Certificate of Analysis

NRC·CNRC Certified Reference Material

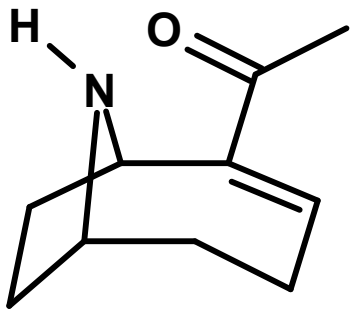
CRM-ATX (Lot# 20100721)

Certified Calibration Solution for Anatoxin-a

Anatoxin-a (ATX-a) is a cyanobacterial toxin linked to animal deaths in North America [1] and globally [2]. CRM-ATX is a certified calibration solution prepared to aid the analyst in the determination of ATX-a. Each ampoule contains approximately 0.5 mL of ATX-a dissolved in methanol/water (9:91, v/v) with 0.01% acetic acid. The concentration is suitable for calibration of liquid chromatography experiments and for standard addition and spike recovery studies.

Table 1: Certified concentration values for CRM-ATX

Compound	µmol/L (at +20 °C)	µg/mL (at +20 °C)	µg/g
Anatoxin-a	30.0 ± 1.1	4.96 ± 0.18	5.03 ± 0.18



(+) Anatoxin-a
CAS registry No.: 64285-06-9
Molecular formula: $C_{10}H_{15}NO$
Molecular weight: 165.232 g/mol
$[M+H]^+$: *m/z* 166.1226

Expiry date: 1 year from date of sale
Storage conditions: In the dark at -20 °C

Intended Use

CRM-ATX is a calibration solution certified reference material (CRM) designed for analytical method development and accurate quantitation of ATX-a. The CRM concentration makes it suitable for preparing a dilution series for calibration of instrumentation, such as liquid chromatography with detection by ultraviolet absorbance (LC-UV), fluorescence (LC-FL) or mass spectrometry (LC-MS), and for spiking control samples for recovery experiments.

Preparation of the CRM-ATX

ATX-a was isolated from an algal culture of *Aphanizomenon issatschenkoi* [3]. The toxin was extracted from the cells, purified by chromatography and then dried *in vacuo* to the anhydrous form [4,5]. The CRM solution was prepared by dissolving the purified ATX-a in degassed methanol/water (9:91, v/v) with 0.01% acetic acid. The solution was thoroughly mixed with a Teflon-coated stir bar and magnetic mixer, under argon. Aliquots were dispensed into amber glass ampoules pre-filled with argon, which were then immediately flame-sealed. Each ampoule contains approximately 0.5 mL of solution.

Structural Confirmation and Purity Assessment

The purity of the toxin was checked by the following techniques: 500 MHz ^{1}H NMR spectroscopy, LC-MS [6], LC-UV, capillary electrophoresis with UV detection (CE-UV) [7], and liquid chromatography with chemiluminescence nitrogen detection (LC-CLND) [8]. The molecular structure of ATX-a was confirmed by NMR spectroscopy [9] and quadrupole time-of-flight (QToF) mass spectrometry. The ^{1}H NMR spectrum of ATX-a is shown in Figure 1. The accurate mass was obtained for the $[M+H]^+$ ion at 166.12269 Da, which is within 0.05 mDa of the theoretical monoisotopic mass of 166.12264 Da (Δ = 0.30 ppm). A tandem MS spectrum of the $[M+H]^+$ ion was also acquired, as shown in Figure 2. NMR, LC-MS, LC-UV and LC-CLND were used to estimate the purity of ATX-a. No major impurities were observed.

Homogeneity

As this CRM is a true solution, there should be insignificant variation between ampoules. Nevertheless, approximately 0.8% of all ampoules produced were randomly selected and the ATX-a concentration was measured by LC-UV. The between-ampoule variation was measured to be no greater than the variation for replicate analyses of one solution, thus demonstrating acceptable homogeneity over the entire ampoule range.

