National Research Council Canada | Conseil national de recherches Canada

Certificate of Analysis

Certified Reference Material

CRM-PSP-Mus (Lot# 200703)

Mussel Tissue Reference Material for Saxitoxin and Related Analogues

Paralytic shellfish poisoning (PSP) is caused by consumption of shellfish that have accumulated saxitoxins produced by dinoflagellates [1]. CRM-PSP-Mus is a blend of whole mussel tissues (*Mytilus edulis*), cultured *Alexandrium spp.* biomass and semi-purified toxins [2]. Certified values and expanded uncertainties are provided for saxitoxin (STX), gonyautoxin-2 (GTX2) and gonyautoxin-3 (GTX3) (Table 1), and information values are provided for additional STX analogues (Table 2). A certified value is also provided for the sum of domoic acid (DA) and C5'-epi-domoic acid (epiDA) (Table 3).

Table 1: Certified concentration values and associated uncertainties for STX analogues in CRM-PSP-Mus.

Compound	µmol/kg	µg STXdiHCl eqv/kg*
Saxitoxin (STX)	4.99 ± 0.33	1860
Gonyautoxin-2 (GTX2)	0.96 ± 0.13	128
Gonyautoxin-3 (GTX3)	0.33 ± 0.05	78

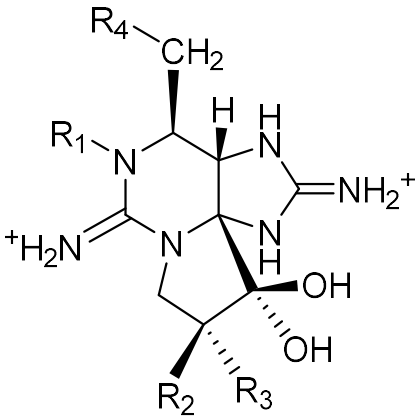
Table 2: Information values for other STX analogues in CRM-PSP-Mus.

Compound	µmol/kg**	µg STXdiHCl eqv/kg*
Gonyautoxin-1 (GTX1)	0.13	49
Gonyautoxin-4 (GTX4)	0.04	12
Decarbamoylgonyautoxin-2 (dcGTX2)	0.38	22
Decarbamoylgonyautoxin-3 (dcGTX3)	0.10	14
Neosaxitoxin (NEO)	0.19	65
Decarbamoylsaxitoxin (dcSTX)	0.27	51
Total µg STXdiHCl eqv/kg:		2278

*Toxicity equivalence information values calculated using toxicity equivalency factors [3]
**Concentrations are not certified

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

Canada



R_1	R_2	R_3	R_4	Toxin
H	H	H		STX
OH	H	H		NEO
H	H	OSO_3^-		GTX2
H	OSO_3^-	H		GTX3
OH	H	OSO_3^-		GTX1
OH	OSO_3^-	H		GTX4
H	H	OSO_3^-		dcGTX2
H	OSO_3^-	H	−OH	dcGTX3
H	H	H		dcSTX

Figure 1: Structure of saxitoxin and related analogues present in CRM-PSP-Mus.

Table 3: Certified value and uncertainty for the sum of DA and epiDA in CRM-PSP-Mus.

Compound	mg/ kg
DA + epiDA	33.6 ± 2.1

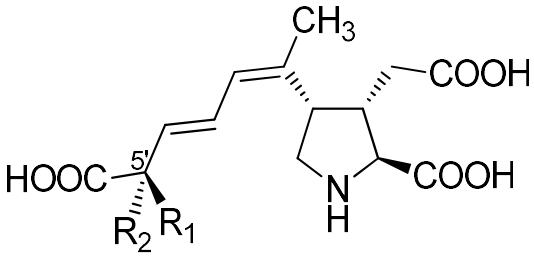


Figure 2: Structure of DA (R_1=CH_3, R_2=H) and C5'-epi-DA (R_1=H, R_2=CH_3).

Intended Use

CRM-PSP-Mus is a matrix CRM with certified values for STX, GTX2, GTX3, and the sum of DA and epiDA. It is designed to test the accuracy of entire analytical methods or to assist in the development of new analytical methods for these toxins. CRM-PSP-Mus also has information values for related STX analogues that can be used in method development for these compounds.

