PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
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SHELLFISH LABORATORY EVALUATION CHECKLIST
Diarrhetic Shellfish Poisoning Toxins (DSP) LC-MS/MS

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "✓"**

C – Critical K - Key O - Other NA - Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
K	1, 7, 8	**1.1 Quality Assurance (QA) Plan**	
			1.1.1 Written Plan adequately covers all the following: (check those that apply) a. Organization of the laboratory b. Staff training requirements c. Standard operating procedures d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established e. Laboratory safety f. Internal performance assessment g. External performance assessment
C	**5**		**1.1.2 QA Plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
C	**3**		**1.2.5 LC-MS Operator must be trained in the operation and maintenance of the specific liquid chromatography-mass spectrometry system used.**
		1.3 Work Area	
O	1		1.3.1 Adequate for workload and storage.
O	1		1.3.2 Clean and well lighted.
O	1		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
C	**3**		**1.4.1 A heat block or water bath capable of heating samples to 76 ± 2 °C.**
K	2		1.4.2 Balances provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	7, 8		1.4.3 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.4 Refrigerator temperature is maintained between 0 and 4 ºC.
K	7		1.4.5 Refrigerator temperature is monitored at least once daily on working days. Results are recorded and records maintained.
K	2		1.4.6 Freezer temperature is maintained at -10 ºC or below.

K	7		1.4.7 Freezer temperature is monitored at least once daily on working days. Results are recorded and records maintained.
C	**10**		**1.4.8 All in-service thermometers are properly calibrated and immersed.**
K	4		1.4.9 All glassware is clean.
K	3, 12		1.4.10 An ultra-performance liquid chromatography system (UPLC) equipped with the following is used: a. mobile phase system capable of delivering a pulse-free flow of 0.12 mL/min b. solvent degasser (optional) c. autosampler (refrigerated preferred) with loop suitable for five (5) µL injections d. column compartment capable of controlling temperature at 40 ºC e. a data collection system (e.g., computer, integrator)
C	**3**		**1.4.11 A mass spectrometer equipped with the following is used:** **a. an electrospray ionization source operating in negative ion mode and** **b. multiple reaction monitoring scan mode capability.** **c. if a divert valve is used to divert LC flow at the beginning and end of each chromatographic run, the switching time should be at least one minute before the first peak elution and at least one minute after the last peak elution.**
K	2		1.4.12 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.13 A centrifuge capable of generating 2000 x g and holding 15 mL and 50 mL polypropylene tubes is used.
		1.5	**Reagents and Reference Solution Preparation and Storage**
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	7		1.5.2 Water contains < 100 CFU/ml determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	7		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.4 The mobile phase system used to analyze DSP toxins consists of: A: 2 mM ammonium formate and 50 mM formic acid in water B: 2 mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water**
O	2		1.5.5 Mobile phase is degassed manually before use if the UPLC does not have a degasser or if the degasser is not in use.
C	**3**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
C	**6**		**1.5.7 All primary standards are stored appropriately as per supplier recommendations.**
C	**6**		**1.5.8 All standards used are within their expiration date.**

C	**2, 3**		**1.5.9 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	**3**		**1.5.10 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 100% methanol).**
		1.6 Collection and Transportation of Samples	
O	5, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	5, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**5, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**
K	2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
		PART II – EXAMINATION OF SHELLFISH FOR DSP TOXINS	
		2.1 Preparation of Sample	
C	**2**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., three (3) geoduck gut balls).**
O	5		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	5		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for five (5) minutes.
K	5		2.1.8 Pieces of shell and drainage are discarded.