Stability Study

Extensive studies have been conducted to determine the stability of CRM-ATX under various conditions. ATX-a is unstable under basic solutions and is sensitive to light [10]. Feasibility studies on previous ATX-a preparations have demonstrated excellent long-term stability of ATX-a solutions stored in acidified methanol/water at +4 °C. A long-term stability study was performed where CRM-ATX was stored at several temperatures (-80 °C, -20 °C, +4 °C, +25 °C and +37 °C). ATX-a exhibited no detectable decomposition when stored at -20 °C and had less than 3% decomposition at +4 °C after one year. Less than 2% degradation was observed at +37 °C after ten days.

Certified Value

The certified value of 30.0 ± 1.1 µmol/L (Table 1) for CRM-ATX is based on results obtained at NRC using two independent analytical methods: LC-CLND and quantitative nuclear magnetic resonance (QNMR) spectroscopy [11]. Calibration of both of these techniques was performed using accurate USP grade caffeine solutions.

The results shown in this certificate are traceable to the SI standard through gravimetrically prepared standards of caffeine 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-ATX includes uncertainties associated with batch characterization (u_{char}), between-bottle variation (u_{hom}), and instability during long-term storage (u_{stab}) [12-13]. These components can be combined as:

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

where k is the coverage factor (generally 2).

Quantitative measurements by CLND and QNMR contributed the most significant uncertainties. When combined they resulted in a relative uncertainty for batch characterization (u_{char}) of 0.018. As this CRM is a true solution, (u_{hom}) was not significant, as solutions are inherently homogenous [14]. Nevertheless, tests on homogeneity were performed (see Section Homogeneity), and the between-bottle variance was determined to be no greater than the measurement variance resulting in a relative value of 0.002 for (u_{hom}). Therefore, no uncertainty contribution from homogeneity testing was included in the combined uncertainty calculation for CRM-ATX. A long-term stability study was performed on this CRM (see Section Stability Study) which showed no observable loss of material when stored under the recommended conditions. The uncertainty due to stability (u_{stab}) [15] was extrapolated in order to reflect a shelf life of 1 year, but was also not significant with a relative value of 0.001. Applying a coverage factor of 2 resulted in a final relative expanded uncertainty in the certified value of 0.036.

Storage Instructions

To ensure the stability of ATX-a, the CRM should be stored in the dark in a freezer (approx. -20 °C). The CRM was found to be stable under these conditions. The CRM has been prepared under conditions that minimize the chance of bacterial contamination, but once the ampoule has been opened there may be a chance of contamination. Therefore, it is recommended that aliquots and dilutions of the CRM be stored in a good freezer to minimize the chances of bacterial degradation.

Expiry

If stored unopened at the recommended storage conditions (section Storage Instructions), the certified concentration of the CRM is valid for 1 year from the date of sale**.**

Instructions for Use

Prior to opening, each ampoule should be allowed to warm to room temperature and the contents should be thoroughly mixed. The ampoule should be inverted several times, then held upright, tapped to ensure that most of the solution drains to the bottom, and 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 on glass surfaces. A useful procedure that ensures accurate dilutions involves using a balance to determine weights of the dispensed aliquot and the final diluted solution, assuming that water is used as diluent (the density of the CRM solution is 0.9851 g/mL at +24 °C). *Note*: The volume of the solution is not certified. Only the concentration is certified.

Safety Instructions

If sufficient quantities are ingested, ATX-a can cause paralysis and even death. 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-ATX.