Instructions for Storage and Use

To ensure the stability of CRM-PSP-Mus bottles should be stored in a freezer (−12 °C or below).

Bottles should be allowed to warm to room temperature prior to opening and the contents thoroughly mixed. Each bottle contains approximately 8 g (± 0.5 g) of tissue. The mass of homogenate is not certified. Remove hermetic seal carefully, mix contents thoroughly and weigh the sample on an analytical balance. It is not recommended that sub-samples be taken from individual bottles of CRM-PSP-Mus; the entire contents of the bottle should be used for analysis.

Preparation of the CRM-PSP-Mus

CRM-PSP-Mus is a blend of cooked mussels (*Mytilus edulis*) with no detectable levels of STX or DA toxins (harvested in Canada and The Netherlands), cooked mussels (*Mytilus edulis*) contaminated with DA (harvested in Prince Edward Island, Canada), cultured toxic *Alexandrium tamarense* and *Alexandrium minutum* biomass, and a small amount of semi-purified STX and NEO [4]. The STX toxin profile in the algal biomass was stabilized after harvesting [5]. All mussel material was combined and homogenized. Processed *A. tamarense* and *A. minutum* were added along with an aqueous solution containing semi-purified STX and NEO. Ethoxyquin was included as a stabilizer (0.02 % w/w). The combined material was passed through a Comitrol food cutter multiple times using deionized water to rinse, resulting in a final water content of approximately 85%. The homogenate was de-aerated, purged with nitrogen, and dispensed into polypropylene bottles which were flushed with nitrogen, heat sealed, and thermally processed in a steam retort (118 °C for 20 min). After cooling, the bottles were frozen before individual units were sealed in trilaminate pouches.

Analytical Methods and Value Assignment

The certified values for STX, GTX2, and GTX3 were obtained using two analytical methods. CRM-PSP-Mus extracts were prepared using a liquid-solid extraction (LSE) method modified from the AOAC extraction procedure [6] (8 g tissue, extracted using 3-step procedure with 0.1 M HCl to a final volume of 25 mL [2]). Analysis was done using a modified version of the LC-pcox-FLD method [7] (Figure 3), and by hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC-MS/MS) (Figure 4). For LC-pcox-FLD, external calibration was performed using accurate dilutions of STX calibration solutions (CRM-STX and CRM-GTX2&3). For HILIC-MS/MS, standard addition with a spiking solution prepared using the same CRMs was used to compensate for matrix effects. Information values for CRM-PSP-Mus (Table 2) were assigned using one or both of the same techniques, using dilutions of NRC calibration solutions (CRM-NEO, CRM-dcSTX, CRM-dcGTX2&3 and CRM-GTX1&4) as the calibrants.

The certified value for DA and epiDA (Table 3) was obtained by extracting samples using an exhaustive liquid-solid extraction method (4 g tissue with 50 mL of 50% aqueous methanol, 4-step procedure). Filtered extracts were analysed by LC-UV and LC-MS/MS (Figure 5). No significant matrix effects were observed in LC-MS/MS. Calibrations were performed using accurate dilutions of CRM-DA. The certified value is comprised of the combined concentration of DA and its

C5'-diastereomer, C5'-epiDA, which has a UV spectrum identical to that of DA. Information values for the individual concentrations of DA and epiDA by LC-UV are 30.8 and 2.8 mg/kg, respectively.

A low concentration of okadaic acid (OA) was measured in CRM-PSP-Mus by LC-MS/MS using CRM-OA as the calibrant. The information values for OA before and after base hydrolysis are 0.03 µg/g and 0.06 µg/g, respectively.

Homogeneity

A representative number of CRM-PSP-Mus bottles were selected from across the fill series. Relative concentrations of STXs and DA were measured by LC-pcox-FLD and LC-UV, respectively. Uncertainty contributions from homogeneity testing are included in the overall uncertainty budget for CRM-PSP-Mus (Table 4).

Stability

The STXs and DA demonstrated good stability in CRM-PSP-Mus when stored at -12 °C. There was no significant degradation of certified analytes at +4 °C over two weeks.

