Single Laboratory Validation (SLV) Protocol for Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

Name of the New Method:

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Name of Method Developer:

Whitney Stutts, Ph.D. and Jonathan Deeds, Ph.D.

Developer Contact Information:

FDA Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

Phone: 240-402-1474 (Deeds) or 240-402-1895 (Stutts)

whitney.stutts@fda.hhs.gov; jonathan.deeds@fda.hhs.gov

A. Need for the New Method

1. Need for Which the Method has been Developed:

Shellfish contaminated with natural toxins can cause consumer illnesses. The Food and Drug Administration has established guidance levels for five groups of natural toxins in shellfish responsible for the following illnesses: amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). Shellfish hazards for domestic products are managed under the National Shellfish Sanitation Program (NSSP), but at present approved and approved limited use methods are only available for ASP, NSP, and PSP. Shellfish harvesting closures have been required due to DSP toxins in excess of the established regulatory guidance level of 16 µg OA eq./100 g shellfish on the Texas Gulf Coast since 2008, in the Puget Sound region since 2011, and in the New England region since 2015. This report describes the validation of an LC-MS/MS method for DSP toxins for use in the NSSP for the control of this hazard in clams. Once found to be fit for purpose for clams, the method will be fully validated for the additional matrices of mussel and oyster. Due to the immediate need for approved methods for this toxin group it was felt that submitting this proposal now, with the available full SLV data for clam, was important. Preliminary data is available for mussel and oyster such that the method can be used for these matrices in an approved limited use capacity.

2. Purpose and Intended Use of the Method: The intention is for this method to be an Approved Method for Biotoxin testing for DSP toxins under the NSSP (for clams) and that it should appear in Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Marine Biotoxin Testing) under the new heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP), and the applications should be (1) Growing Area Survey and Classification and (2) Controlled Relaying with the sample type of Shellfish for both. Preliminary data is also provided for the additional matrices of mussel and oyster such that the method should be included in Table 4 (Approved Limited Use Methods for Biotoxin Testing) for these matrices while additional SLV data is generated.

3. Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:

The regulatory guidance level in the U.S. for diarrhetic shellfish poisoning toxins (DSTs) is 16 µg total (free plus esterified) toxins/100 g shellfish. The European Union (EU) recently adopted LC-MS/MS as the reference method for lipophilic shellfish toxins, which include DSTs.[1] However, because the EU harmonized protocol also measures a number of additional lipophilic toxins, including pectenotoxins, yessotoxins, and azapriracids, the protocol contains multiple method modifications and variations to account for this, depending on the needs of individual laboratories. This work optimized the EU lipophilic toxin reference method specifically for the quantitation of DSTs in clams for use in the NSSP. Some labs in the U.S. are already using best available science based on the EU LC-MS/MS reference method; thus, an NSSP-validated method for use in the U.S. is urgently needed. This LC-MS/MS method would be complimentary to other available testing methods such as the *in-vitro* protein phosphatase inhibition assay (PPIA), which has also been submitted for approval to the ISSC under a previous proposal, and comparative data is provided between these two methods in the comparability section.

4. Type of Method (Chemical, Molecular, or Culture):

Chemical Confirmatory Method: Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) specifically measures okadaic acid, dinophysistoxin 1, and dinophysistoxin 2.

B. Method Documentation

Method Title:

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Method Scope:

This method is fully validated for the determination of diarrhetic shellfish poisoning toxins in clams. Preliminary and comparative data only is provided for mussels and oysters.

References:

1. European Union Reference Laboratory for Marine Biotoxins. Harmonized Standard Operating Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS. Version 5, January 2015.
2. Gerssen, A.; McElhinney, M.A.; Mulder, P.P.J.; Bire, R.; de Boer, J. Liquid chromatograph-tandem mass spectrometry method for the detection of marine lyophilic toxins under alkaline conditions. Journal of Chromatography A, 1216 (2009) 1421-1430.
3. ICH Q2B, Validation of Analytical Procedures: Methodology, Fed. Regist. 1997, 62 (96), 27463-27467.
4. McNabb, P.; Selwood, A. I.; Holland, P. T. Multi-residue method for determination of algal toxins in shellfish: single-laboratory validation and inter-laboratory study. Journal of AOAC International, 88 (2005) 761.
5. AOAC International "Appendix K: Guidelines for Dietary Supplements and Botanicals, Part 1 AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals", 2013.
6. Smienk et al. 2012 Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. Toxins, 5, 339-352.
7. Smienk et al. 2013. Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal of AOAC International Vol. 96, No. 1.

Principle:

Liquid chromatography tandem mass spectrometry is an analytical technique that provides both physical separation (liquid chromatography) and mass analysis (mass spectrometry) of sample components. In this work, a Waters ACQUITY® Ultra Performance Liquid Chromatography system was coupled with an AB Sciex 5500 QTrap® triple quadrupole mass spectrometer by electrospray ionization. Pre-validation studies were performed to assess the impacts of acidic versus basic chromatography and the use of neat versus matrix matched standard curves on overall method performance (data included). For targeted quantitation of DSTs, structurally informative transitions were specified for multiple reaction monitoring (MRM). In MRM mode, an ion of interest (precursor ion) is preselected in the first quadrupole and fragmented in the second quadruple. Multiple product ions resulting from fragmentation of the precursor ion are then mass analyzed in the third quadrupole. Two analyte specific transitions, one for quantitation and one for confirmation, are specified for each analyte and monitored in both the calibration standard solutions and in the extracted shellfish matrices. A linear fit is applied to the peak area data for the quantitation ion collected for the calibration standards, and the equation for this line is utilized to calculate the concentration of each analyte in the spiked matrix samples. The enhanced resolving power afforded by LC and the selectivity gained by tandem mass spectrometry permitted the accurate detection and quantitation of DSTs in complex shellfish matrices. Individual toxin values are converted to a single integrated okadaic acid equivalents value through the use of established

toxicity equivalency factors (OA: 1, DTX1: 1, DTX2: 0.6). Only OA and DTX1 have been found to date in the U.S.

Analytes of Interest:

Diarrhetic shellfish poisoning toxins: Okadaic Acid (OA), Dinophysistoxin-1 (DTX1), and Dinophysistoxin-2 (DTX2). Shellfish metabolites (fatty acid acyl-esters for all 3 toxins, collectively referred to as DTX3) are included through the use of a required alkaline hydrolysis step.

Proprietary Aspects: None

Cost of the Method:

Capital equipment purchases:

1. Ultra Performance Liquid chromatograph (UPLC) or High Performance Liquid Chromatograph (HPLC), capable of running in gradient mode [example: Waters Corporation Acquity UPLC system (Manchester, UK) (approx. $60,000, new with government (GSA) discount)]

2. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities [example: AB Sciex QTrap 5500 equipped with a Turbo V ionization source (Framingham, MA) (approx. $320,000, new with government (GSA) discount)]

Cost per sample (approx. for consumables): $10/sample

Sample Turn Around Time: Analysis of 10 samples, including extraction, hydrolysis, sample analysis, and quantitation can be accomplished in approx. 6 -7 hours. Analysis time increases by 2.5 hours for every 10 additional samples.

