Certificate of Analysis

NRC·CNRC Certified Reference Material

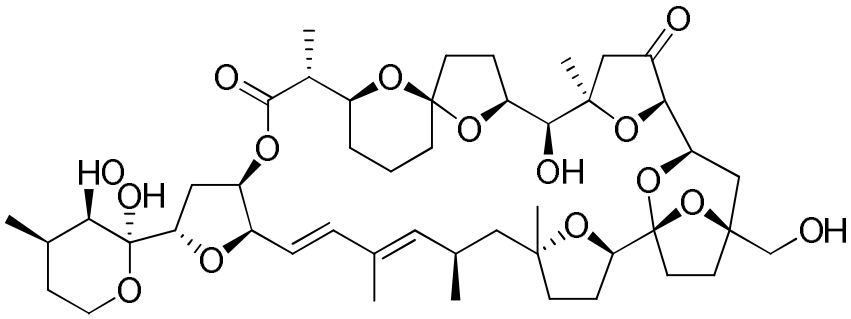
CRM-PTX2-b (Lot# 20120516)

Certified Calibration Solution for Pectenotoxin-2

Pectenotoxins (PTXs) are a group of polyether lactone toxins produced by several dinoflagellate species such as *Dinophysis fortii* and *Dinophysis acuta* [1,2]. PTXs have been associated with diarrhetic shellfish poisoning due to their co-occurrence in shellfish and plankton with the okadaic acid group and because of their toxicity to mice via intraperitoneal injection. However, they have not yet been established to be orally toxic to mice and have not been implicated in human illness [3]. Nonetheless, PTXs are regulated by the European Union at 160 µg/kg of tissue equivalent in combination with okadaic acid and dinophysistoxins [4]. CRM-PTX2-b is a certified calibration solution of PTX2 in methanol with a concentration of 5.13 µmol/L.

Table 1: Certified concentration values for CRM-PTX2-b.

Compound	µmol/L (at +20 °C)	µg/mL (at +20 °C)	µg/g
Pectenotoxin-2	5.13 ± 0.15	4.40 ± 0.13	5.58 ± 0.16



Pectenotoxin-2
CAS registry No.: 97564-91-5
Molecular formula: $C_{47}H_{70}O_{14}$
Molecular weight: 859.07
$[M+NH_4]^+$: *m/z* 876.5104

Expiry date: 1 year from date of sale
Storage conditions: -12 °C or below

Intended Use

CRM-PTX2-b is a certified calibration solution designed for analytical method development and accurate quantitation of PTX2. The concentration of CRM-PTX2-b makes it suitable for preparing a dilution series for calibration of liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC-MS) instrumentation, as well as for spiking shellfish control samples for recovery experiments.

Preparation of CRM

PTX2 was isolated at the Cawthron Institute, New Zealand, from a phytoplankton bloom where *Dinophysis acuta* was the predominant species. The phytoplankton were harvested 3-4 km off the coast of Buller Bay, New Zealand, in early February 2001, and was purified through a series of chromatographic steps as described previously [2]. A stock solution was prepared by dissolving purified PTX2 in CD_3OH (~4 mM). The concentration of PTX2 in this stock solution was then determined by quantitative NMR [5], after which the CRM solution was prepared by accurately diluting the PTX2 stock solution in degassed, high-purity methanol and making to a final volume of 2 L. This solution was thoroughly mixed with a Teflon stir bar and magnetic mixer while being cooled in an ice bath under an argon atmosphere. Aliquots were dispensed into amber glass ampoules and flame-sealed under argon. Ampoules were then labeled, packaged, and immediately transferred to long-term storage at -80 °C. Each ampoule contains approximately 0.5 mL of solution.

Structural Confirmation and Purity Assessment

The structure and purity of PTX2 used in the preparation of CRM-PTX2-b was confirmed by ^1H nuclear magnetic resonance (NMR) (Figure 1) and LC-MS/MS. A product ion spectrum showed a fragmentation that is characteristic of PTX2. LC-MS was also used to estimate the general purity of the PTX2-b stock solution (column: 2.5 µm Luna C18(2), 100 × 2 mm; water (A), acetonitrile/water (95/5) (B) each containing 2 mM ammonium formate and 50 mM formic acid (pH 2.3); 5 to 100% B over 15 min at 200 µL/min; +20 °C column temperature). A partially resolved, unidentified isomer and PTX2c were observed (Figure 2). Using an isocratic LC method (column and mobile phase as above; 55% B at 250 µL/min; +20 °C column temperature) the retention times of the impurities relative to that of PTX2 were 1.07 and 2.05 respectively. The CRM solution contains approximately 2.5% of the unresolved isomer and 2.6% of PTX2c when monitored under isocratic elution and selected ion monitoring (SIM) conditions. The proportions of these minor compounds are provided as information values only and are not certified.

