

**National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish: 2019 Revision**

<p><b>PUBLIC HEALTH SERVICE U.S. FOOD AND DRUG ADMINISTRATION OFFICE OF FOOD SAFETY SHELLFISH AND AQUACULTURE POLICY BRANCH 5001 CAMPUS DRIVE COLLEGE PARK, MD 20740-3835 TEL. 240-402-2151/2055/4960 FAX 301-436-2601 <a href="mailto:CFSANDSSLEOS@FDA.HHS.GOV">CFSANDSSLEOS@FDA.HHS.GOV</a></b></p>		
<p><b>SHELLFISH LABORATORY EVALUATION CHECKLIST Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV</b></p>		
LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:
LABORATORY REPRESENTED BY:		TITLE:
LABORATORY EVALUATION OFFICER:		SHELLFISH SPECIALIST:
		REGION:
OTHER OFFICIALS PRESENT:		TITLE:
Items which do not conform are noted by:		
<p><b>C – Critical      K - Key      O - Other      NA - Not Applicable      Conformity is noted by a “1”</b></p>		

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<b>PART I – QUALITY ASSURANCE</b>		
Code	REF	Item Description
		<b>1.1 Quality Assurance (QA) Plan</b>
K	5, 8	1.1.1 Written Plan adequately covers all the following: (check '1' 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.
C	5	<b>1.1.2 QA Plan is implemented.</b>
		<b>1.2 Educational/Experience Requirements</b>
C	State's Human Resources Department	<b>1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.</b>
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	<b>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 years of laboratory experience.</b>
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 months of experience in laboratory sciences.
C	5	<b>1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.</b>
		<b>1.3 Work Area</b>
O	5, 8	1.3.1 Adequate for workload and storage.
O	8	1.3.2 Clean and well lighted.
O	8	1.3.3 Adequate temperature control.
O	8	1.3.4 All work surfaces are nonporous and easily cleaned.
		<b>1.4 Laboratory Equipment</b>
K	6	1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5	1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8	1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment. [Only applicable if using the sample cleanup procedure]

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K	8	1.4.4 The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded. [Only applicable if using the sample cleanup procedure]
K	5, 11	1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure. [Only applicable if using the sample cleanup procedure]
K	6, 2	1.4.6 The balances being used 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	8	1.4.7 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	1.4.8 Refrigerator temperature is maintained between 0 and 4 °C.
K	8	1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	4,15	1.4.10 Freezer temperature is maintained at -10 °C or below.
K	8	1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	13	<b>1.4.12 All in-service thermometers are properly calibrated and immersed.</b>
K	5	1.4.13 All glassware is clean.
K	4	1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 1.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 20 µL injections, d. temperature controlled column compartment capable of controlling temperature at 40 °C, e. ultraviolet detector/diode array detector able to achieve the required sensitivity at a wavelength (A) of 242 nm, and f. a data collection system (e.g., computer, integrator).
K	2	1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	4	1.4.16 A solid phase extraction (SPE) vacuum manifold capable of holding 3 mL cartridges is used. [Only applicable if using the sample cleanup procedure]
O	4	1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
		<b>1.5 Reagents and Reference Solution Preparation and Storage</b>
C	4, 15	<b>1.5.1 All solvents and reagents used are analytical or LC grade materials.</b>
O	8	1.5.2 Water contains < 100 CFU/ml as 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.)

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K	8	1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	4, 15	<b>1.5.4 The mobile phase system used to analyze domoic acid consists of: 10% aqueous acetonitrile (v/v) and 0.1% trifluoroacetic acid (TFA).</b>
O	4	1.5.5 Mobile phase is filtered before use if the HPLC does not have a degasser.
C	7	<b>1.5.6 Only certified reference materials are used for standard solutions.</b> <b>Source of the reference standard: _____</b>
K	4, 15	1.5.7 A cartridge wash solution is made up of 1 volume acetonitrile to 9 volumes of water (i.e., 10% aqueous acetonitrile). [Only applicable if using the sample cleanup procedure]
K	4	1.5.8 Citrate buffer (0.5 M, pH 3.2) is made up by dissolving 40.4 g citric acid monohydrate and 14 g triammonium citrate in 400 mL water, then adding 50 mL acetonitrile and diluting the total to 500 mL with water [or equivalent buffer]. [Only applicable if using the sample cleanup procedure]
C	7	<b>1.5.9 NRC CRM Zero-Mus or a negative control is used as a blank to ensure that there is no carry over between samples/standards. Source of the negative control: _____</b>
C	7	<b>1.5.10 All primary standards are stored appropriately as per supplier recommendations.</b>
C	7	<b>1.5.11 All standards used are within expiration date.</b>
C	2	<b>1.5.12 All standards are prepared either gravimetrically or using positive displacement pipettes.</b>
C	4, 15	<b>1.5.13 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 50% aqueous methanol). Dilution with toxin-free, cartridge wash solution (aqueous acetonitrile) is allowed if using the diluted crude sample or the sample cleanup procedure.</b>
C	7	<b>1.5.14 Zero-Mus is stored according to manufacturer's instructions.</b>
C	2	<b>1.5.15 Quality Control shellfish tissues are stored frozen.</b>
		<b>1.6 Collection and Transportation of Samples</b>
O	6, 1	1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6, 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	6, 1	<b>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.</b>