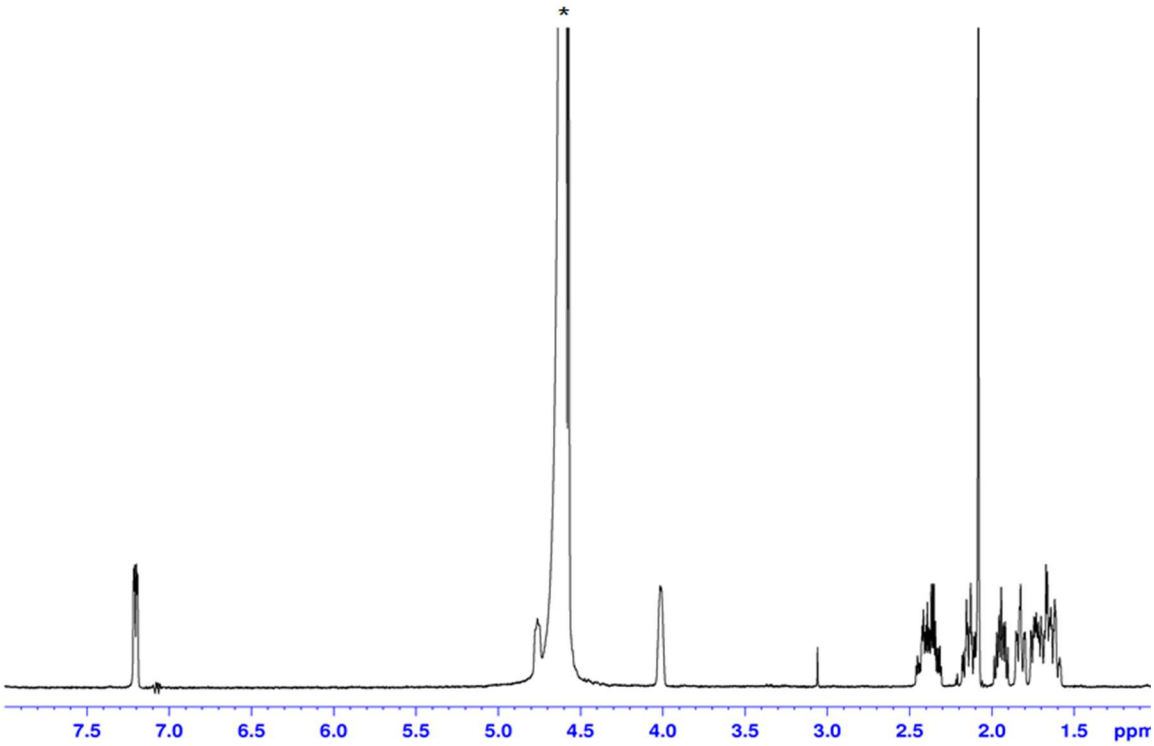


Figure 1: Proton NMR spectrum of ATX-a. The resonance marked with * is due to water.

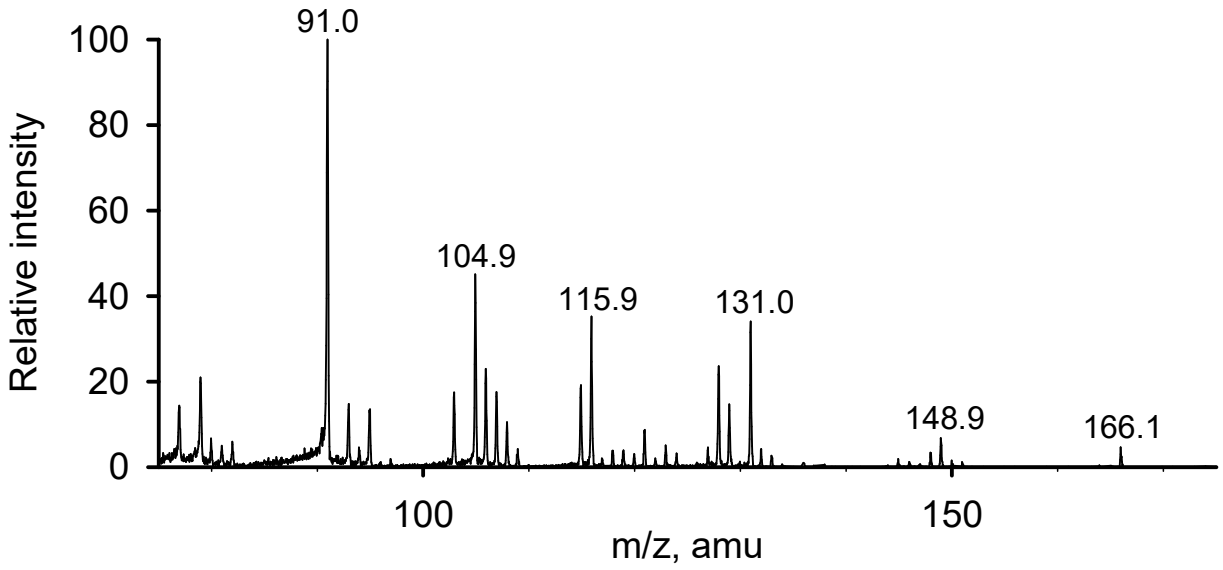


Figure 2: Product ion spectrum of $[M+H]^+$ ion (*m/z* 166) of ATX-a from an LC-MS/MS analysis of CRM-ATX. Conditions: AB Sciex API4000 QTRAP; collision energy = 40 V; declustering potential = 50 V.

