.
5. Pauwels J, Lamberty A, Schimmel H (2000). Evaluation of uncertainty of reference materials. *Accred Qual Assur* 5: 95-99.
6. Pauwels J, Lamberty A, Schimmel H (1998). The determination of the uncertainty of reference materials certified by laboratory intercomparison. *Accred Qual Assur* 3: 180-184.
7. Ellison SLR, Burke S, Walker RF, Heydorn K, Mansson M, Pauwels J, Wegscheider W, te Nijenhuis B (2001). Uncertainty for reference materials certified by interlaboratory study: Recommendations of an international study group. *Accred Qual Assur* 6: 274-277.
8. Evaluation of measurement data – Guide to the expression of uncertainty in measurement (GUM), JCGM 100:2008 (corrected version 2010), [www.bipm.org](http://www.bipm.org). Printed as ISO/IEC Guide 98-3:2008, ISO Geneva.
9. Abal P, Louzao MC, Antelo A, Alvarez M, Cagide E, Vilariño N, Vieytes MR, Botana LM (2017). Acute Oral Toxicity of Tetrodotoxin in Mice: Determination of Lethal Dose 50 (LD50) and No Observed Adverse Effect Level (NOAEL). Vasconcelos V, ed. *Toxins* 9(3): 75.
10. EFSA CONTAM Panel (Knutsen HK et. al) 2017. Scientific opinion - Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. *EFSA Journal* 15(4): 4752.



**Figure 1:** Full scan (A) and collision induced dissociation (MS/MS) (B) LC-HRMS spectra of TTX in CRM-TTX analyzed on a QExactive-HF mass spectrometer. Full scan data was acquired at a resolution setting of 120 000 and MS/MS data was acquired in parallel reaction monitoring scan mode at a resolution setting of 30 000 and a normalized collision energy of 57 V.



**Figure 2:** LC-HRMS analysis of CRM-TTX using an Agilent 1200 LC connected to QExactive-HF mass spectrometer. LC conditions: Acquity UPLC BEH Amide (100 mm  $\times$  2 mm i.d.) at +35  $^{\circ}$ C; 0.3 mL/min gradient elution from 10% A (water) to 75% B (95% acetonitrile) over 10 min, both with 2 mM ammonium formate and 50 mM formic acid.

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**This Certificate is only valid if the corresponding material was obtained directly from the NRC or an Authorized Reseller.**

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