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K	14, 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.
<b>PART II – EXAMINATION OF SHELLFISH FOR ASP TOXINS</b>		
<b>2.1 Preparation of Sample</b>		
C	6, 1	<b>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., 3 geoduck gut balls).</b>
O	6	2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6	2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6	2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6	2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	6	<b>2.1.6 Damage to the body of the mollusk is minimized in the process of opening.</b>
O	6	2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6	2.1.8 Pieces of shell and drainage are discarded.
C	2, 6	<b>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).</b>
<b>2.2 Sample Extraction</b>		
K	4,6	2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 °C or below.
C	4	<b>2.2.2 Four (4) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.</b>
C	4	<b>2.2.3 The sample homogenate is extracted with 16 mL extraction solvent (1:1 methanol:water [also referred to as 50% aqueous methanol]).</b>
K	4, 15	2.2.4 Homogenate/extract mixture is centrifuged and filtered before analysis.
K	4, 15	2.2.5 The filtered extract is injected into the HPLC or loaded into the autosampler immediately.

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K	4		2.2.6 When crude samples are diluted, dilutions are made by diluting 1 mL of filtered sample supernatant into a 5 mL volumetric flask and diluted with water to 5 mL.
K	4, 15		2.2.7 Crude extracts are sealed tightly and stored at -10 °C or below.
			<b>2.3 Sample Cleanup (Optional)</b>
O	4, 15		2.3.1 Three (3) mL SAX cartridges (500 mg silica derivatized with quaternary ammonium silane) are used for cleanup.
K	4		2.3.2 The SAX cartridge is conditioned with 6 mL methanol, followed by 3 mL water, followed by 3 mL extraction solvent (1:1 methanol:water).
C	4, 15		<b>2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.</b>
K	4, 15		2.3.4 Five (5) mL of filtered extract is loaded onto the cartridge and flowed slowly (~1 drop/s) until sample meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.5 Five (5) mL of wash solution (1:9 acetonitrile:water) is loaded to the cartridge and washed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4		2.3.6 0.5 mL of citrate buffer (0.5 M, pH 3.2) is loaded to the cartridge and flowed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.7 A 2 mL volumetric tube is placed under the cartridge and any domoic acid is eluted into the tube by loading and flowing as much citrate buffer as needed slowly (~ 1 drop/s) until the 2 mL mark is reached on the tube.
C	4, 15		<b>2.3.8 The solution is thoroughly mixed before withdrawing an aliquot for analysis.</b>
K	4, 15		2.3.9 The cleaned up extract is injected into the HPLC or loaded into the autosampler immediately.
			<b>2.4 Analysis</b>
C	2		<b>2.4.1 A standard calibration curve (of at least six concentrations) is performed daily. Results are recorded and records are maintained.</b>
K	4, 15		2.4.2 Twenty (20) µL of extract is injected for analysis.
K	2		2.4.3 Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 9 hours if the autosampler is held at room temperature.
K	4, 15		2.4.4 A column heater is used and the temperature is maintained at 40 °C during the analysis.
C	4		<b>2.4.5 The appropriate analytical column is used: 25 cm x 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica or equivalent.</b>
K	2		2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column (e.g., 201GCC54T) are/is used, rejection criteria are established to determine when to change the filter/guard column.

