Technical Report

Multi-Laboratory Validation Study for Quantitation of Total Saxitoxin (STX) by CAC-STX-1.0

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Technical questions concerning this method should be addressed to:

John Kelly, Ph.D., Lead Method Development and Multilaboratory Validation
john.kelly@cdfa.ca.gov
California Department of Food and Agriculture, Center for Analytical Chemistry (CAC)
3292 Meadowview Rd.
Sacramento, CA 95832

and

Mohammadreza Chehelamirani, Ph.D., Research and Development Supervisor
mohammadrez.chehelamirani@cdfa.ca.gov
California Department of Food and Agriculture, Center for Analytical Chemistry (CAC)
3292 Meadowview Rd.
Sacramento, CA 95832

CAC Approvals for use:

_____ _____
Teresa Bowers Date
CAC Environmental Program Manager I

_____ _____
Sarvamangala Balachandra Date
CAC Quality Assurance Officer

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1. Introduction:

1.1. Background

Saxitoxins (STX), also known as paralytic shellfish poisoning (PSP) toxins, are alkaloids produced by some marine dinoflagellates and a variety of cyanobacteria species. They were originally found in mollusks after poisonings of humans following consumption of seafood but have also been found in both marine and fresh water during the occurrence of some harmful algal blooms (HABs).

STX exposure in water primarily comes from consuming untreated surface water, either from water systems that don't sufficiently treat their surface water or through unintentional swallowing during recreational exposure in lakes and rivers.

1.2. Method Summary

The analytical method for this study was validated and the single laboratory validation results are summarized in the following section. Refinements were made to that method based on the comments and results of the participating three laboratories in the multilaboratory validation study (MLVS). Those updates are released as CAC-STX-1.1. This complete method is attached to this report as Appendix A.

The analytical method includes sample preparation and sample analysis procedures for source- and finished-surface water. The limited sample preparation includes a chlorine quenching agent for finished water and a buffer for sample stability. The method utilized enzyme-linked immunosorbent assay (ELISA) for the quantitative analysis of total saxitoxins in raw and finished surface-sourced drinking water.

1.3. Summary of the Single-Laboratory Study

The single-laboratory validation was performed by CDFA's Center for Analytical Chemistry Research and Development Unit (Sacramento, CA) (CAC-R&D), the laboratory originally contracted by State Water Resources Control Board, Division of Drinking Water (DDW) to develop a total saxitoxin analytical methodology, and validate its use in this MLVS.

A large variety of aqueous matrix samples, including surface water-sourced raw and finished drinking water, were spiked at five concentration levels from 0.050 ppb to 0.300 ppb and analyzed in the single-laboratory validation study; all of which had recoveries between 70-130%. The single-laboratory validation proposed a minimum reporting limit (MRL) of 0.03 ppb STX, after calculating an estimated lowest concentration minimum reporting limit (LCMRL) of 0.017 ppb STX. The LCMRL was calculated as an estimate, but was unable to be properly bracketed and confirmed due to it being below the lowest calibration point that was provided with the ELISA kits.

While automated plate washers and microplate pipetting workstations can simplify this method, the required plate reader has become commonplace in many full-service environmental laboratories. This means that this method should be accessible and possible to be implemented in a typical mid-sized full-service environmental laboratory.

2. Study Management, Objectives, Design, and Implementation:

While this MLVS is not designed to support an alternate test procedure (ATP) application, the number of matrices and statistical analyses of the data reflect, as much as possible, what would be required for an ATP for national use.

2.1. Study Management

Three laboratories (two commercial contract laboratories and one municipal water district laboratory) volunteered to participate in the study, under the authorization and stewardship from DDW. The three laboratories are listed in Table 2-1. All three laboratories contributed to the analysis of the aqueous matrices in this report (surface water, raw and finished). For the purposes of this study, the laboratories were randomly assigned numbers, which were used to maintain the anonymity of the results, these numbers do not correspond to their order of appearance in Table 2-1.

The CAC Quality Assurance unit (CAC-QA) managed the sample spiking, shipment, and received all data packages from the laboratories. Analytical standards and ELISA kits were provided to the participating laboratories by CAC. CAC-QA, with an established operational set-up and expertise to conduct Proficiency Testing (PT) schemes, prepared the PT samples shipped as part of this study, while CAC prepared the aqueous sample matrices.