Equipment Required:

1. Instrumentation for sample preparation: knives for shucking, stainless steel laboratory spatulas, sieve for draining
2. 4 oz. plastic screw top specimen cups (if a sample of unextracted homogenate is to be saved) (e.g., Covidien #17099)
3. Balance, accuracy to the nearest 0.01 g
4. Blender or food processor
5. Laboratory homogenizer (e.g., Polytron, Ultraturax, etc.) equipped with a generator appropriate for shellfish (e.g. 20 mm saw toothed)
6. Vortex mixer (either single or multi-tube)
7. Centrifuge, capable of 2000 x *g*, with adaptors for 50 mL and 15 mL centrifuge tubes
8. Heat block or water bath capable of maintaining 76 ± 2 °C
9. Volumetric flasks or graduated cylinders capable of accurately measuring 20, 100, 500, and 1000 mL

10. Disposable 50 mL centrifuge tubes
11. Disposable glass Pasteur pipettes with bulbs
12. 20 mL glass scintillation vials with coned polyethylene lined caps (if a sample of non-hydrolyzed methanolic extract is to be saved) (e.g., Wheaton #986560)
13. Disposable 16 × 100 mm glass screw cap tubes with phenolic PTFE lined caps (e.g., Fisher #14-959 35AA (tubes), Corning #9998-15 (caps))
14. 0.01, 0.05, 0.1, 0.5, and 1 mL positive displacement microdispensers or syringes (e.g., Drummond #3-000-510, 3-000-575, 3-000-590 or Hamilton #80530, 80630, 81330, 81365)
15. 1 mL disposable syringes (e.g., Becton Dickinson #309602)
16. PTFE syringe tip membrane filters, 13 mm, pore size 0.2 µm (e.g., Pall #PN4542)
17. LC-MS autosampler vials with pre-slit caps (e.g., Agilent #5182-0715)
18. Reversed Phase HPLC Column (e.g., Acquity UPLC BEH C18 1.7 µm particle size, 1.0 × 150 mm)
19. Ultra Performance Liquid chromatograph (UPLC) or High Performance Liquid Chromatograph (HPLC), capable of running in gradient mode
20. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities

Reagents Required:

1. Certified Reference Standards

Okadaic acid: e.g. (NRC-CRM-OAc) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-1: e.g. (NRC-CRM-DTX1) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-2: e.g. (NRC-CRM-DTX2) NRC-CNRC Institute for Marine Biosciences, Canada

2. Chemicals

Acetonitrile, HPLC Optima Grade
Methanol, HPLC Optima Grade
Water, HPLC Optima Grade
Ammonium formate (≥98% purity)
Formic Acid (≥98% purity)
Hydrochloric acid (37%)
Sodium hydroxide (≥98% purity)
Hexanes (Certified ACS, ≥98.5%)

Solution Preparation

1. Extraction solvent: 100% methanol

2. Hydrochloric Acid 2.5 M: Add 60 mL of water to a 100 mL volumetric flask or graduated cylinder. To the water, add 20 mL of hydrochloric acid and then dilute with water to 100 mL. Place in an appropriate glass jar for storage marked with the date of creation. This solution may be stored at room temperature and can be used for up to three months.

3. Sodium hydroxide 2.5 M: Dissolve 10 grams of sodium hydroxide in 75 mL of water in a glass beaker and add to a 100 mL volumetric flask or graduated cylinder. Make up to 100 mL with water and transfer to an appropriate storage vessel marked with the date of creation. This solution may be stored at room temperature and used for up to 3 months.

Matrix or Matrices of Interest:

Clam, mussel, and oyster. Full SLV validation data is provided here for clam but pre-validation and method comparability studies (data provided) indicate that the method will also be applicable to additional shellfish matrices such as mussel and oyster. Data for additional matrices will be provided upon approval of the method for clam.

Sample Collection, Preservation, and Storage requirements:

CRM-DTX1, CRM-DTX2, CRM-OA stock solutions (in MeOH in sealed ampules) were purchased from the National Research Council Canada and stored at −20 °C according to manufacturer's recommendations.

For each bivalve type, animals were collected from four different growing areas (Table 1). Shellfish were shucked, rinsed and drained to remove salt water. Ten to twelve animals were combined and homogenized using a blender/food processor and/or a Polytron homogenizer. Homogenized tissues were stored in plastic screw top specimen containers at −20 °C until used.

Table 1. Sources of blank shellfish matrices for spiking experiments

Source	Clam (*Mercenaria mercenaria*)	Mussel (*Mytilus edulis*)	Oyster (*Crassostrea virginica*)
A	Provided by Maryland Department of Natural Resources	Provided by MD Department of Natural Resources (used for pre-validation studies)	Provided by Maryland Department of Natural Resources (used for pre-validation studies)
B	Purchased live directly from harvester in Virginia	Washington Department of Health (used for method comparability data)	Provided by Texas Department of State Health Services (used for method comparability data)
C	Purchased live from retail. Harvest location Northport, Maine	Not performed yet	Not performed yet
D	Provided by Florida Wildlife Research Institute. Collection location Cedar Key, Florida.	Not performed yet	Not performed yet

Safety Requirements:

Proper precautions should be taken to avoid inhalation of harmful reagents or contact with skin or eyes. Analyst should wear a lab coat, gloves and safety glasses when working with chemicals. Chemical reagents that are flammable and/or toxic should be used within a chemical fume hood to protect laboratory workers.

Other Information:

Technical Skills Required: General laboratory skills (i.e., ability to accurately pipette small volumes, etc.). Experience with operation and general maintenance of liquid chromatography mass spectrometry equipment is required.

Abbreviation and acronyms:

DTX1: Dinophysistoxin-1
DTX2: Dinophysistoxin-2
OA: Okadaic acid
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

Step by Step Test Procedure:

Shellfish Preparation:

1. Clean outside of the shellfish with fresh water.
2. Cut the adductor muscles to open and rinse the inside with fresh water to remove any debris.
3. Remove the meat from the shell and drain tissue using a sieve to remove salt water.
4. Combine 10–12 animals and homogenize using a blender/food processor or a laboratory homogenizer.

Extraction Procedure:

1. Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a 50 mL disposable centrifuge tube.
2. Add 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min.
3. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
4. Transfer the supernatant to a clean 20 mL graduated cylinder, volumetric flask, or glass scintillation vial.
5. Re-extract the residual tissue pellet with an additional 9.0 mL of methanol and homogenize using a laboratory stick homogenizer (e.g. Polytron, Ultraturax).
6. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
7. Transfer the supernatant to a 20 mL volumetric flask or graduated cylinder and combine with the first extract. Adjust total extract volume to 20 mL with 100% methanol.
8. Transfer the 20 mL of total extract back into the 20 mL scintillation vial for storage (if desired).

