PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
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SHELLFISH LABORATORY EVALUATION CHECKLIST
Paralytic Shellfish Poisoning-PSP (OMA 2005.06, HPLC-FLD)

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:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "1"

PART I – QUALITY ASSURANCE			
Code	REF		Item Description
			1.1 Quality Assurance (QA) Plan
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		**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 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 months of experience in laboratory sciences.
C	5		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
			1.3 Work Area
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.
			1.4 Laboratory Equipment
O	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.

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.
K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt 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, 9		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 ºC.
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	1		1.4.10 Freezer temperature is maintained at -20 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	13		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	3,4		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 2.0 mL/min, b. injection loop suitable for 25-100 µL injections, c. fluorescence detector with excitation set at 340 nm and emission at 395 nm d. a data collection system (e.g., computer, integrator) Not required by OMA2005.06, but recommended: e. solvent degasser f. refrigerated autosampler that can maintain 6 °C.
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	3		1.4.16 A solid phase extraction (SPE) vacuum manifold or automated SPE system capable of holding 3 mL cartridges is used.
O	3		1.4.17 A centrifuge capable of holding 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.**
C	3, 8		**1.5.2 Water is glass distilled or deionized and exceeds 0.5 megaohm resistance or is less than 2 µSiemens/cm conductivity at 25 ºC to be tested and recorded monthly for resistance or conductivity and the results are recorded. (Not required for bottled HPLC grade water when used immediately upon opening.)**
K	8		1.5.3 Water is analyzed for residual chlorine monthly and is at a nondetectable level (≤0.1 ppm) Results are recorded and records are maintained.

K	8		1.5.4 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	3		**1.5.5 The binary mobile phase system used to analyze PSP toxins consists of: A: 0.1 M ammonium formate in water and B: 0.1 M ammonium formate in 5% acetonitrile/95% water, both adjusted to pH 6.0.**
C	3		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: ___**
C	7		**1.5.7 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.8 All standards used are within expiration date.**
C	**7**		**1.5.9 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	3		**1.5.10 Analytical standard working stocks are made up from primary standard by dilution with deionized water.**
C	3		**1.5.11 Working standard mixes, if used, are made up from working stocks by dilution with water and are adjusted to approximately pH 4 with 0.1 M acetic acid.**
C	3		**1.5.12 Periodate oxidant is prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4 and adjusting the pH to 8.2 with 0.2 M NaOH using a pH meter.**
C	3		**1.5.13 Hydrogen peroxide (H_2O_2), 10% aqueous solution is prepared daily by mixing 1 volume of 30% H_2O_2 with 2 volumes of deionized water (1:2, v/v)**
			1.6 Collection and Transportation of Samples
O	6		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6		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		**1.6.3 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	14		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	6		**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 PSP TOXINS			
			2.1 Preparation of Sample
C	6, 1		**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).**
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		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
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	6		**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	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	3		**2.2.2 Five (5.0 ± 0.1 g) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and 3ml of 1% acetic acid is added.**
C	3, 4		**2.2.3 Sample is mixed on a vortex for 2 minutes and heated in a boiling water bath at 100 °C for 5 minutes.**
K	3		2.2.4 After boiling, sample is allowed to cool in a refrigerator or ice bath for 5 minutes, remixed on a vortex, and then centrifuged for 10 minutes at 3600 xg. Supernatant is transferred to a graduated 15 ml conical tube.
K	3		2.2.5 3 mL of 1% acetic acid solution is added to the centrifuge tube containing the solid residue. The sample is mixed with a Vortex mixer for 2 minutes and centrifuged again for 10 minutes at 3600 xg.
K	3		2.2.6 The supernatant is transferred into the same graduated conical test tube that contains the first portion of extract, and the volume is made up to 10 mL with water.
			2.3 Sample Cleanup
K	3		2.3.1 SPE C18 cartridges: Supelclean LC-18 cartridges (500 mg/3 mL volume) (Supelco) or equivalent are used.
K	3		2.3.2 The SPE cartridge is conditioned with 6 mL methanol, followed by 6 mL water.
K	3		2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.