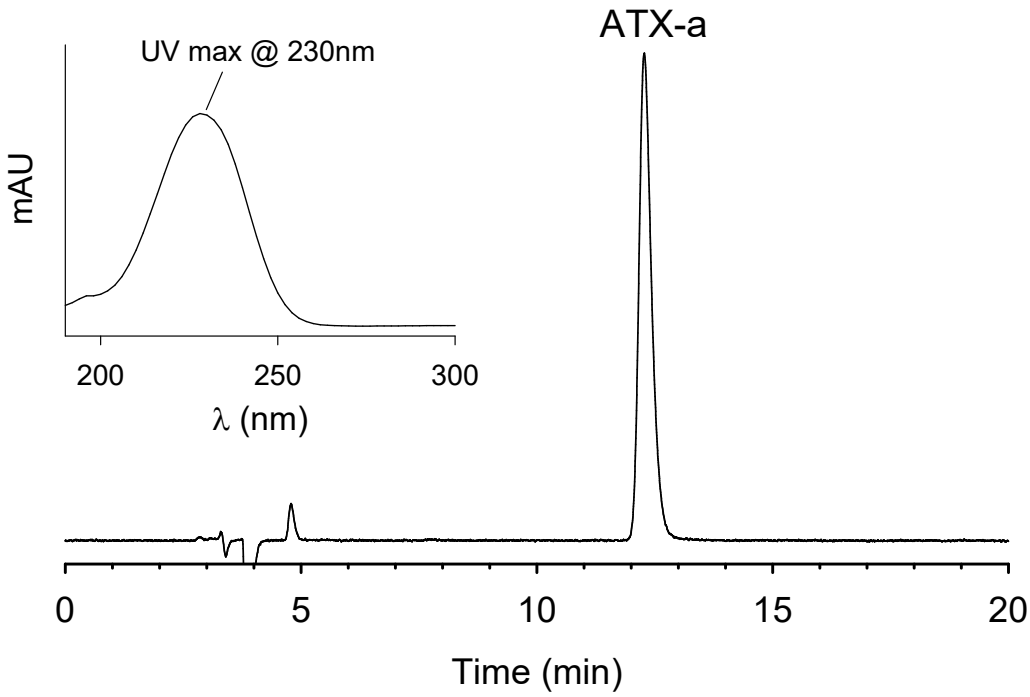


Figure 3: Analysis of CRM-ATX by LC-UV (230 nm). Conditions: Agilent 1100 LC with a Hewlett Packard 1050 UV detector with a standard flow cell; 250 mm × 2.1 mm i.d. column with 3 µm Phenomenex Columbus C18 at +30 °C; 0.2 mL/min $CH_3OH/H_2O/CF_3COOH$ (10:89.9:0.1); 10 µL injection. Inset shows a UV spectrum acquired separately on a diode-array detector.