Hydrolysis:

Note: *For this procedure, total DSP toxins (i.e. free toxin plus toxin fatty acid esters) must be quantified. To do this, all samples must go through an alkaline hydrolysis step prior to analysis.*

Accurately transfer a 2 mL aliquot of the 20 mL methanolic extract to a 16 × 100 mm glass tube with a phenolic PTFE lined screw cap using a positive displacement microdispenser or syringe. Add 250 µL of 2.5 M NaOH to the 2 mL extract aliquot. Homogenize with a vortex mixer for 30 seconds and record the total weight of the tube. Make sure caps are securely fastened and heat the mixture at 76 °C for 40 minutes. [Note: 76 °C is above the boiling point for methanol, therefore sample loss will occur if tube caps do not fit well and are not securely fastened.] Dry the water from the outside of the tubes, allow the tubes to cool to room temperature (approx. 5-10 minutes), and then re-weigh each tube. If the weight has dropped by more than 0.1 g, replace lost weight using 100% MeOH. Finally, neutralize samples with 250 µL of 2.5 M HCL, mix by vortexing for 30 seconds.

Sample Clean-up:

Partitioning of the methanolic shellfish extracts with hexane is performed to remove nonpolar lipids such as triglycerides, which could contaminate the source region of the instrument and/or suppress the responses of the DSTs.[4] Add 5 mL of hexanes directly to the 2.5 mL hydrolyzed methanolic extract. Mix by vortexing for 1 minute. Partition by centrifuging at 2,000 *g* for 10 min. Using a disposable glass pasture pipette, collect the hexanes (top layer) and discard to an appropriate waste container. Transfer approximately 1 mL of the methanolic extract (bottom layer) into a 1 mL disposable syringe equipped with a 0.2 µm PTFE syringe tip filter. Filter directly into an LC-MS certified glass vial and cap.

Quality Control Steps:

1. If available, a divert valve should be used to divert LC flow at the beginning and end of each chromatographic run. If a divert valve is not used, the ion source region and curtain plate will need to be cleaned between each batch (approximately every 24 hours) to maintain adequate sensitivity.
2. Use a new calibration curve each day of analyses. The calibration curve should be analyzed before and after each set of samples, and the data from both curves should be fitted with a line using least squares linear regression. Each calibration curve should be derived from at least six calibration points and the linear regression should yield a correlation coefficient (R^2) greater than or equal to 0.98. Analysts should also visually inspect the plot of the calibration to confirm linearity. If a calibration curve yields a correlation coefficient less than 0.98 or if non-linearity is visually observed, a new calibration curve should be prepared and samples should be reanalyzed.
3. The variation in the calibration curve slopes between the first and second set of calibration standards should not exceed 25%.
4. Reagent Blanks (methanol solvent) should be analyzed after the high calibration standard and periodically after fortified samples to insure that analyte carryover is not occurring; toxins

should not be detected above 10% of the lowest calibration point or should be below LOD. If carryover is observed, the LC gradient should be extended to allow for a longer wash at high organic (99% B).

5. Procedural Blanks (methanol carried through sample preparation process at the same time as the samples) should be analyzed before and after extracted samples.
6. One mid-scale calibration standard (e.g., 10 ng/mL) should be analyzed bracketing at least every 10 samples to assure that no retention time shifts (possibly due to column failure) or loss in signal intensity (due to fouling of the column or mass spectrometer) has occurred.
7. The retention time of analytes in all matrix solution should be within 3% that of the neat toxin standards.
8. Each chromatographic peak must be defined by at least 10 data points.
9. To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio(S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3.
10. Ion ratios between the quantitative and qualitative ion transitions should be within ±20% that of the relative ion abundance of the neat toxin standards.
11. Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (R_s) of OA/DTX2 should be calculated using the equation below and must be ≥ 1 for correct identification).

$$R_s = \frac{2x(RT_2 - RT_1)}{W_1 - W_2}$$

LC-MS/MS Method

Instrumentation Used for Validation: AB Sciex QTrap 5500 equipped with a Turbo V ionization source (Framingham, MA) and a Waters Corporation Acquity UPLC system (Manchester, UK).

LC Parameters:

UPLC column used for validation: Waters BEH C18 (1.7 µm, 1.0 mm × 150 mm)
Column Temperature: 40 °C
Autosampler Temperature: 10 °C
Injection Volume: 5µL

LC Gradient:

Mobile phase A: 2mM ammonium formate and 50 mM formic acid in 100% water.

Preparation of 1000 mL: dissolve 128 ± 10 mg ammonium formate in water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with water and add 1.9 mL of formic acid. Fill to mark with water.

Mobile phase B: 2mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water.

Preparation of 1000 mL: dissolve 128 ± 10 mg ammonium formate in 48.1 mL water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with acetonitrile and add 1.9 mL of formic acid. Fill to mark with acetonitrile.

Weak needle* and strong needle* wash solvent composition matched that of mobile phase A and B, respectively (*specific to Waters Acquity UPLC).

For the gradient in Table 2, LC flow should be diverted to waste from time 0.0–3.5 min and from 9.0–15.0 min.

Table 2. LC Gradient

Time (min)	Flow Rate (mL/min)	%A	%B
0.0	0.120	50	50
2.0	0.120	50	50
6.0	0.120	30	70
8.0	0.120	1.0	99
10.0	0.120	1.0	99
10.5	0.120	50	50
15.0	0.120	50	50

Analyte retention times for this gradient and column can be seen in Figure 1.

MS Ion Source Parameters:

Turbo V ion source parameters were optimized in negative ionization mode for all analytes under the acidic chromatographic conditions listed above. These parameters will vary between different instrument platforms or ionization sources.

Table 3. Turbo V ion source parameters

Source Temperature	550 °C
Ion Spray Voltage	−4500 V
Curtain Gas	25 au
Gas 1	40 au
Gas 2	40 au

MRM Parameters:

Manual or automatic compound optimization must be performed by every laboratory to determine the optimal settings for the MRM parameters as these may vary between different instrument platforms. Ruggedness studies (detailed later in the document) found that compound re-optimization was required even for detector replacement with the same make and model. Instrument must be correctly calibrated for negative ionization mode. OA, DTX1, DTX2 should be monitored in negative ionization mode. The precursor and product ion mass-to-charge (*m/z*) should be confirmed by acquiring full scan MS1 and MS/MS spectra for each toxin. At least two product ions must be monitored for each toxin, one for quantitation and one for confirmation. Dwell times for transitions should be set such that at least 10 data points are acquired across each peak. Declustering Potential (DP), Entrance Potential (EP), Collision gas (CAD), Collison Energy (CE), and Collision Cell Exit Potential (CXP) should be optimized for each MRM transition monitored. Table 4 lists the compound-dependent parameters optimized for two different AB Sciex QTRAP 5500 detectors. For method validation, two confirmatory ions were monitored; however, for routine analysis monitoring the product ion at *m/z* 151 is not required.