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C	2	2.4.8 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.
<b>2.5 System Suitability</b>		
C	2	2.5.1 The correlation coefficient for the linear regression of the calibration standards must be $\geq 0.990$ for domoic acid.
C	3	2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of L-tryptophan and domoic acid.
K	2	2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C	2	2.5.4 The column is replaced when a measure of peak asymmetry becomes $<0.9$ or $>1.3$ .
C	2,4	2.5.5 Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.
C	2	2.5.6 Repeated injections of calibrated standards/samples agree within $\pm 5\%$ (as determined through the use of the coefficient of variation).
<b>2.6 Calculation of Sample Toxicity</b>		
C	4, 15	<p>2.6.1 The toxicity of the individual toxins is calculated as follows:</p> $\mu\text{g/g domoic acid (DA)} = \text{DA injected} \times \frac{V}{W} \times (F)$ <p>where:</p> <p>DA injected = the concentration in <math>\mu\text{g/ml}</math> of the extract injected;  V = total volume of homogenate and extraction solvent (mL);  W = weight (g) of tissue homogenate extracted (e.g., 4 g); and  F = dilution factor (e.g., if SAX cleanup or crude sample dilution are performed).</p> <p>The concentration of DA injected may be determined using the nearest standard or the equation of the day's standard curve.</p>
C	4, 15	2.6.2 Calculated domoic acid concentrations include the sum of domoic acid and epimer peaks, when the epimer represents 5% or more of the peak area.
C	12	2.6.3 Any value at or above 20 ppm (mg/kg or $\mu\text{g/g}$ ) domoic acid is actionable.
<b>REFERENCES</b>		
1. American Public Health Association. 1984. <i>Compendium for the Microbiological Examination of foods</i> , 2 <sup>nd</sup> Edition. APHA. Washington D.C.		
2. Good Laboratory Practice. 21 CFR 58.		
3. AOAC Official Method 991.26 Domoic Acid in Mussels. Liquid Chromatography Method. First Action 1991. Final Action 1999.		
4. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1995. J. AOAC Int. 78(2): 543-554.		



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6. American Public Health Association. 1970. <i>Recommended Procedures for the Examination of Sea Water and Shellfish</i> , 4 <sup>th</sup> Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. <i>Standard Methods for the Examination of Water and Wastewater</i> , 18 <sup>th</sup> Edition. APHA, Washington, D.C.
9. American Public Health Association. 192. <i>Standard Methods for the Examination of Dairy Products</i> , 16 <sup>th</sup> Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. <i>American Laboratory</i> 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. <i>NSSP Guide for the Control of Molluscan Shellfish</i> . FDA/ISSC, Washington, D.C. and Columbia, S.C.
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14. Compendium of Methods for the Microbiological Examination of Foods, 3 <sup>rd</sup> Edition, pg. 901.
15. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1991. A Rapid Extraction and Clean-up Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Bioscience, Technical Report #64, National Research Council Canada #33001.





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<b>LABORATORY STATUS</b>	
<b>LABORATORY</b>	<b>DATE</b>
<b>LABORATORY REPRESENTATIVE:</b>	
<b>AMNESIC SHELLFISH POISON (ASP or domoic acid) COMPONENT: PARTS I AND II</b>	
<b>A. Results</b> Total # of <b>Critical (C)</b> Nonconformities Total # of <b>Key (K)</b> Nonconformities Total # of <b>Critical, Key, and Other (O)</b> Nonconformities	_____ _____ _____
<b>B. Criteria for Determining Laboratory Status of the ASP (domoic acid) Component</b> <ol style="list-style-type: none"> <li>1. <b>Conforms Status:</b> The ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.                         <ol style="list-style-type: none"> <li>a. <b>No Critical nonconformities.</b></li> <li>b. <b>and &lt;6 Key nonconformities.</b></li> <li>c. <b>and &lt;12 Total nonconformities.</b></li> </ol> </li> <li>2. <b>Provisionally Conforms Status:</b> The ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.                         <ol style="list-style-type: none"> <li>a. the number of critical nonconformities is <math>\geq 1</math> but <math>&lt; 4</math>.</li> <li>b. and <math>&lt; 6</math> Key nonconformities.</li> <li>c. and <math>&lt; 12</math> Total nonconformities.</li> </ol> </li> <li>3. <b>Does Not Conform Status:</b> The ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.                         <ol style="list-style-type: none"> <li>a. The total # of Critical nonconformities is <math>\geq 4</math>.</li> <li>b. or the total # of Key nonconformities is <math>\geq 6</math>.</li> <li>c. or the total # of Critical, Key, or Other is <math>\geq 12</math>.</li> </ol> </li> </ol>	
<b>C. Laboratory Status (circle appropriate)</b> <b>Does Not Conform – Provisionally Conforms – Conforms</b>	

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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: _____