K	3		2.3.4 The flow rate is kept between 2-3 mL/min for all elutions.
K	3		2.3.5 The supernatant is transferred into the same tube that contains the first portion of extract and the volume is made up to 10.0 mL with water.
K	3		2.3.6 SPE C18 cartridges: Supelclean LC-18 cartridges (500 mg/3 mL volume) (Supelco) or equivalent are used.
C	**3**		**2.3.7 The SPE cartridge is conditioned with 6 mL methanol, followed by 6 mL water.**
K	3		2.3.8 The cartridge is not allowed to run dry during conditioning through sample loading.
K	3		2.3.9 The flow rate is kept between 2-3 mL/min for all elutions.
C	3		2.3.10 One (1) mL of crude extract (0.5g shellfish equivalent) is loaded onto the cartridge, and effluent is collected in a 15 ml conical test tube.
C	3		2.3.11 Cartridge is washed with 2 mLs water and combined with the effluent.
K	3		2.3.12 Flow rate is kept between 2 and 3 mL/min for all elutions.
C	3		2.3.13 pH of extract is adjusted to 6.5 with 1.0 M NaOH and diluted to exactly 4.0 ml with deionized water and thoroughly mixed before withdrawing an aliquot for analysis.
K	3		2.3.14 Extracts found to contain N-1 Hydroxylated PSP analogues after C18 cleanup are fractionated using SPE-COOH ion exchange.
K	3		2.3.15 Bakerbond Carboxylic Acid silane (COOH) bonded to silica gel (500 mg/3 mL volume) (J.T. Baker) or equivalent are used.
C	3		2.3.16 The 3 mL SPE-COOH cartridge is conditioned by passing 10 mL of 0.01 M ammonium acetate through it. Keep the flow rate between 2-3 mL/min for all elutions. Do not let the cartridge run dry.
K	3		2.3.17 The cartridge is not allowed to run dry during conditioning through sample loading.
K	3		2.3.18 The flow rate is kept between 2-3 mL/min for all elutions.
C	3		**2.3.19 Fraction #1 (contains C toxins): 2 (two) ml of SPE C18 cleaned extract is passed through the COOH cartridge and collected in graduated tube marked Fraction #1. Then 4.0 mL of water is passed through the cartridge and collected into the same tube. The volume is brought to exactly 6.0 ml with water.**
C	3		**2.3.20 Fraction #2 (contains GTX 1,4; GTX2,3; B1; B2; and dcGTX 2,3): 4.0 mL of 0.05 M NaCl is passed through the same cartridge used for Fraction #1 and collected in a graduated tube marked as Fraction #2. The final volume is adjusted to 4.0 mL if necessary.**
C	3		**2.3.21 Fraction #3 (contains STX, NEO and dcSTX): 5.0ml of 0.3 M NaCl is passed through the same cartridge and collected in a graduated tube marked Fraction #3. The final volume is adjusted to 5.0 mL if necessary.**
K	3		2.3.22 If fractions need to be concentrated to achieve required sensitivity, fractions are evaporated in a 50 mL round bottom flask to approximately 1 mL on a rotary evaporator with a water bath set at 45 °C.
O	3		2.3.23 If concentrating fractions, the reduced solution is transferred to a 2 or 5 mL volumetric flask or graduated tube using a Pasteur pipet. The 50 mL round bottom flask is rinsed 3 times with 0.2-0.3 mL of water each time,

			transferring the rinse into the graduated tube. The final volume of extract is adjusted to 2.0 mL.
			2.4 Analysis
C	2		2.4.1 **A standard calibration curve (of at least six concentrations) of each toxin or mix of toxins is performed upon initial instrument set up, following any major hardware maintenance activity, changes to the analytical system, or with new standard lots. Results are recorded and records are maintained.**
K	3		2.4.2 50-100 µL of extract is injected for periodate oxidation.
K	3		2.4.3 25-50 µL of extract is injected for peroxide oxidation.
O	4		2.4.4 If a column heater is used, the temperature is maintained at 35°C during the analysis.
C	3		2.4.5 **The appropriate analytical column is used: reversed phase C18 column, 15 cm x 4.6 mm id, 5 µm particle size (Supelcosil LC-18 or equivalent).**
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 are/is used, rejection criteria are established to determine when to change the filter/guard column.
K	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.
			2.5 System Suitability
C	2		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.98 for each individual toxin.**
C	3		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of PSP toxins.**
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, 14		**2.5.4 The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.**
C	2, 14		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards based on an assessment of individual standard toxin variability. Variability in response of peaks bracketing samples must be less than 10% for calculation of toxicity in samples.**
			2.6 Calculation of Sample Toxicity
C	3		2.6.1 The toxicity of the individual toxins is calculated as follows: $$\frac{A_x \times C_s \times V_x \times D \times 100}{A_s \times M}$$ where: A_x = peak area of the toxin in the extract analyzed A_s = peak area of the nearest standard

			C_s = concentration of standard (µg/mL) **V_x = final volume of extract analyzed (mL): 4 mL for C18 SPE; 6 mL for SPE-COOH Fraction 1; 4 mL for Fraction 2; and 5 mL for Fraction 3.** **D = dilution factor (for those extracts where PSP toxins are above the highest standard and require dilution and reanalysis)** **M = amount of test portion (g) carried through cleanup procedure: 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup** **100 = factor to convert results from µg/g to µg/100 g**
C	3		**2.6.2 If both NEO and dcSTX are present, the concentration of NEO is calculated by difference in accordance with AOAC OMA 2005.06.**
C	3, 10		**2.6.3 The total toxicity in saxitoxin equivalents (STX eq) is calculated by multiplying each toxin analog concentration by its toxicity equivalent factor (TEF) as determined by Oshima (1995), corrected for its molecular weight, and summing them.** **Total toxicity in shellfish (µg STX eq/100 g) =** $$\sum TEF_i \times C_{xi}$$ **where:** **TEF_i = toxicity equivalent factor for each analog detected:** ***STX = 1; NEO = 0.88; dcSTX = 0.6; GTX1,4 = 0.63; GTX2,3 = 0.38; B1 = 0.051; C1,2 = 0.032; C3,4 = 0.022*** **C_{xi} = concentration of each toxin analog measured in the shellfish sample (µg/100g)**
C	12		**2.6.4 Any value at or above 80 µg STX equivalents/100 g of meat is actionable.**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. AOAC Official Methods of Analysis (2005). AOAC Official Method 2005.06 Paralytic Shellfish Poisoning Toxins in Shellfish. Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection.
4. European Union Reference Laboratory for Marine Biotoxins. (2020). Standard operating procedure for the analysis of paralytic shellfish toxins (PST) by precolumn HPLC-FLD according to OMA AOAC 2005.06.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 192. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Oshima, Y. (1995). Postcolumn derivation liquid chromatographic method for paralytic shellfish toxins. Journal of AOAC International, 78(2), 528–532.
11. Consult pH electrode product literature.
12. 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.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. *Compendium of Methods for the Microbiological Examination of Foods*, 3rd Edition, pg. 901.