Homogeneity

A representative number of ampoules (n=30) were selected across the fill series to determine the homogeneity of CRM-PTX2-b. Analyses were performed by LC-MS (Figure 3). The between-bottle variation was measured to be smaller than the variation for replicate analyses of one solution (RSD <5% for n=16), thus demonstrating acceptable homogeneity over the entire ampoule range.

Stability

The short- and long-term stability of CRM-PTX2 (Lot # 20021127) in methanol was previously assessed using a classical stability study design. CRM-PTX2-b was prepared in the same manner and equivalent stability is reasonably assumed. Over a period of 30 days, no degradation of PTX2 was observed at temperatures up to +20 °C, indicating excellent stability over the short-term. However, degradation of PTX2 did become significant after 30 days at +37 °C. Regressions for the 1 year long-term stability data were calculated for each storage temperature and tested for significance. The regressions were

found to be insignificant for all conditions up to +4 °C. An uncertainty associated with the long-term stability assessment was determined using the standard error of the -12 °C slope (see section Uncertainty). The relative uncertainty due to long-term stability (μ_{lts}) is 0.77%. The recommended storage temperature for CRM-PTX2-b is -12 °C.

Certified Value

The certified value for CRM-PTX2-b, 5.13 ± 0.15 µmol/L (Table 1), is based on the results obtained at the National Research Council of Canada (NRC) using quantitative NMR (qNMR) spectroscopy [5] and LC-MS in SIM mode. For qNMR, three NMR tubes of the purified PTX2-b stock solution were analyzed and quantified using a high purity caffeine standard as the calibrant. For LC-MS, six ampoules were selected from across the fill series and injected in triplicate using CRM-PTX2 (Lot # 20021127) as a calibrant.

The results shown in this certificate are traceable to the SI standard through gravimetrically prepared standards of established purity. This product serves as a suitable reference material for laboratory quality assurance programs.

Uncertainty

The overall uncertainty estimate (U_{CRM}) for CRM-PTX2-b includes uncertainties associated with batch characterization (μ_{char}), between-bottle variation (μ_{hom}), and stability in long-term storage (μ_{stab}) [6]. These components can be combined and expanded according to:

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

where *k* is the coverage factor (generally 2 or 3).

All reasonable sources of error related to the characterization of CRM-PTX2-b were considered and quantitated. Quantitative analysis of CRM-PTX2-b by qNMR and LC-MS yielded a characterization uncertainty (μ_{char}) value of 0.065 µM. An extensive homogeneity study was performed on CRM-PTX2-b and no significant heterogeneity was detected. The uncertainty for long-term stability (μ_{stab}) was established to be 0.039 µM when expressed as µmol/L. All these sources of uncertainty were combined with a coverage factor of 2 to estimate the final uncertainty associated with the certified value of CRM-PTX2-b, as summarized in Table 2.

Table 2: Uncertainty budget for the certified value of CRM-PTX2-b.

Uncertainties	µmol/L
μ_{hom}	*negligible*
μ_{stab}	0.039
μ_{char}	0.065
U_{CRM} (k=2)	0.15

Storage Instructions

To ensure the stability of CRM-PTX2-b, this material should be stored unopened at -12 °C or below. Likewise, any dilutions of this material should be stored in low-headspace vials at -12 °C.

Expiry

If stored unopened at the recommended storage condition, the certified concentration of CRM-PTX2-b is valid for 1 year from the time of sale.

Instructions for Use

Prior to opening, each ampoule should be allowed to warm to room temperature and the contents mixed thoroughly. The ampoules 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. Increases in concentration due to evaporation of methanol will occur if the solution is left opened for more than a few minutes. It is recommended that solutions of CRM-PTX2-b should not be evaporated to dryness, due to potential decomposition on glass surfaces. *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.