C	**2, 5**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	2		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**3**		**2.2.2 Two (2.00) ± 0.05 g of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**3**		**2.2.3 The sample homogenate is extracted with 9 mL of 100% methanol and vortexed to mix.**
K	3		2.2.4 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant decanted into a clean container (e.g. new polypropylene tube or glass vial).
C	**3**		**2.2.5 The tissue pellet is reextracted with nine (9) mL of 100% methanol and homogenized to mix.**
K	3		2.2.6 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant combined with the supernatant in 2.2.4.
K	3		2.2.7 The total extract volume is adjusted to 20 mL with 100% methanol.
K	3		2.2.8 The crude extract is hydrolyzed or stored in the freezer at < -10 °C.
		2.3 Sample Hydrolysis and Cleanup	
K	3		2.3.1 A two (2) mL aliquot of the sample extract is transferred to an appropriately size* glass tube with a phenolic PTFE lined screw cap using a positive displacement pipette or syringe. *Note: A 16 x 100 mm tube will have sufficient volume to perform the hydrolysis and hexane wash steps and fit in a standard 15 mL centrifuge tube adaptor.
K	3		2.3.2 The sample extract is hydrolyzed by adding 250 µL of 2.5 M NaOH and the sample is homogenized with a vortex mixer for 30 seconds.
C	**3**		**2.3.3 Sample tube caps are securely fastened to prevent extract loss, and the weight of the sample tube is recorded. The sample tube is heated at 76 °C for 40 minutes, then allowed to cool to room temperature, dried, and re-weighed. If the weight has dropped by more than 0.1 g, lost volume is replaced using 100% MeOH.**
K	3		2.3.4 Samples are neutralized with 250 µL of 2.5 M HCL and vortexed to mix.
O	3		2.3.5 Nonpolar lipids are removed by adding seven (7) mL of hexanes to the hydrolyzed sample extract and vortexing to mix (2.3.5 – 2.3.7 Preferred).
K	3		2.3.6 The sample extract/hexane mixture is partitioned by centrifuging for 10 minutes at 2000 x g (2.3.5 – 2.3.7 Preferred).
K	3		2.3.7 The hexane layer is removed with a glass pipette and one (1) mL of the hydrolyzed methanolic extract is removed and filtered into an LC-MS glass autosampler vial using a 0.2 µm PTFE syringe tip filter (2.3.5 – 2.3.7 Preferred).

<table>
<tr><td>K</td><td>2, 3</td><td></td><td>2.3.8 The cleaned-up extract is loaded into the autosampler immediately for analysis.</td></tr>
<tr><td colspan="2"></td><td colspan="2">2.4 Analysis</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.1 Analytes are detected in standards and samples using the mass transitions in the table (negative ion mode).
<table>
<tr><th>Compound</th><th>Q1 (m/z)</th><th>Q3 (m/z)</th></tr>
<tr><td>OA</td><td>-803.5</td><td>-255.2</td></tr>
<tr><td>OA</td><td>-803.5</td><td>-113.1</td></tr>
<tr><td>OA</td><td>-803.5</td><td>-151.1</td></tr>
<tr><td>DTX2</td><td>-803.5</td><td>-255.2</td></tr>
<tr><td>DTX2</td><td>-803.5</td><td>-113.1</td></tr>
<tr><td>DTX2</td><td>-803.5</td><td>-151.1</td></tr>
<tr><td>DTX1</td><td>-817.5</td><td>-255.2</td></tr>
<tr><td>DTX1</td><td>-817.5</td><td>-113.1</td></tr>
<tr><td>DTX1</td><td>-817.5</td><td>-151.1</td></tr>
</table></td></tr>
<tr><td>K</td><td>3</td><td></td><td>2.4.2 Other system parameters such as collision energy are optimized for the specific system using standards before analysis.</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.3 A standard calibration curve of at least six (6) concentrations is performed at the beginning of each run. An additional curve is required if a run lasts longer than 24 hours.</td></tr>
<tr><td>K</td><td>3</td><td></td><td>2.4.4 Five (5) µL of extract is injected for analysis.</td></tr>
<tr><td>K</td><td>2, 12</td><td></td><td>2.4.5 Samples are stored in the sample compartment of the autosampler at ≤ 10°C during analysis. Otherwise the samples must be analyzed within 24 hours if the autosampler is held at room temperature.</td></tr>
<tr><td>K</td><td>3</td><td></td><td>2.4.6 A column heater is used and the temperature is maintained at 40 °C, with a tolerance as specified by the manufacturer, during the analysis.</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.7 An Acquity UPLC BEH C18 1.0 × 150 mm, 1.7 µm particle size (or equivalent) analytical column is used for analyte separation</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.8 Analytes are separated on the LC column using gradient elution.</td></tr>
<tr><td>K</td><td>2</td><td></td><td>2.4.9 The column is stored following the manufacturer’s instructions when not in use.</td></tr>
<tr><td>K</td><td>2</td><td></td><td>2.4.10 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.11 A matrix or procedural blank (i.e. NRC CRM Zero-Mus or equivalent negative matrix, or methanol carried through sample preparation process at the same time as the samples) should be included in each analytical batch.</td></tr>
<tr><td colspan="2"></td><td colspan="2">2.5 System Suitability</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.5.1 The retention time of analytes in all samples are within 3% that of one of the intermediate toxin standards, measured from the apex of the peak.</td></tr>
</table>