Table 4. MRM Parameters used for Method Validation on an AB Sciex QTrap 5500 Mass Spectrometer

Compound	Polarity	Q1 (*m/z*)	Q3 (*m/z*)	Dwell Time (ms)	DP† (V)	EP (V)	CAD Gas	CE (V)	CXP (V)
OA	Negative	803.5	255.2	100	−110	−10	High	−70	−15
OA	Negative	803.5	113.1	100	−110	−10	High	−100	−19
OA*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	255.2	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	113.1	100	−110	−10	High	−100	−19
DTX2*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	255.2	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	113.1	100	−110	−10	High	−100	−19
DTX1*	Negative	817.5	151.1	100	−110	−10	High	−70	−15

*One additional confirmatory ion transition was monitored for method validation purposes.

† Compound re-optimization for ruggedness testing using a second AB Sciex QTRAP 5500 detector found that the declustering potential had to be changed to -5 V for optimum method performance.

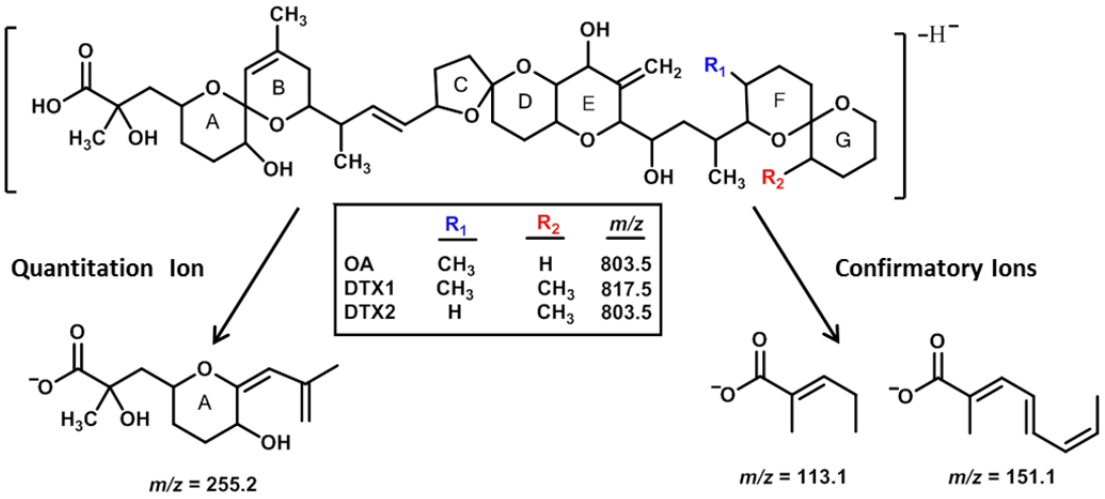


Figure 1. Structure of precursor ions and proposed product ion structures for DSTs.

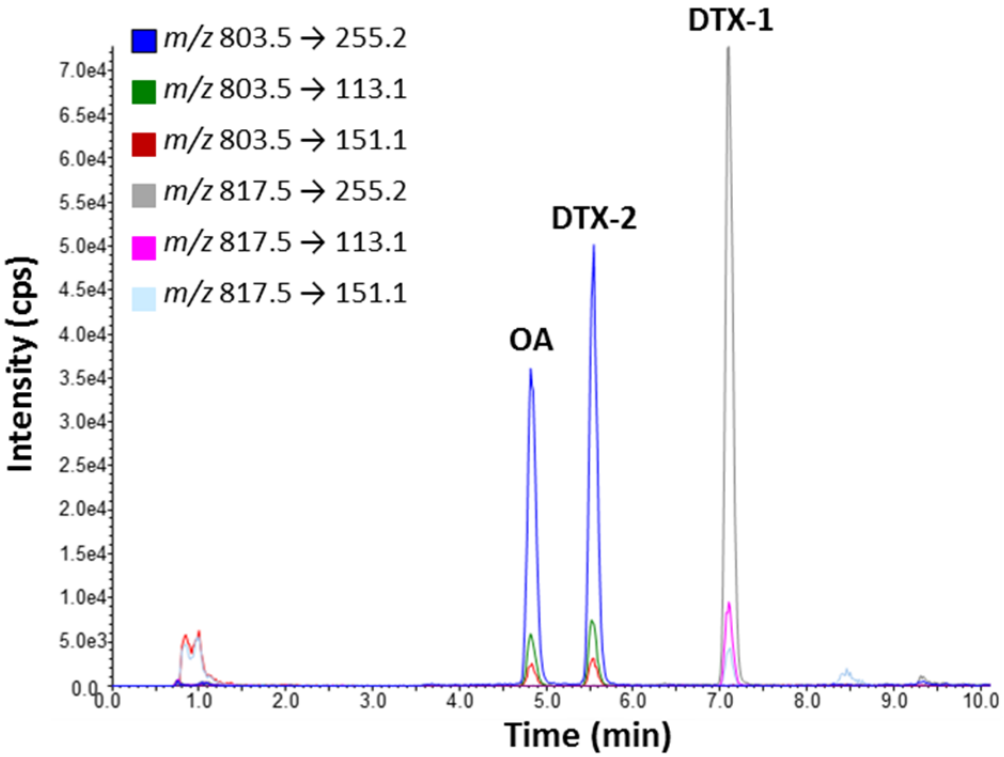


Figure 2. MRM chromatogram for the 12.5 ng/mL (regulatory level = 12.8 ng/mL) spike of OA, DTX1, and DTX2 into blank clam matrix.

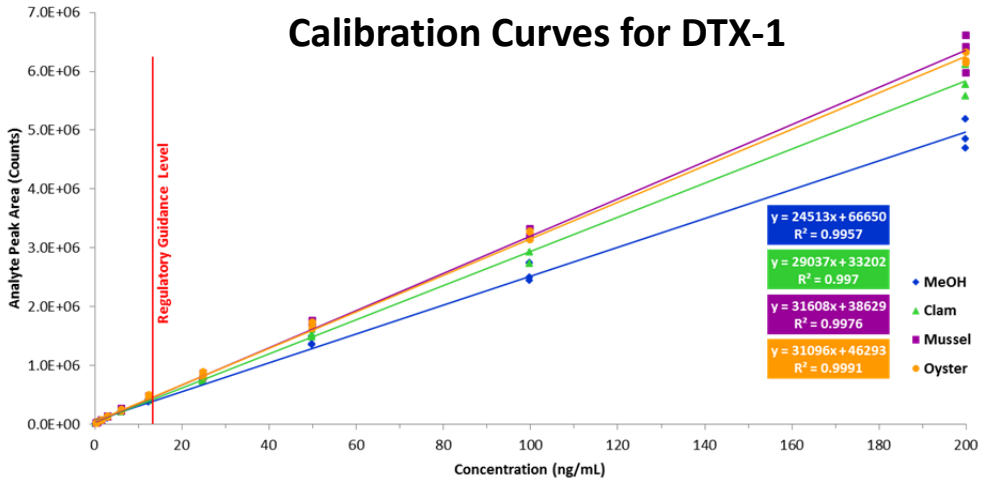
Pre-Validation Study Results (Testing for potential shellfish matrix effects for clam, mussel, and oyster, and ruggedness testing for acidic versus basic chromatography)