Safety Instructions

PTXs were originally classified as diarrhetic shellfish toxins due to their co-occurrence in plankton and shellfish with okadaic acid group toxins; however, they have not yet demonstrated oral toxicity in mouse feeding studies and have not been directly implicated in any human poisoning events [3]. Nevertheless, precautions should be taken when handling CRM-PTX2-b. Inhalation and ingestion of methanol is harmful; ingestion may cause blindness or fatality, while prolonged skin contact may result in dermatitis and/or kidney damage. Only qualified personnel should handle the solution and appropriate disposal methods should be used. Heavy gloves and eye protection should be used when opening the ampoule in the event the glass shatters. A material safety data sheet (MSDS) is available for CRM-PTX2-b.

References

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4. Anonymous. (2004). Regulation (EC) No 853/2004 of the European parliament and of the council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Off. J. of the European Union* L 139 of 30 April 2004.
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6. Pauwels, J., Lamberty, A., Schimmel, H., (2000). "Evaluation of uncertainty of reference materials." *Accred. Qual. Assur.* 5, 95-99.

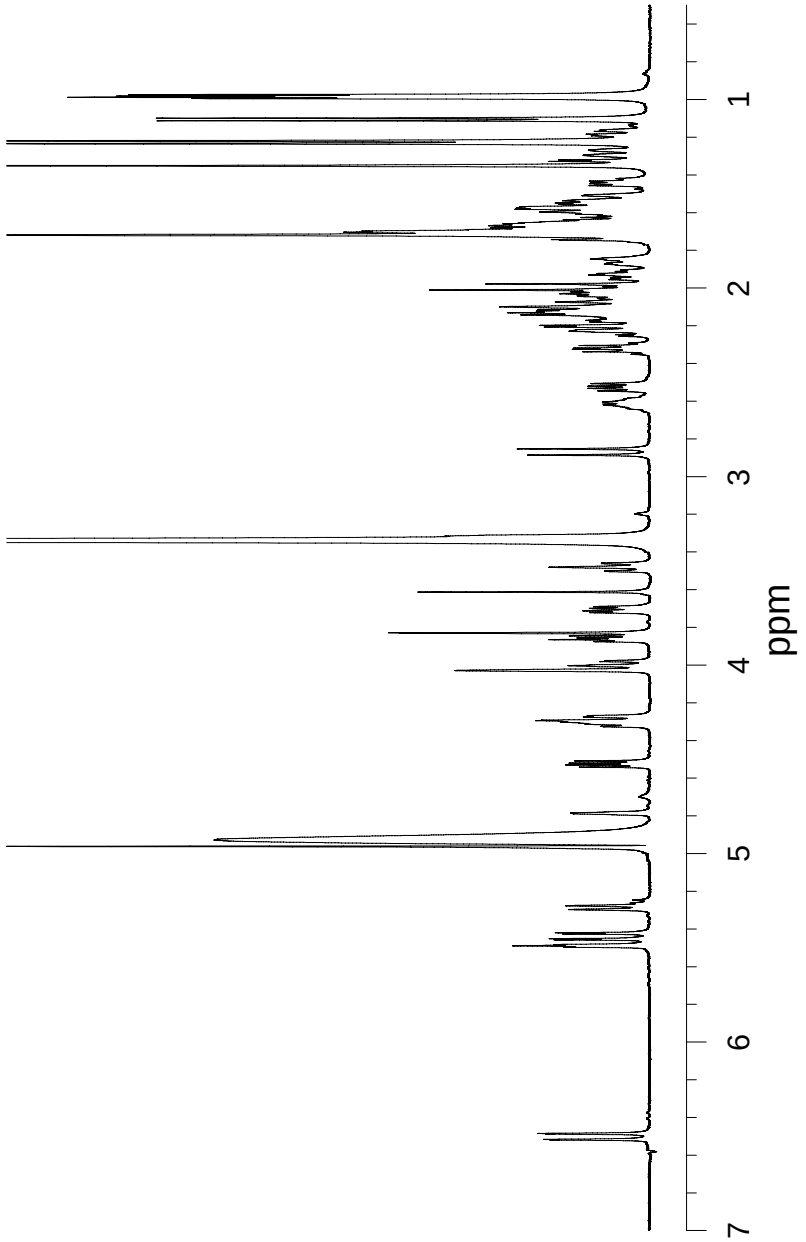

Figure 1: Proton NMR spectrum of PTX2 used for preparation of CRM-PTX2-b dissolved in methanol-d_3.