Uncertainty

All reasonable sources of error related to the characterization of CRM-PSP-Mus were considered and measured. The overall uncertainty estimate (U_{CRM}) includes uncertainties associated with batch characterization (u_{char}), between-bottle variation (u_{hom}) and instability during storage (u_{stab}) [8-11]. These components are listed in Table 4, 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).

Table 4: Relative uncertainty components for the certified values in CRM-PSP-Mus.

Compound	u_{char}	u_{hom}	u_{stab}
STX	0.031	0.011	0.003
GTX2	0.036	0.057	0.006
GTX3	0.031	0.069	0.002
DA + epiDA	0.026	0.009	0.016

Safety Instructions

CRM-PSP-Mus contains STXs and DA, toxins responsible for incidents of paralytic shellfish poisoning and amnesic shellfish poisoning, respectively. Only qualified personnel should handle the material and appropriate disposal methods should be used. A safety data sheet (SDS) is available for CRM-PSP-Mus.

Period of Validity

If stored unopened at the recommended storage condition of -12 °C or below, the certified concentrations of CRM-PSP-Mus are 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 NRC certified reference materials for STX (CRM-STX-e), GTX2 and GTX3 (CRM-GTX2&3-c) and DA (CRM-DA-f).

Quality 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.

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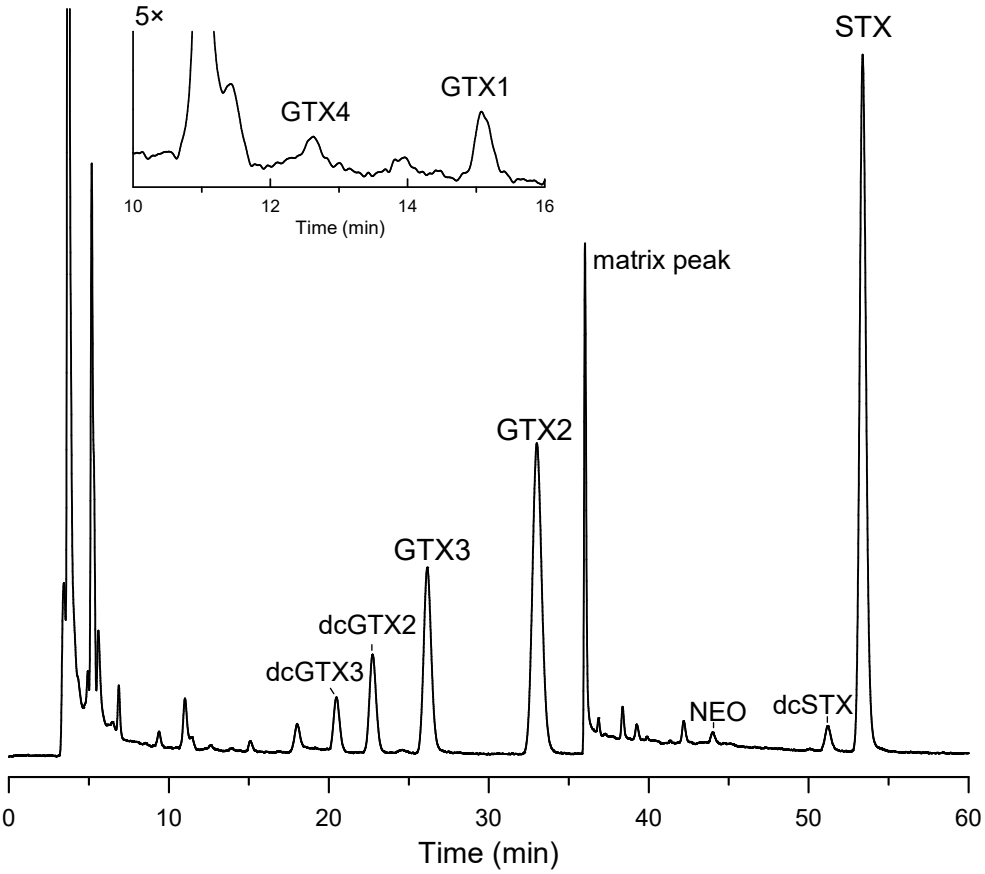