CAC served as the method consultant to the MLVS and was available to clarify any questions or concerns.

Table 2-1. Participating Laboratories

Laboratory	**Location**	**Role**
Participating MLVS Labs		
Bend Genetics, LLC	Sacramento, CA	MLVS participant
L.A. Department of Water and Power	Pasadena, CA	MLVS participant
Weck Laboratories	Industry, CA	MLVS participant
Ancillary Labs		
CDFA Center for Analytical Chemistry – Research & Development	Sacramento, CA	Single laboratory validation, preparation of matrix spike standards
CDFA Center for Analytical Chemistry – Quality Assurance	Sacramento, CA	Preparation of PT standards, evaluation of data

2.2. Study Objective and Design

The focus of the MLVS is to generate the necessary data to document the precision and accuracy and overall performance of the analytical method for quantitation of STX in aqueous matrices. The primary objectives of this MLVS are to:

- Obtain data from aqueous matrices that are representative of the method's intended use.
- Obtain data from laboratories that are representative of those likely to use the method, but that were not directly involved in its development.
- Obtain feedback from laboratory users on the specifics of the method SOP.
- Use study data to evaluate the performance of the method.
- Develop QC acceptance criteria – average recovery and relative standard deviation – that will reflect method performance capabilities in real-world situations.

A brief description of the key points of this study design include:

- At least three laboratories, at least one of which is a municipal water laboratory.
- Two aqueous matrix samples from source and finished surface water.
- Initial calibration of STX by each laboratory.
- Initial Demonstration of Capability (IDOC) by each laboratory.
- Analyses of PT sample and matrix spike samples from each aqueous matrix.

This MLVS was conducted in two phases. The IDOC, which includes the initial calibration and verification of MRL level, and the method evaluation in the chosen aqueous matrices.

2.3. Matrix and Sample Selection

Two surface water samples (one source and one treated) were used for this phase of the MLVS. These were chosen to be representative of the expected real-world matrices analyzed by this method. These samples were collected from the Sacramento River Water Treatment Plant.

The MLVS was designed so that for each sample the following would be analyzed: an 'unspiked' (native) sample, two replicates spiked at low concentration, and two replicates spiked at a mid-level concentration.

2.4. Selection of Spiking Levels and Aqueous Media

The sample matrices were collected by DDW. The chosen sample matrices were raw and finished water from the West Sacramento River Water Treatment Plant. The saxitoxin spiking levels were chosen by CAC to be at the proposed MRL for the low-spike, and just above the center of the calibration range for the mid-spike. CAC-QA separately selected the PT spike level so that it would be blind to everyone participating, including all other CAC units.

2.5. Preparation of Study Samples

Due to the short hold time of the saxitoxin samples, the study samples were prepared by the participating laboratories. They were provided with a guidance document detailing the necessary preparations (included as Appendix B). Each laboratory was provided with working standards for both the matrix samples and the PT sample. The working standards were prepared by CAC-R&D for the matrix samples and CAC-QA for the PT sample.

The laboratories were instructed to fortify 1 mL samples using the working standards provided by CAC. The standards were frozen to -80°C, packed in insulated shipping containers (such as ThermoSafe® or similar) to ensure standard integrity during transport, and shipped to the laboratories.

2.6. Storage Stability Study

During method development, a storage stability study was conducted to determine the hold time of saxitoxin samples in finished surface-sourced drinking water. The matrix samples were spiked in triplicate for this study, and varying concentrations of chlorine quenching agent (ascorbic acid) were also tested to see the effect on saxitoxin stability. This data and the chosen concentration of chlorine quenching agent can be found in the method SOP in Appendix A, and the hold time was determined to be six days. Past day six, the recoveries begin to drop below the QC acceptance criteria.

3. Data Management, Data Validation, and Data Rules for Statistical Analysis:

3.1. Programmatic Overview

The data management process involved documented and approved instructions, meetings, consultation and communication when required, and review of laboratory packages. A “kickoff” meeting over Teams was scheduled to explain the expectations for the MLVS to the participating laboratories. Laboratories were provided with the method prior to the meeting in preparation and given the opportunity to clarify any questions or concerns. They were also encouraged to ask questions via email or to schedule a one-on-one meeting throughout the MLVS as necessary. A written document containing the MLVS guidelines and passing QC criteria was also provided to the laboratories along with a data reporting template Excel workbook, to ensure uniform reporting.