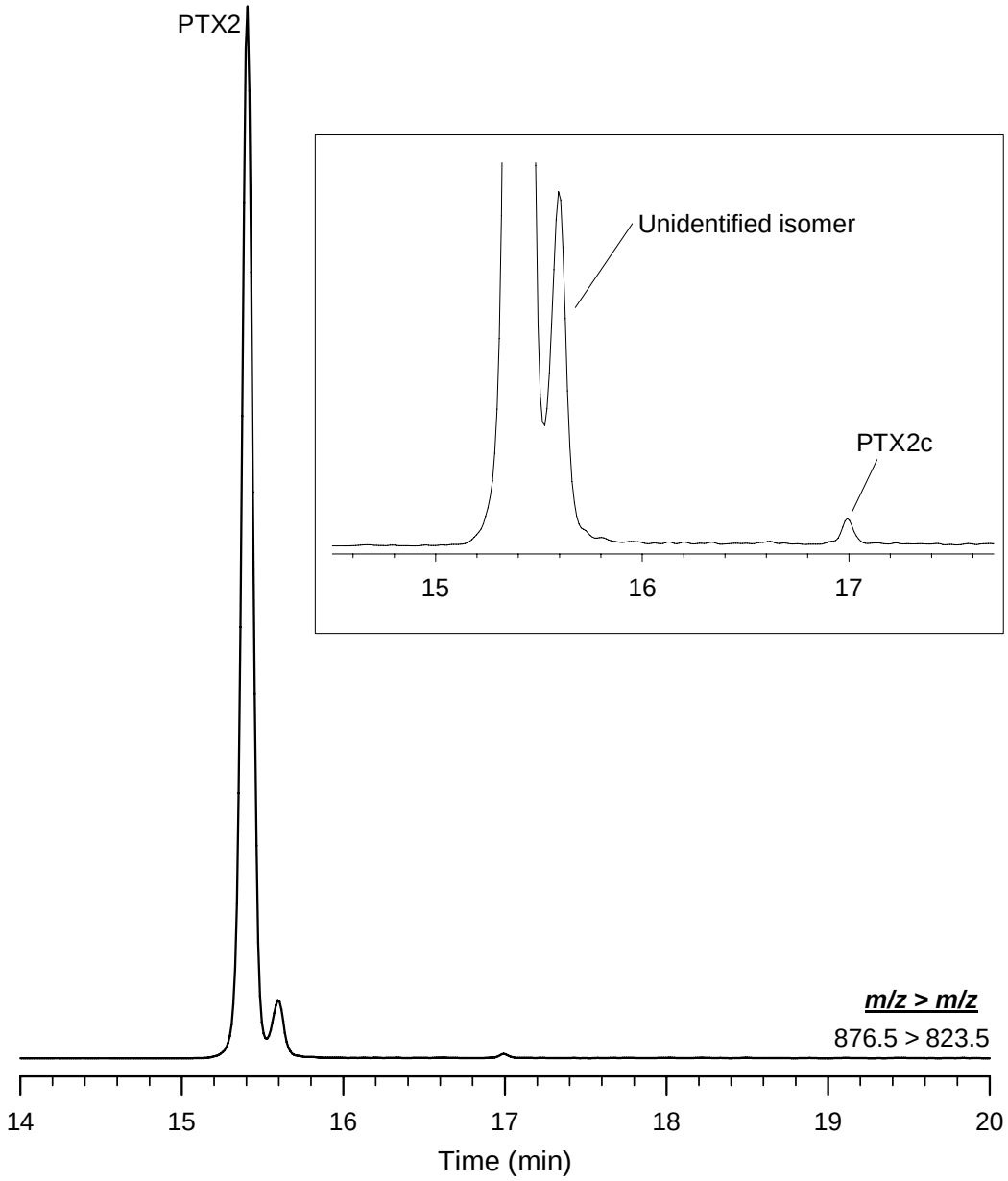

Figure 2: Purity analysis of PTX2-b stock solution by LC-MS/MS shows the presence of two PTX2 isomers. Conditions: 2.5 µm Luna C18(2), 100 × 2 mm, maintained at +20 °C; mobile phase: water (A), acetonitrile/water (95:5, v/v) (B), each containing 50 mM formic acid and 2 mM ammonium formate, gradient 5-100% B over 15 min, 200 µL/min; 0.1 µL injection; MS operated in positive selected reaction monitoring mode.