C	**3**		**2.5.2 Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (Rs) of OA/DTX2 should ≥ 1 when calculated using the equation below (RT is retention time and W is peak width at baseline; Peak 1 is OA and Peak 2 is DTX2).** $$Rs = 2 \times (RT2 - RT1)/(W1 \mp W2)$$
K	2, 3		2.5.3 Each chromatographic peak must be defined by at least 10 data points.
C	**3**		**2.5.4 Reagent blanks (methanol) are analyzed after the high calibration standard, periodically (as determined by the laboratory's internal verification), and after fortified samples to ensure that analyte carryover is not occurring. Analyte carryover is defined as a confirmed peak > LOD.**
C	**3**		**2.5.5 To confirm the presence of each DST, two (2) mass transitions must be observed above the limit of detection (LOD).** **The transition yielding the highest signal-to-noise ratio (S/N) is used for quantitation (i.e. 817.5 → 255.2 for DTX-1, 803.5 → 255.2 for OA and DTX-2). The transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation is ≥ 3.**
C	**3**		**2.5.6 The ratio of the abundance of the confirmation ion transition to the quantitation ion transition is calculated for each toxin. These ion ratios must be within ± 20% of that of the toxin standards in order to confirm toxin identity.**
C	**3**		**2.5.7 When ≥ 10 samples are analyzed, analysts must show that the calibration has not significantly drifted using an option provided below:**
		OPTION 1 – USE OF BRACKETING CALIBRATION CURVES	
C	**3**		**2.5.7.1 A second standard curve is analyzed at the end of the analytical sequence and the averaged peak areas are used for the linear regression.**
C	**3**		**2.5.7.2 The linear regression of the averaged calibration curves must yield an $R^2 \geq 0.98$. Results are recorded and records are maintained.**
C	**3**		**2.5.7.3 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The average of bracketing calibration curves yields an $R^2 < 0.98$.** **b) The difference in the slope between bracketing calibration curves exceeds 25%.** **c) The difference in retention times of the standards in the bracketing standard curves exceeds 3%.**

		OPTION 2 – USE OF A CONTINUING CALIBRATION VERIFICATION (CCV) STANDARD	
C	**3**		**2.5.7.4 The linear regression of the single calibration curve must yield an R^2 ≥ 0.99.**
C	**3**		**2.5.7.5 A continuing calibration verification (CCV) standard, matching on of the intermediate standards from the calibration curve, is analyzed after every 10 samples and at the end of a run.**
C	**3**		**2.5.7.6 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The calibration curve yields an R^2 < 0.99.** **b) The retention time of a CCV sample exceeds 3% of the corresponding standard.*** **c) The peak area of a CCV sample exceeds ± 15% compared to the corresponding standard in the calibration curve.*** *** Samples immediately preceding and post the failed CCV shall be reanalyzed with a new standard curve.**
C	**3**		**2.5.8 Repeated injections of calibration or control samples at a concentration near the action level agree within ± 10% (as determined through the use of the coefficient of variation).**
		2.6 Calculation of Sample Toxicity	
C	**3, 11**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $\frac{ug}{g} toxin = C \times \frac{V}{W}$ **X Hyd X ReTx** **where:** **C = the concentration in µg/ml of the extract injected, determined using the standard curve** **V = total volume of homogenate and extraction solvent (mL)** **W = weight (g) of tissue homogenate extracted** **Hyd = dilution factor for hydrolysis (1.25)** **ReTx = relative toxicity of toxin vs. Okadaic Acid** **Relative Toxicity Values**

Toxin	ReTx
OA	1
DTX1	1
DTX2	0.6

The individual toxicities for each toxin are summed to obtain the overall sample toxicity in ppm OA equivalents.

C	**9**		**2.6.2 Any value at or above 0.16 ppm OA equivalents (mg/kg or µg/g) of the sum of any analytes present is actionable. Shellfish Program Management is made aware of positive result. Laboratory action is_____ .**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
3. Interstate Shellfish Sanitation Conference (ISSC), Proposal 17-103 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish
4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
5. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
6. Consult reference standard product literature.
7. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
8. American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
9. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
10. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
11. The EFSA Journal. 2009. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. 1306, 1-23.
12. Deeds, J.R. and M.D. Celiz, Personal Communication, Addendum to proposal 19-136 DSP LC MS/MS Additional Ruggedness Testing; effect of the sample storage temperature during analysis (24 hrs), Email Received by ISSC Checklist Subcommittee 2/18/2021.

LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DIARRHETIC SHELLFISH POISON (DSP) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Conforms Status: The DSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The DSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The DSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥4**.
   - b. or the total # of Key nonconformities is **≥6.**
   - c. or the total # of Critical, Key, or Other is **≥12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____