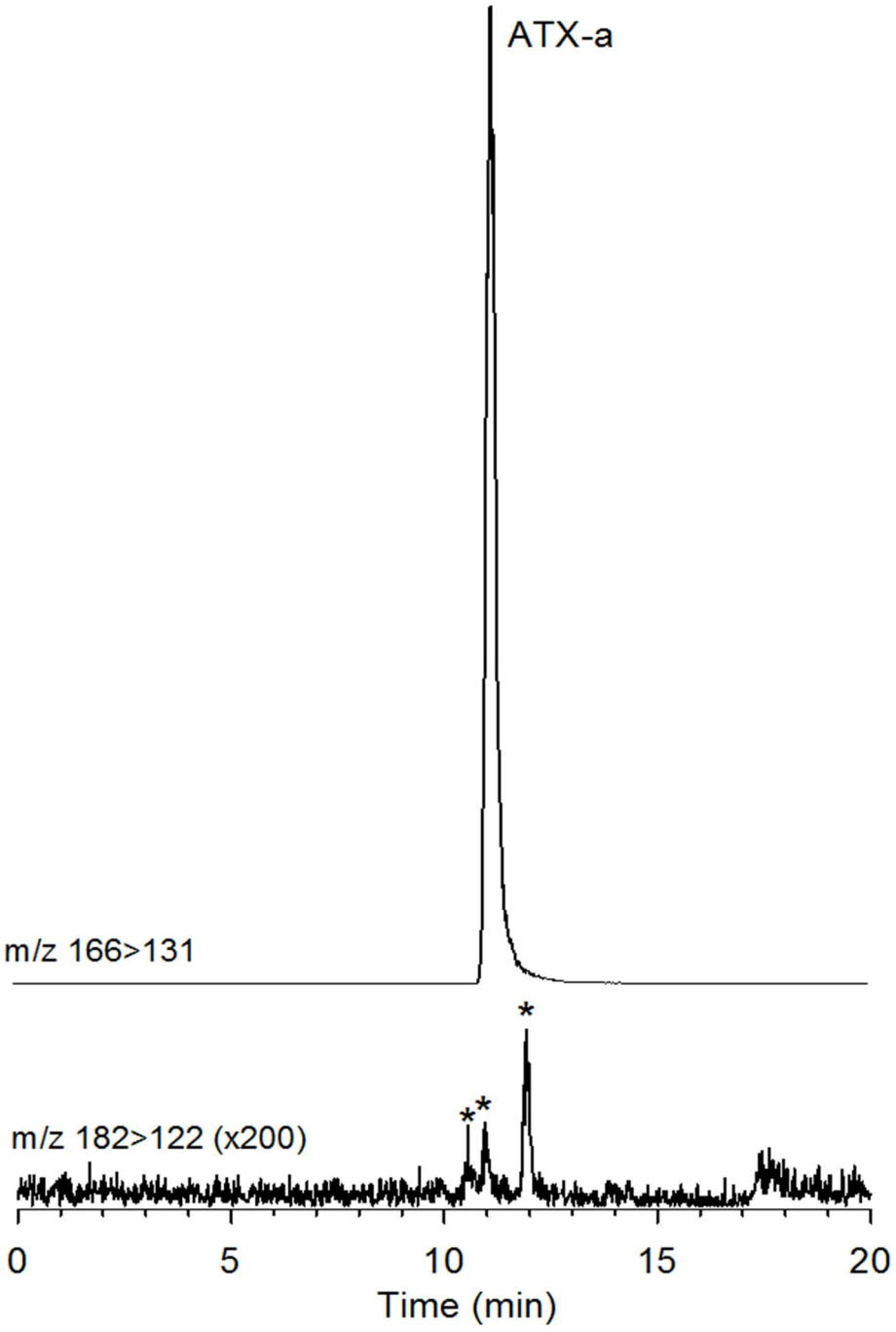


Figure 4: Analysis of CRM-ATX by LC-MS with selected reaction monitoring. Impurities (*) with an $[M+H]^+$ ion at m/z 182 were detected only at very low levels. Conditions: Agilent 1200 LC and AB Sciex API4000 QTRAP; 150 mm × 2 mm i.d. 3 µm Toso-Haas Amide-80 column at +40°C; gradient elution 90 to 70% B over 25 min, where A = H_2O with 50 mM NH_4COOH and 2 mM HCOOH, and B= CH_3CN. Flow rate = 0.2 mL/min.