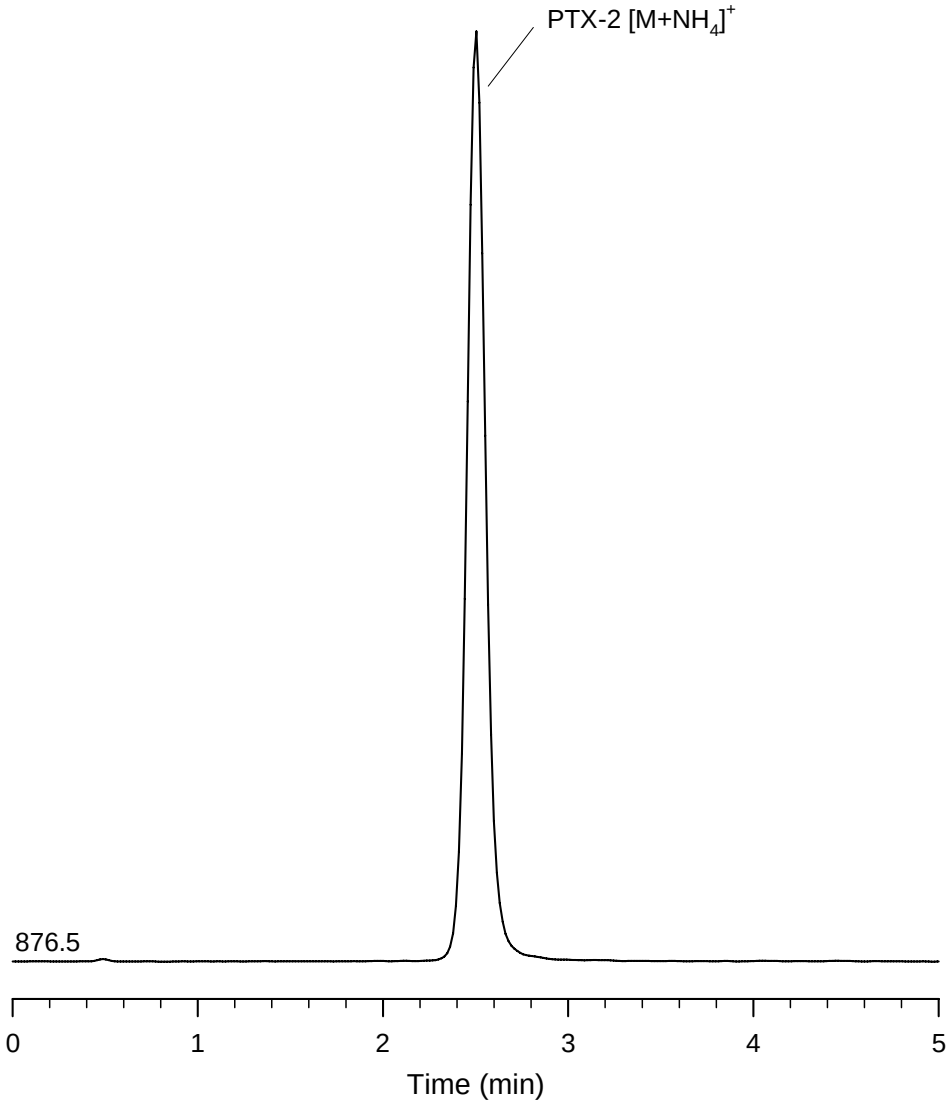

Figure 3: LC-MS analysis of CRM-PTX2-b. Conditions: 2.5 µm Luna C18(2), 50 × 2 mm, maintained at +20 °C; mobile phase: 50 mM formic acid and 2 mM ammonium formate in both (A) and (B); water (A), acetonitrile/water (95/5) (B), isocratic elution at 50% B, 300 µL/min; 0.8 µL injection; MS operated in positive SIM mode.

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Signed: 

Michael A. Quilliam, Ph.D.
Group Leader, Biotoxin Metrology
Measurement Science and Standards

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Comments, information and inquiries should be addressed to:

National Research Council Canada
Measurement Science and Standards
1411 Oxford Street
Halifax, Nova Scotia B3H 3Z1

Telephone: 1-902-426-8281
Fax: 1-902-426-5426

Email: CRM-MRCBiotoxin-Biotoxines@nrc-cnrc.gc.ca

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