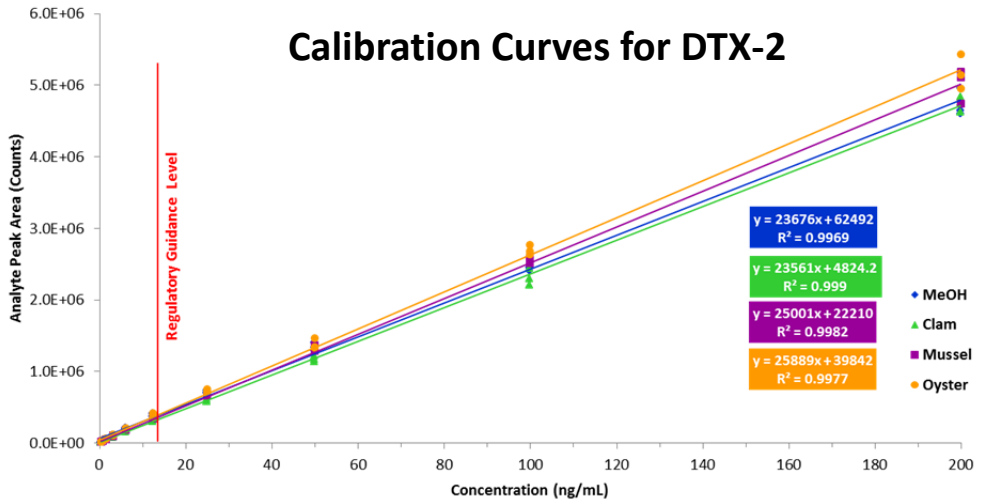
Initial pre-validation testing investigated the impact of mobile phase pH on method performance, and potential matrix effects (i.e. MS signal suppression or enhancement) for clam (*Mercinaria mercinaria*), mussel (*Mytilus edulus*), and oyster (*Crassostrea virginica*) in neat versus matrix matched spiked standard curves. Various laboratories in the United States and abroad are using different chromatographic mobile phase conditions, based on individual lab preference and need. Specifically, some labs are utilizing acidic chromatography (pH 2.4) while others are using basic chromatography (pH 11). Acidic chromatography is routinely used for quantifying total DSP toxin following hydrolysis. However, for methods that require polarity switching to investigate many classes of lipophilic toxins, basic chromatography is advantageous in that analytes that are preferentially ionized in positive mode are sufficiently separated from those that are preferentially ionized in negative mode.[2] Basic chromatography has also been reported to enhance sensitivity for certain lipophilic toxins, but due to matrix effects often requires the use of matrix matched standard curves, which adds additional cost and time to the method.

Figures 3 and 4 illustrate calibration curves from neat standard solutions in methanol compared to spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic and basic chromatographic conditions, respectively. For the preparation of blank shellfish extracts, homogenates from 5 previously tested composite shellfish samples (10-12 animals each) for each species, found to have no detectable DSTs, were re-extracted, hydrolyzed, and hexane washed following the procedure described above. These 5 extracts were then pooled per species and used for the preparation of matrix-matched standard curves. Ten fortification concentrations: 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL (equivalent to 0.49-250 µg/100g) of each toxin (from 2 µg/mL stocks) were used to construct each calibration curve. Fortification with DSTs was done post extraction to evaluate potential matrix interferences without having to account for analyte recovery. Each curve was analyzed in triplicate. All calibrations curves were shown to be linear ($R^2 \geq 0.99$) within the range of 0.391–200 ng/mL using both acidic and basic chromatography.

Our results indicate that under acidic chromatographic conditions over an extended working range, the slope of the calibration curves for OA and DTX2 in the presence of matrix is within 10% that of the neat toxin standards in methanol for all three matrices. DTX1 response, on the other hand, showed signal enhancement in matrix compared to neat standards, especially at high toxin concentrations (>50 ng/mL in the hydrolyzed shellfish extract). The percent difference in the slope of the solvent-only calibration curve and the matrix-matched calibration curve for DTX1 was 19% in clam, 29% in mussel, and 27% in oyster. However, at the regulatory guidance level (16 µg/100g shellfish, 12.8 ng/mL on column, in the hydrolyzed shellfish extract), the difference in peak areas for DTX1 in matrix versus solvent is <20% for all three matrices. In comparison, under basic chromatographic conditions OA and DTX2 signals were suppressed in the presence of matrix, resulting in significantly lower calibration curve slopes (up to 19% lower) for matrix-matched calibration curves. At the regulatory guidance level, matrix suppression resulted in response differences as high as 40% for OA and 37% for DTX2. LODs and LOQs were comparable for both acidic and basic chromatography, and both were well below the level of concern for these toxins (additional information below). Based on this information, acidic chromatographic conditions were chosen for the SLV study because they would allow the use of neat standard curves in methanol, saving both time and expense. In addition, the greatest risk from the use of acidic chromatography with non-matrix-matched standard curves is potentially overestimating DTX1 concentrations (increased chance of false positive result of exceeding guidance level), while the greatest

risk from basic chromatography with non-matrix matched standard curves is underestimating OA or DTX2 concentrations (increased chance of false negative result of exceeding guidance level). From a public health perspective, overestimation is more protective than underestimation and it was felt that this slight risk was acceptable when weighted against the additional burden in both time and expense in requiring the use of matrix-matched standard curves. Furthermore, additional validation including the use of matrix matched standard curves from multiple matrix sources would likely be required for accurate quantitation of DSTs when using mobile phases at high (basic) pH.