References

1. Devlin JP, Edwards OE, Gorham PR, Hunter NR, Pike RK, Stavric B (1977) Anatoxin-a, a toxic alkaloid from *Anabaena flos-aquae* NRC-44h. *Can J Chem* 55:1367-1371.
2. Osswald J, Rellán S, Gago A, Vasconcelos V (2007) Toxicology and detection methods of the alkaloid neurotoxin produced by cyanobacteria, anatoxin-a. *Environ Int* 33:1070-1089.
3. Selwood AI, Holland PT, Wood SA, Smith KF, McNabb PS (2007) Production of anatoxin-a and a novel biosynthetic precursor by the cyanobacterium *Aphanizomenon issatschenkoi*. *Environ Sci Technol* 41:506-510.
4. Namikoshia M, Murakamia T, Watanabe MF, Odac T, Yamadac J, Tsujimurad S, Nagaia H, Oishie S (2003) Simultaneous production of homoanatoxin-a, anatoxin-a, and a new non-toxic 4-hydroxyhomoanatoxin-a by the cyanobacterium *Raphidiopsis mediterranea Skuja*. *Toxicon* 42: 533-538.
5. Harada K, Kimura Y, Ogawa K, Suzuki M, Dahlem AM, Beasley VR, Carmichael WM (1989); A new procedure for the analysis and purification of naturally occurring anatoxin-a from the blue-green alga *Anabaena Flos-Aqua*. *Toxicon* 27:1289-1296.
6. Dell'Aversano C, Eaglesham GK, Quilliam MA (2004) Analysis of cyanobacterial toxins by hydrophyllic interaction liquid chromatography-mass spectrometry. *J Chrom A* 1028 :155-164.
7. Vasas G, Gaspar A, Pager C, Suranyi G, Mathe C, Hamvas MM, Borbely G (2004) Analysis of cyanobacterial toxins (anatoxin-a, cylindrospermopsin, microcystin-LR) by capillary electrophoresis. *Electrophoresis* 25:108-115.
8. Quilliam MA, Fraser H: manuscript in preparation.
9. Koskinen AMP, Rapoport H (1985) Synthetic and Confirmational Studies on Anatoxin-a: A potent Acetylcholine Agonist. *J Med Chem* 28:1301-1309.
10. Stevens DK, Krieger RI (1991) Stability studies on the cyanobacterial nicotinic alkaloid anatoxin-a. *Toxicon* 29:167-179.
11. Burton IW, Quilliam MA, Walter JA (2005) Quantitative ^{1}H NMR with external standards: Use in preparation of calibration solutions for algal toxins and other natural products. *Anal Chem* 77:3123-3131.
12. Pauwels J, Lamberty A, Schimmel H (2000) Evaluation of uncertainty of reference materials. *Accred Qual Assur* 5:95-99.
13. 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.
14. 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.
15. Guide to the Expression of Uncertainty in Measurement, ISBN 92-67-10188-9, 1st edition ISO, Geneva, Switzerland (1993).

Acknowledgements

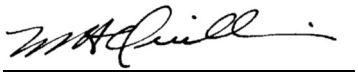
The following staff members of Measurement Science and Standards at NRC contributed to the production and certification of CRM-ATX: E. Bond, S. Crain, K. Bekri, I. Burton, Y.M. Chen, J. Frazer, Y. Gao, W. Hardstaff, D. Marciniak, P. McCarron, K. Reeves, S. Regier, K. Thomas, M. Quilliam and J. Walter. The following staff members of the Cawthron Institute in New Zealand contributed to the production, isolation and characterization of ATX-a used in CRM-ATX: P. Holland, A. Selwood, P. Terazaki, and S. Wood. A scholarship for Yi-Min Chen from the government of Taiwan is greatly appreciated.

This document should be cited as:

K. Thomas, A. Selwood, S. Wood, Y.M. Chen, S. Crain, J.A. Walter and M.A. Quilliam. “CRM-ATX, a certified calibration solution reference material for anatoxin-a”, Biotoxin Metrology Technical Report CRM-ATX-20100721, National Research Council Canada, Halifax, February 2011 (NRCC No.: 51829). DOI https://doi.org/10.4224/crm.2011.atx.20100721

First certification completed: March, 2011
Document version: 20220315

Revised: March 2022 (DOI added and editorial updates)

Signed **:** 

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

This Certificate is only valid if the corresponding product was obtained directly from NRC or one of our qualified vendors.

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

Également disponible en français sur demande.