Figure 3: LC-pcox-FLD analysis of a CRM-PSP-Mus extract. Chromatographic conditions: Zorbax Bonus-RP column (150 × 4.6 mm, 5 µm); mobile phase: (A) water with 11 mM heptane sulfonate, 5.5 mM phosphoric acid (pH 7.1), and (B) water with 11 mM heptane sulfonate, 16.5 mM phosphoric acid (pH 7.1) and 11.5 % acetonitrile; step gradient 100% A, switch to 100% B at 18 min; 0.8 mL/min at +30 °C; 20 µL injection; post-column oxidation: 0.4 mL/min 5 mM periodic acid, 100 mM phosphoric acid in water at pH 7.8 with reaction coil at 80 °C; effluent acidified with 0.4 mL/min 0.75 M nitric acid; detection: fluorescence with excitation at 330 nm and emission at 390 nm.

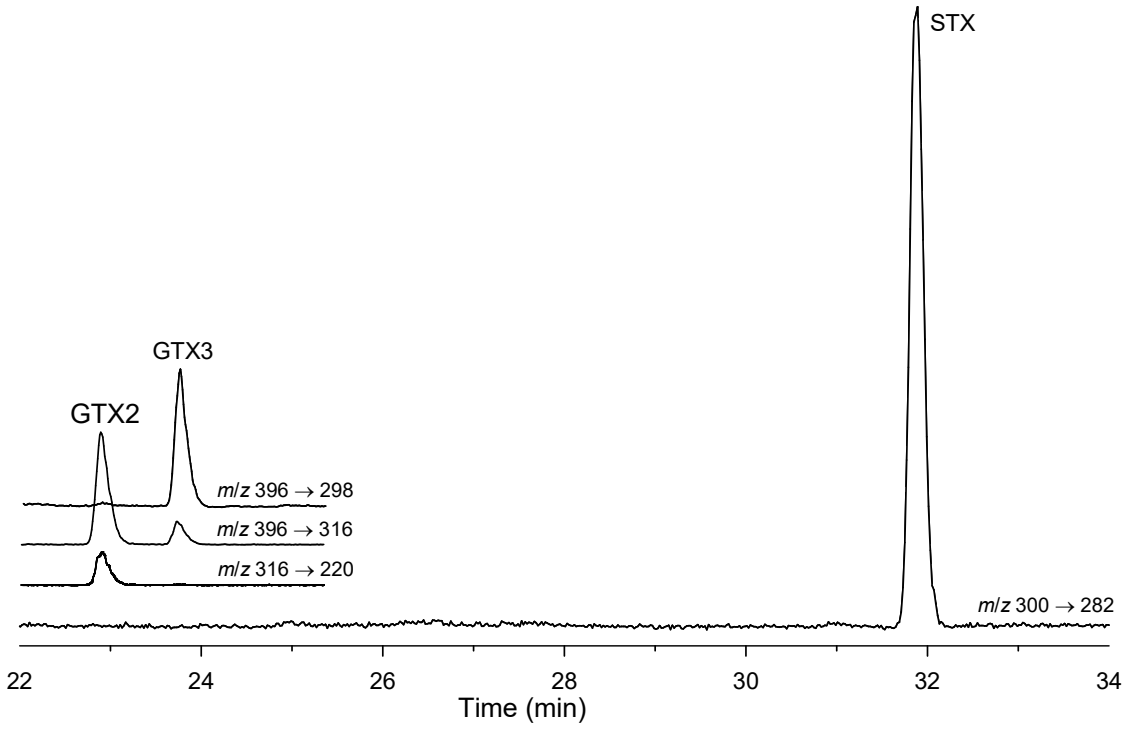

Figure 4: LC-MS/MS analysis of STX, GTX2 and GTX3 in a CRM-PSP-Mus extract using an Agilent 1290 LC connected to a Sciex 5500 QTRAP with positive electrospray ionization. Chromatographic conditions: Toso-Haas Amide-80 column (250 × 2.1 mm, 5 µm); mobile phase: (A) water with 50 mM formic acid 2 mM ammonium formate, and (B) MeCN; gradient: 90 to 55% B over 25 min; 0.2 mL/min at +40 °C; 5 µL injection. MS operated in positive ion selected reaction monitoring mode used previously described conditions [2].