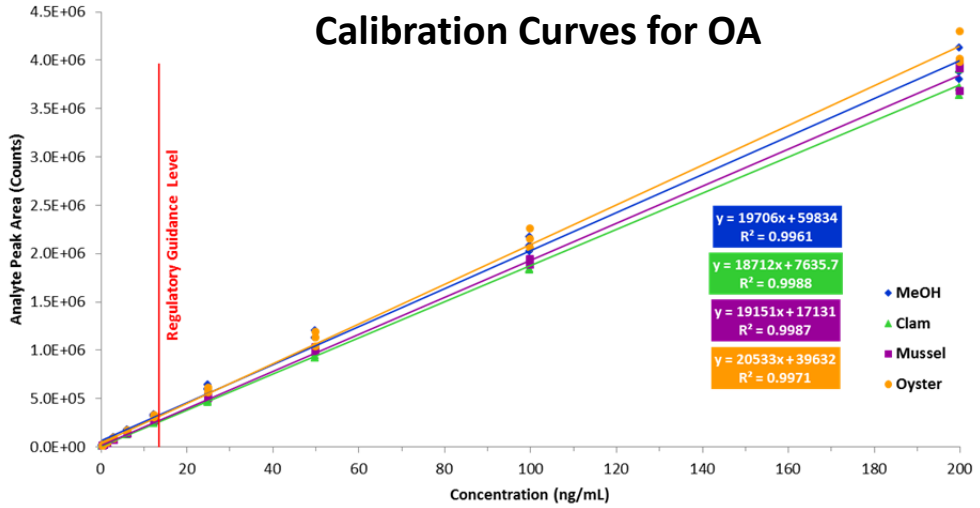
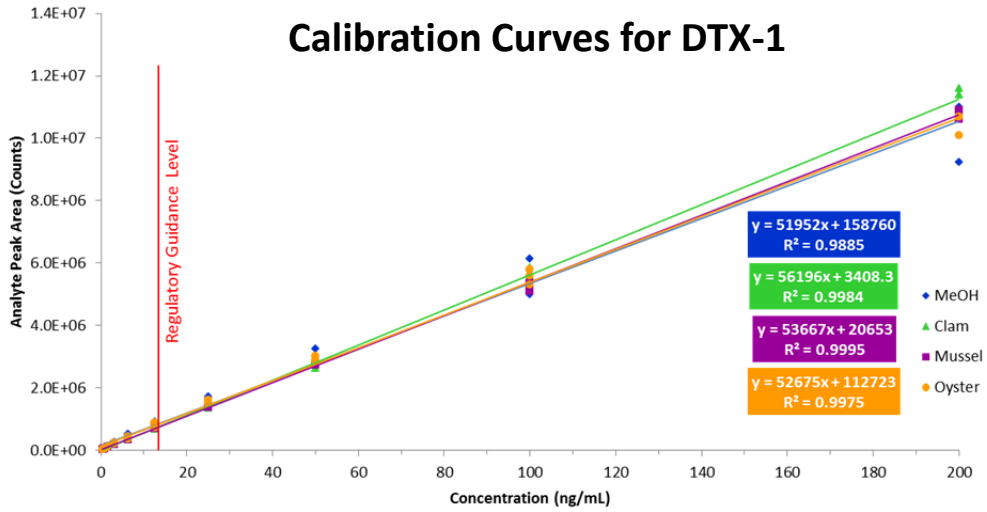


Figure 3. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.



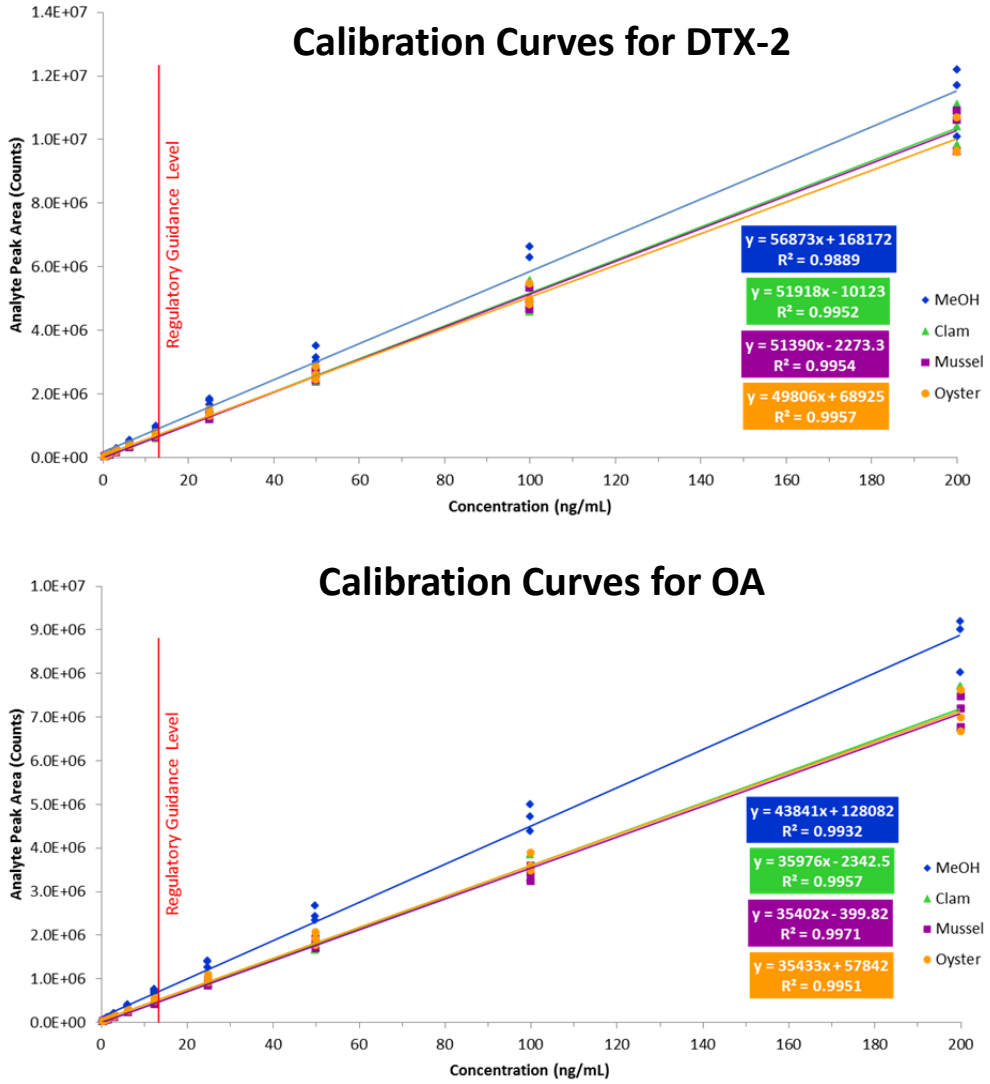


Figure 4. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under basic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.