3.2. Data Management

All result reporting forms, and raw data were submitted to CAC-QA via email. The labs were provided with an Excel workbook template to report their analysis batch data and results in, as well as a sample handling sheet to note how samples were prepared. The Excel template had password protected cells for any formulas to prevent accidental corruption or unintended changes. The laboratories also provided both their raw absorbance data as well as a plate map to identify the locations of each sample on the 96-well plate.

3.3. Data Validation

All data packages were reviewed by CAC-QA for completeness, compliance with the MLVS guidelines, and performance according to the QC metrics, see QC acceptance criteria in the SOP in Appendix A.

4. Calibration and Quantification:

Aqueous samples were analyzed by ELISA. As this method calls for the use of a commercial ELISA kit manufactured for use in analysis of water samples, the participating laboratories used the supplies provided in those kits for the calibration of the method. Each laboratory was advised to follow the manufacturer's instructions for the absorbance calibration. While manufacturer specifications may vary, the kits used in this study comprised of five calibration standards (in addition to a zero standard) that ranged from 0.02 ppb to 0.4 ppb in concentration.

4.1. Quantification

The preferred calculation for quantification using these ELISA kits is a Four Parameter Logistic (4PL) regression. This regression method is detailed in the method SOP in Appendix A.

4.2. Calibration Verification

A control standard was included in each of the ELISA kits. These standards were approximately at the mid-level (IC_{50} for 4PL) of the calibration set provided in the kits. The laboratories ran this control in each batch. The kit-provided controls were required to recover within ±30% of their true value. No sample results were eliminated from the study due to CV failures.

4.3. Instrument Sensitivity Check

Each laboratory created Low-CV spikes at the method MRL, from the standards provided in the ELISA kits. A Low-CV was analyzed in each batch. Low-CVs were required to recover within ±50% of their true value. No sample results were eliminated from the study due to Low-CV failures.

5. Initial Demonstration of Capabilities:

In addition to performing the initial calibration, laboratories submitted an (IDOC. The IDOC included a precision and accuracy analysis, system background check, and MRL confirmation.

5.1. Demonstration of Precision and Accuracy (P&A) Results

Laboratories were required to spike seven replicate Laboratory Fortified Blanks (LFBs) at 0.070 ppb STX. These LFBs were prepared and analyzed in the same manner as study samples, per the method. A percent relative standard deviation (%RSD) of less than 15% and a mean percent recovery of ±30% of the true value were the required criteria for this demonstration. All laboratories met these criteria.

5.2. Acceptable System Background

Five Laboratory Reagent Blanks (LRB) were run in the same batch as the LFBs for the P&A. The laboratories were required to have a background level of less than one-third of their MRL. All laboratories satisfied this requirement.

5.3. Minimum Reporting Limit Verification Analyses

The Minimum Reporting Limit for STX using this method was set by the method development (MD) laboratory at 0.030 ppb. The participating laboratories were required to confirm their MRLs using the MD level as an initial reference point. Seven LFBs were to be spiked at the proposed MRL for confirmation using the formulas in the method.

Three laboratories confirmed 0.030 ppb as the MRL, while the remaining lab confirmed 0.015 ppb as their MRL. It should be noted for future use of the method that the confirmed MRL should not be below the lowest standard provided in the ELISA kits. Any finding below the lowest standard level will be considered a ND in real samples.

5.4. Quality Control Sample (QCS)

A QCS prepared from a standard from a different source to that of the calibration standards was also required as part of the IDOC. The QCS was not required to be analyzed in the same batch as the P&A LFBs, and this requirement was satisfied by the PT samples sent to the laboratories (see section 6.3). Each laboratory analyzed a PT sample prepared from a standard sourced from a different vendor than the ELISA kit they were supplied with.

Table 5-1. IDOC Results

Laboratory	Precision (%RSD)	Accuracy (Mean %)	System Background (Mean ppb)	MRL (ppb)	UPIR	LPIR
1[#]	5.5%	79.0%	0.003	0.015	137.9%	71.6%
2	4.5%	108.4%	0.006	0.030	141.3%	82.5%
3	6.0%	100.6%	0.001	0.030	124.7%	77.2%

[#]during the MRL verification, the laboratory did not run the MRL and the LRB together as was required, originally resulting in LPIR below 50%. Upon being directed to follow the guidelines, the lab repeated the MRL study with an acceptable metric of PIRs, however, the MRL was studied below the lowest available standard, 0.02 ppb, which is to be considered ND in real sample situations.