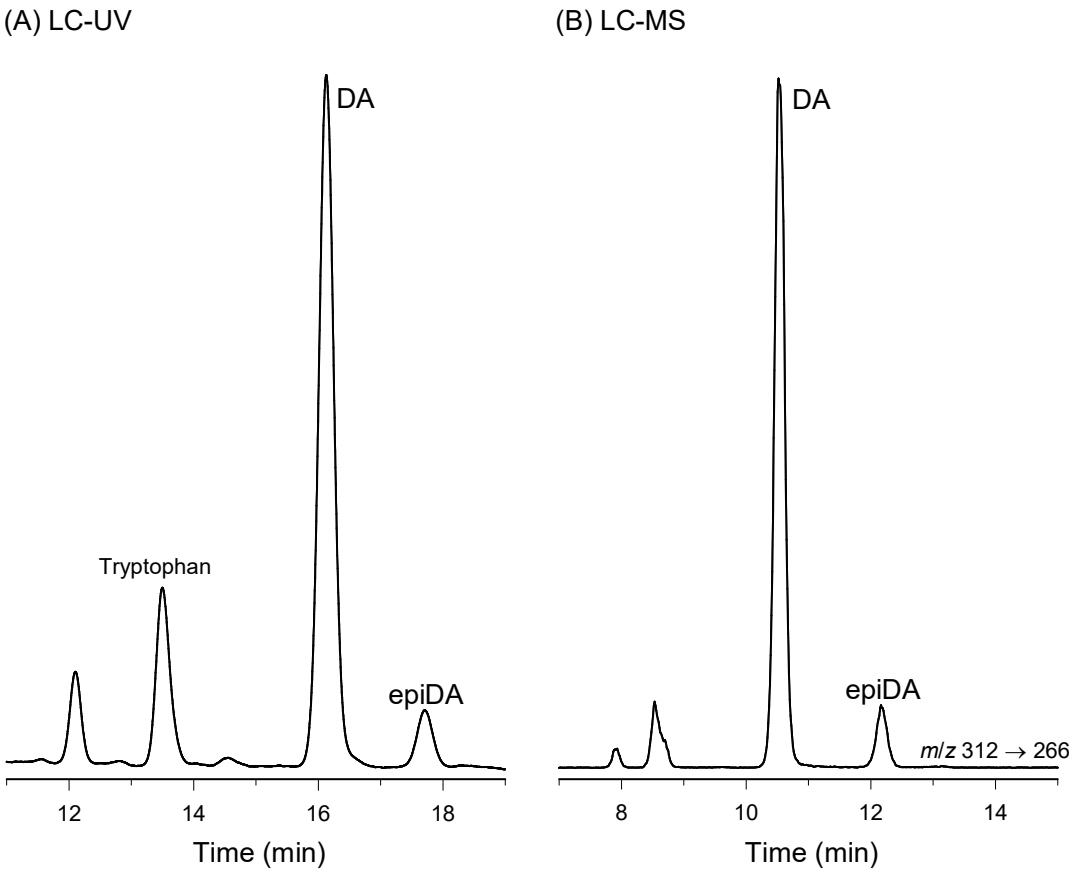

Figure 5: LC-UV (A) and LC-MS/MS (B) analysis of DA and epiDA in CRM-PSP-Mus extracts using Phenomenex Luna C18 column (150 × 4.6 mm, 3 µm). LC-UV conditions: Agilent 1100 LC with 1290 diode array detector; wavelength: 242 nm; mobile phase: water with 10% MeCN and 0.1 % TFA; 0.9 mL/min at +35 ºC; 10 µL injection. LC-MS conditions: Agilent 1200 with a Sciex 4000 QTRAP; mobile phase: water with 10% MeCN and 0.1 % formic acid; 0.9 mL/min at +35 ºC; 5 µL injection; MS operated in positive ion selected reaction monitoring mode; MS collision energy: 25 V, MS declustering potential: 50 V.

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