Pre-validation Limits of Detection (LODs) and Quantitation (LOQs):

LODs and LOQs are based on the standard deviation of the response and the slope.[3] The equations for each are expressed as:

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

Where σ = the standard deviation of the response from five blank matrix samples
S = the slope of the calibration curve

	LOD (ng/mL)			**LOQ (ng/mL)**		
	OA	DTX-2	DTX-1	OA	DTX-2	DTX-1
	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic
Clam	0.055 / **0.040**	0.010 / **0.003**	**0.032** / 0.043	0.166 / **0.120**	0.031 / **0.009**	**0.096** / 0.129
Mussel	0.019 / **0.007**	0.007 / **0.006**	0.018 / **0.008**	0.057 / **0.020**	0.022 / **0.018**	0.053 / **0.023**
Oyster	0.017 / **0.015**	**0.008** / 0.011	0.018 / **0.016**	0.050 / **0.046**	**0.025** / 0.034	0.054 / **0.049**

Method Validation

Preparation of Stock Solutions for Validation:

Certified reference materials from the National Research Council Canada are supplied in sealed glass ampoules containing at least 0.5 mL of MeOH containing each toxin at a certified concentration that differs between toxins and lots. Thus, different volumes of each toxin standard, in 0.5 mL aliquots transferred using a 0.5 mL positive displacement Hamilton syringe, were transferred to a 20 mL glass scintillation vial and diluted with methanol to achieve stock standard solutions for each toxin at a concentration of 2 µg/mL. Stock solutions were stored at −20 °C.

Table 4. Toxin Stock Solution Preparation for Clam Validation Study

Certified Reference Material	Certified Concentration (µg/mL)	Lot #	Volume (mL)	Solvent (ml)	Total Volume	Final Concentration (µg/mL)
CRM-DTX1	15.1 ± 1.1	20071024	2	13.10	15.10	2
CRM-DTX2	7.8± 0.4	20071121	4	11.60	15.60	
CRM-OA-c	13.7 ± 0.6	20070328	2.5	14.625	17.125	

Validation Criteria:

Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

Accuracy/Trueness

Each shellfish sample used for this study was screened for DSTs using the described LC-MS/MS method to assure that each matrix did not contain any naturally accumulated DSP toxins. Once these samples were established to be free of contamination, twenty sample homogenates (five each from matrix sources A through D from Table 1) were spiked at five concentrations ranging from 8 µg/100 g to 32 µ/100 g. Concentrations for spiking were selected to cover one half to two times the regulatory limit for DSTs in shellfish which is 16 µg of toxin per 100 g of shellfish tissue. Detailed procedures are described below.

1. For each sample, ten-twelve animals were rinsed, shucked, drained, and homogenized in a commercial food processor.
2. 2.0 ± 0.05 g of each homogenate was weighted into a 50 mL disposable centrifuge tube using a stainless steel laboratory spatula.
3. 9.0 mL of 100% methanol was added to the centrifuge tube

4. Individual DSP toxin standards were added into the tube, using positive displacement pipettes, so that the final concentration of each toxin was 8, 12, 16, 24, or 32 µg/100 g
5. Each sample was mixed for 3 min using a vortex mixer
6. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
7. The supernatant was transferred to a 20 mL glass scintillation vial.
8. The residual tissue pellet was re-extracted with 9.0 mL of methanol and homogenized using a Polytron homogenizer, followed by vortexing for 3 min.
9. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
10. The supernatant was transferred to a clean 25 mL graduated cylinder, combined with the first extract, and the total extract volume was adjusted to 20 mL by adding 100% methanol. The adjusted extract was then transferred back into the original 20 mL glass scintillation vial.
11. A 2 mL aliquot of the 20 mL extract was transferred to a 16 × 100 mm glass tube using a 1 mL positive displacement Hamilton syringe and 250 µL of 2.5 M NaOH was added. Each tube was sealed with a phenolic PTFE lined screw cap and vortexed to mix for 30 seconds. Tube weights were recorded, then placed in a 76 ± 2 °C water bath for 40 minutes.
12. Sample tubes were dried, allowed to cool to room temperature for 5–10 minutes, and re-weighted to assess any evaporative sample loss. No samples required volume adjustment during these experiments. Samples were then neutralized with 2.5 M HCL, followed by vortex mixing for 30 seconds.
13. Approx. 5 mL of hexane was added to each 2.5 mL hydrolyzed methanolic extract and vortexed for 30 seconds to mix. Samples were partitioned by centrifuging at 2,000 *g* for 10 min at 20 °C. Using a disposable glass Pasteur pipette, the upper hexane layer was removed and discarded into an appropriate waste container, and approximately 1 mL of the remaining methanolic extract (bottom layer) was transferred into a 1 mL disposable syringe equipped with a 13 mm, 0.2 µm syringe tip filter using a clean disposable glass Pasteur pipette. Each filtrate was collected directly into an LC-MS certified glass vial for analysis.

Data for OA, DTX1, and DTX2 in clam are reported in tables 4, 5, and 6 respectively. These data were collected on four different days over the course of nine days.

Precision and Recovery

Matrix fortification and extraction were conducted as described above for Accuracy/Trueness. However, each of the ten samples (five samples each from sources A and B from Table 1) was fortified at concentrations of 8, 16, and 32 µg/100 g. Even number samples, comprised of clams from two source locations, were prepared on the same day and ran within 24 h. Odd number samples also consisting of clams from the two sources were prepared and analyzed nine days later than the even number samples. Data for OA, DTX1, and DTX2 are reported in tables 7, 8, and 9, respectively. Precision is reported as percent relative standard deviation (%RSD).

Specificity

All three DSTs were analyzed in the presence of the potentially co-occurring lipophilic toxins azaspiracids (NRC-CRM-AZA 1, NRC-CRM-AZA 2, NRC-CRM-AZA 3), pectenotoxin (NRC-CRM-PTX2) and yessotoxin (NRC-CRM -YTX). For each sample three aliquots of blank tissue matrix were used. One aliquot served as a control blank, one sample contained a ½ action level spike (8 µg/100 g) of each DSTs, and one

contained the same concentration of DSTs and also a moderate to high concentration of each potential interfering compound. Five technical replicates of each aliquot, excluding the negative control blank, were analyzed. The specificity index is reported for each DST.

Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity

Matrix fortification and extraction were conducted as described above for Accuracy/Trueness. However, each of ten replicate clam samples (five each from source locations A and B from Table 1) were fortified at five concentrations spanning 50-150% of the range of interest (4, 8, 16, and 32, 48 µg/100 g). Two replicate injections of each fortified sample were analyzed. The linear range for OA, DTX1, and DTX2 extracted from fortified clam is shown in figures 5, 6, and 7. These data were collected on four different days over the course of ten days.

Ruggedness

In addition to pre-validation ruggedness testing of acidic versus basic chromatography and neat verses matrix matched calibration curves (data presented earlier in this report), two additional factors were assessed: 1. Effect of the hexane washing step on accuracy/trueness, and 2. Effect of using a different mass spectrometer of the same make and model on method performance.

To assess the effect of the hexane washing step on accuracy/trueness, two sub-samples from each of 10 extracts from previously spiked samples, representing two different matrix sources (5 samples each from matrix sources A and B), each spiked at 5 different concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were hydrolyzed following the procedure above. For each sample, one hydrolyzed extract was put through the above described hexane washing step, while the other was filtered straight into an LC vial for analysis. This entire procedure was repeated on different days so that in total 20 samples were tested both with and without the hexane washing step. The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results.