6. Water Matrix Results:

Each participating laboratory was sent two 10 mL water matrix samples from the West Sacramento River Water Treatment Plant. One sample was raw surface water, the other was finished drinking water from the same treatment plant. The laboratories were provided with instructions on how to spike the required samples for this phase of the MLVS, as the short hold time for STX (six days) left limited feasibility for shipping pre-spiked samples.

6.1. STX Concentrations in Unspiked Matrices

Due to the laboratories being shipping bulk water matrix instead of prepared samples, each lab was required to analyze unspiked samples separately before preparing and running the matrix spikes. All laboratories reported native concentrations of STX well below one-third of their MRLs. These levels are included in Table 6-1.

6.2. Matrix Spike Results

The spike recovery data from all laboratories is also shown in Table 6-1. The raw and finished water matrices were spiked in duplicate at the proposed MRL-level (0.030 ppb) and at a mid-level (0.100 ppb). The results from all laboratories at all levels were acceptable with recoveries ranging from 73.3%-126.7% recovery.

6.3. PT Results

Each laboratory was provided with a PT working standard, prepared from a standard sourced from either Gold Standard Diagnostics or Creative Diagnostics. Each laboratory was sent the working standard prepared from the vendor that did not manufacture the ELISA kit they were supplied with, and instructions on how to prepare the PT sample for analysis. PT sample ensemble with matrices were packaged in an insulated shipping box (ThermoSafe® or similar) with ice packs ensuring standard integrity. All PT samples were shipped together to ensure process integrity and uniformity. Participating labs were allotted a two-week period to perform the analyses and submit results to the CAC - QA. Table 6-2 denotes the results. Since laboratory 2 did not initially report satisfactory results, CAC-EA laboratory, an independent unit of the CAC, was reached out as an additional laboratory participant and the final PT results from all laboratories are provided below in Table 6-2.

Table 6-1. Unspiked and Spiked Matrix Results

Laboratory	Raw (%Recovery)					Finished (%Recovery)				
	Unspiked (ppb)	MRL Rep. 1	MRL Rep. 2	Mid-Level Rep. 1	Mid-Level Rep. 2	Unspiked (ppb)	MRL Rep. 1	MRL Rep. 2	Mid-Level Rep. 1	Mid-Level Rep. 2
1	0.010	80.0%	100.0%	83.0%	84.0%	0.006	113.3%	110.0%	95.0%	90.0%
2	ND	93.3%	90.0%	81.0%	84.0%	ND	83.3%	73.3%	82.0%	79.0%
3	0.010	116.7%	113.3%	124.0%	119.0%	0.002	123.3%	126.7%	111.0%	110.0%

Table 6-2. PT Results

Laboratory	Reagent Water PT			Tap Water PT			
	Result (ppb)	Target (ppb)	% Recovery	Result (ppb)	Target (ppb)	% Recovery	Mean % Rec.
1	0.064	0.074	87.1	0.081	0.074	110.2	98.7
2*	0.051	0.074	69.4	0.051	0.074	69.4	69.4
3	0.073	0.074	99.5	0.075	0.074	102.5	101.0

*laboratory analyzed PT samples with multiple dilutions and the results could not be computed in the initial run, the results presented are from their reanalysis.

7. Summary:

7.1. Preparatory Batch QC

Per the CAC-STX-1.0 method, an analysis batch consists of the calibration standards, field samples, a method blank, low-range calibration verification standard, laboratory fortified blank, and a laboratory fortified sample matrix and laboratory fortified sample matrix duplicate pair. This method is run on 96-well ELISA plates, and while the entire plate may be used in one batch, an analysis batch cannot consist of more than a single ELISA plate.

7.1.1. Method Blank

Method blanks, referred to as Laboratory Reagent Blanks (LRB) in this method, are included in the method to evaluate the potential for background contamination to be introduced during sample preparation in the laboratory. The saxitoxin concentration in the LRB must be less than one-third the MRL. This requirement was met by all participating laboratories.

7.1.2. Ongoing Precision and Accuracy Analyses

Ongoing Precision and Accuracy samples, represented in this method by the batch LFB requirements, are included to evaluate the efficiency of the sample preparation processes over time. A LFB prepared in the same manner as the study samples is included in every preparation batch.

This method also calls for the inclusion of Laboratory Fortified Sample Matrix (LFSM) and Laboratory Fortified Sample Matrix Duplicate (LFSMD). LFSM and LFSMD are field samples, spiked with a known amount of saxitoxin. These are included to check for matrix effects on the samples in each batch.

7.1.3. Low-Level Ongoing Precision and Accuracy Analyses

Low-Level Ongoing Precision and Accuracy samples, Low-CV samples in the method, are included in each analysis batch to evaluate method performance at the at the MRL.

7.2. Matrix Spike Analyses

Ongoing QC criteria of ±40% (Appendix A, Section 9.2.4) was applied to evaluate the results, and all participating labs performed the analyses matrix spikes satisfactorily, presented in Table 6-1.

The matrix results from the fourth laboratory (CAC-EA Lab, which was not a part of the initial MLV design) were not included in the final results as it was unable to be identified if the non-passing QC results were due to the laboratory staff performing the analyses or the potential degradation of the water matrices used, as the water matrices had been stored for approximately 2.5 months prior to CAC-EA preparing and analyzing the matrix samples.

7.3. PT Result Analyses

Ongoing QC criteria of ±40% (Appendix A, Section 9.2.4) was applied to evaluate the results, and all participating labs performed the analyses of the PT samples satisfactorily, presented in Table 6-2. The overall average recovery is 89.7% with an RSD of 19.3 % across the three participating labs.

PT results from the fourth laboratory (CAC-EA Lab, which was not a part of the initial MLV design) could not be used as the storage criteria for the PT samples were not met since the EA lab could not accommodate the analysis of the PT samples in a timely manner.

8. Conclusions:

The objectives of this MLVS were achieved: validation of the CAC-STX-1.0 method and the determination that the method can be implemented at a typical mid-sized full-service environmental laboratory. Overall, the data from the MLVS demonstrates that CAC-STX-1.0, as written, is robust and suitable for laboratories with similar instruments of different manufacturers and models. Points of additional clarity have been added to the SOP as version CAC-STX-1.1. Specifically, it has been noted that there must be a calibration curve run in each batch, and that any detection below the lowest calibration level is considered to be non-detect in real samples. The single-laboratory LCMRL results were also added to the SOP.

The results of the participating labs in this study have met the requirements stated in the method for:

- Calibration verification and sensitivity check
- Initial Precision and Accuracy
- Confirmation of MRL
- Batch QC samples

The suitability of the method to detect and quantify saxitoxin in surface-sourced drinking water (both raw and finished) was successfully demonstrated through the analysis of spiked samples of those real-world matrix types. Method blank results demonstrated no bias from background contamination during sample preparation. The Initial and Ongoing Recoveries and the Low-CV recoveries demonstrated that the QC acceptance criteria in the method were satisfactory for inclusion in the finalized method. ELISA kits from Gold Standard Diagnostics Horsham, LLC and Creative Diagnostics were used during this MLVS. It is noted that while kits from both vendors performed satisfactorily, Gold Standard Diagnostics was much more responsive and able to provide replacement kits on short notice. This could be an important consideration when determining a supplier to use, especially if unplanned or emergency sampling events are within the scope of the study this method is being used for.

9. References:

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9.3. Sasaki, Diane and Mitchell, Robert A. How to Obtain Reproducible Quantitative ELISA Results. Oxford Biomedical Research website. https://www.oxfordbiomed.com/sites/default/files/2017-02/How%20to%20Obtain%20Reproducible%20Quantitative%20ELISA%20results.pdf (accessed February 2023).

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9.5. Eurofins Abraxis Excel Solver, 2020. https://www.goldstandarddiagnostics.us/media/9257/18urofins_abraxis_excel_solver-website.xlsm(accessed 2023 January 25)

9.6. U.S. EPA. August 2016. Method 546: Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay.

Appendix A: Standard Operating Procedure for CAC-STX-1.1

[Changes from CAC-STX-1.0 to CAC-STX-1.1 have been underlined.]

Appendix B: Guidelines for Interlaboratory Validation of Method CAC-STX-1.0

Appendix C: Data Reporting Template for MLVS of Method CAC-STX-1.0