To assess the effect of using a different mass spectrometer of the same make and model on method performance (i.e. method transferability), ten samples (spiked at either 8, 16, or 32 µg/100 g) were analyzed using two different AB Sciex QTrap 5500 detectors equipped with a Turbo V ionization source (same make and model but purchased several years apart). The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results.

Matrix Effects

See Pre-Validation Study Results above.

Comparability

Method comparability**,** typically accomplished through comparison to a reference method, was not directly possible due to the fact that no reference method has been established under the NSSP. LC-MS/MS is the established reference method for the measurement of lipophilic shellfish toxins in the EU. The method described here is a version of the EU reference method optimized specifically for DSP toxins. One of the biggest differences between the method described here and the EU reference method is that the EU reference method is designed to detect multiple groups of lipophilic toxins in addition to DSP such as pectenotoxins, azaspiracids, and yessotoxins. But the analysis of these additional toxin groups in a single chromatographic run requires switching between positive and negative ion modes and initial analysis without sample hydrolysis as hydrolysis destroys several of these toxin groups. Analysis of total DSP toxins (free plus esterified) requires a second sample injection after alkaline hydrolysis of a sub-sample of the shellfish extract. Pectenotoxins and yessotoxins are not required to be analyzed for under the NSSP as they have not been proven to cause human illness, and while azaspiracids are required to be monitored, an optimized LC-MS/MS method for azaspiracids would be performed in positive ion mode and without sample hydrolysis. Therefore we are treating the LC-MS/MS method for azaspiracids as a separate method, even though the same extract, mobile phase, and equipment can be used for the analysis of both toxins. Different labs in the EU do run different versions of the "reference method" including some analyzing with acidic chromatographic conditions and some using basic, but pre-validation studies performed here found that analysis under basic chromatographic conditions would require the use of matrix matched standards. Furthermore, the two regulatory laboratories in the U.S. currently running a version of the EU reference LC-MS/MS method for lipophilic shellfish toxins as best available science are both using acidic chromatographic conditions. Therefore, running a different version of the EU reference LC-MS/MS method, such as basic chromatography or analysis without hydrolysis did not seem relevant for the method comparability requirement.

Analysis of certified reference materials would be another way to assess method performance as these naturally contaminated materials are certified to contain a known amount of all three of the target compounds. Until recently the only source of CRMs for DSP toxins, NRC Canada, only certified these materials for free toxins (i.e. toxins present pre-hydrolysis not taking into account the potential presence of fatty acid acyl ester shellfish metabolites (DTX3), which are known to be present in naturally contaminated shellfish samples. But new materials produced by NRC, both a frozen shellfish homogenate and a lyophilized material, are now provided with informational concentrations for total toxins (free plus esterified). Five aliquots of the frozen CRM-DSP-Mus-c (at $185.00 each) and one aliquot of the lyophilized NRC-FDMT1 (at $1,175 each) were purchased from the NRC Canada Certified References Materials Program. The frozen CRM contains 4 g ± 0.5 g of homogenized material therefore can only be reliably tested once using the required 2 g extraction method. The lyophilized material reportedly contains enough material for approximately 8 extractions. This material was extracted and tested 5 times, for a total of 10 CRM replicate tests (5 frozen and 5 lyophilized). Each extract was hydrolyzed and tested twice on separate days (for a total of 20 analyses) to assess the methods performance.

Although LC-MS/MS is the only reference method currently accepted in the EU for the analysis of lipophilic shellfish toxins, EU regulations do allow for the use of supplementary methods if they are shown to be equally protective (Commission Regulations (EC) No.853/2004 and No.15/2011). One such supplementary method that has been both single[6] and multi-laboratory[7] validated and is recognized as equally protective for DSP toxins in the EU is the OkaTest, produced by ZEU Inmunotec in Spain. The colorimetric protein phosphatase inhibition assay (PPIA), OkaTest, complies with the criteria stipulated by the European Reference Laboratory on Marine Toxins and Commission Regulation 15/2012 for determination of OA-group toxins in molluscs, according to the European Commission (DG-SANCO). But since this method specifically detects DSP toxins while the EU reference LC-MS/MS method detects a number of different lipophilic shellfish toxins, the OkaTest is considered a supplementary method for the detection of DSP and not a full alternative to LC-MS/MS for the lipophilic shellfish toxins by the EU. The OkaTest is now sold in the United States by Abraxis LLC as the Protein Phosphatase Inhibition Assay (PPIA) for DSP toxins. Since the PPIA OkaTest provides a composite toxicity score for all DSP toxins present, it was not possible to test all of the spiked samples generated during this validation directly by both methods as all samples in this study were spiked with all three of the target compounds. Furthermore, the linear range of the OkaTest is 6.3 – 35.4 µg OA eq./100 g. Only two of the spiking levels used in this study were within the linear range of the OkaTest, the 4 and 8 µg/100 g spiking levels (n=10 each; 20 samples total) from the liner range determination, which equated to 12 and 24 µg/100 g in total DSP toxins, closely bracketing the regulatory level of 16 µg OA eq./100 g. In addition, all shellfish homogenates (n = 10 each; 30 samples total) from the three species of shellfish used in the pre-validation matrix effect studies (clams, *Mercinaria mercinaria*; mussels, *Mytilus edulus*; and oysters, *Crassostrea virginica*), that had been previously tested and found to be <LOD for DSP toxins by LC-MS/MS were also tested using the OkaTest to show that they were also negative by this alternative method. Lastly, naturally contaminated shellfish from a variety of species and geographic locations including softshell clams (*Mya arenaria*) from New York (n=9) and containing both OA and DTX1 (range <LOD – 37.3 µg OA eq. /100 g), blue mussels (*Mytilus edulus*) from Washington (n=12) containing DTX1 only (<LOD – 52.5 µg/100 g), and eastern oysters (*Crassostrea virginica*) from Texas (n=11) containing OA only (1.6 – 56.3 µg/100g) were tested by both methods to assess comparability of the LC-MS/MS method with PPIA.

Results

To be provided to the LMRC with sufficient time to be reviewed prior to the 2017 ISSC meeting.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
Name of the Method Developer	Jonathan Deeds
Developer Contact Information	Jonathan.deeds@fda.hhs.gov; 240-402-1474 US FDA, 5001 Campus Drive, College Park, MD 20740

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	
2. What is the intended purpose of the method?	Y	
3. Is there an acknowledged need for this method in the NSSP?	Y	
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	
B. Method Documentation		
1. Method documentation includes the following information:	Y	
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	Y	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	

8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	Y	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	

Submitters Signature Jonathan R. Deeds -S Digitally signed by Jonathan R. Deeds -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, 0.9.2342.19200300.100.1.1=1300218767, cn=Jonathan R. Deeds -S Date: 2017.05.31 12:46:30 -04'00'	Date: 5/31/2017
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

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6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.