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

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
AMNESIC SHELLFISH POISON (ASP or domoic acid) 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 ASP (domoic acid) Component

1. **Conforms Status: The ASP 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 ASP 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 ASP 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:

AOAC Official Method 2005.06
Paralytic Shellfish Poisoning Toxins in Shellfish

phic Oxidation and Liquid Chromatography vith Fluorescence Detection
First Action 2005

[Applicable to the determination of saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 2 and 3 (GTX 2, 3; together), gonyautoxins 1 and 4 (GTX 1, 4; together), decarbamoyl saxitoxin (dcSTX), B-1, C-1 and C-2 together, and C-3 and C-4 (together) in shellfish (mussels, clams, oysters and scallops). In this application, statistics were acceptable with HorRat values <2.0 observed for total paralytic shellfish poisoning (PSP). The method is applicable to quantifying saxitoxin, >22 μg/kg; GTX 2,3 together, >125 μg/kg (lowest concentration tested); B-1, >27 μg/kg; dcSTX, >8 μg/kg; NEO, >40 μg/kg; GTX 1,4 together, >50 μg/kg; C1,2 together, >93 μg/kg; and C3,4 together, >725 μg/kg.]

See Tables **2005.06A–R** for the results of the interlaboratory orting acceptance of the method.

A. Principle

Test portions are extracted by heating with acetic acid solution. Extracts are cleaned up using solid-phase exraction (SPE) C18 cartridges. After periodate and peroxide oxidation, they are analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection. Most toxins (STX, C1,2, B-1, dcSTX, and GTX2,3) can be quantified after simple SPE-C18 cleanup. Extracts containing the toxins NEO, GTX1,4, C3,4, and B-2 must be further purified by using SPE-COOH cleanup/separation. PSP toxin concentrations are calculated by comparison of peak areas or heights in the test samples with those of the standards.

B. Apparatus and Materials

(a) *LC system.*—Producing a binary gradient; equipped with an injection port capable of injecting up to 100 μL solution. Use a reversed-phase C18 column with dimensions of 15 × 4.6 mm id, 5 μm particle size (for example, Supelcosil LC-18) for separation of the toxin oxidation products. If a column of different dimensions is used, the gradient and flow rate conditions must be altered to obtain the necessary separation of the PSP oxidation products within an approximately 15 min chromatographic run. The suggested mobile phase gradient used to elute the PSP oxidation products consists of 2 mobile phases [*see Reagents* (o)] under the following conditions: 0–5% mobile phase B in the first 5 min, 5–70% B for the next 4 min and back to 0% B over the next 2 min; then at 0% B for another 3 min before the next injection.

The flow rate is 2 mL/min. For monitoring the LC effluent, a dual monochromator fluorescence detector, with excitation set to 340 nm and emission to 395 nm. The detector must be sensitive enough to produce a peak height response of at least 3 cm with a peak-to-peak baseline noise of 3 mm (10:1, signal-to-noise ratio) for an injection of 400 pg STX standard carried through the peroxide oxidation procedure under the chromatographic conditions described above.

Table 2005.06A. Interlaboratory study results for the determination of STX in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, μg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	11(1)	471.00		41.16	8.74	115.25	0.49
1	Scallop	15(0)	1048.07		229.23	21.87	641.84	1.38
2	Clams	15(0)	338.27		90.88	26.87	254.46	1.43
3	Oyster	15	ND					
4	Mussel	15	ND					
5	Clams	15(0)	522.20		69.39	13.29	194.29	0.75
5D	Clams	15(0)	517.87		73.89	14.27	206.89	0.81
6	Clams	15	ND[c]					
7	Mussel	14(0)	23.36		8.48	36.30	23.74	1.29
8	Mussel	15(0)	309.60		78.07	25.22	218.60	1.32
8D	Mussel	15(0)	315.53		70.28	22.27	196.78	1.17
9	Mussel	15(0)	132.47		33.38	25.20	93.46	1.16
10	Oyster	15(0)	136.13		46.14	33.89	129.19	1.57
10D	Oyster	15(0)	144.47		41.64	28.82	116.59	1.35
11	Oyster	15(0)	257.67		59.49	23.09	166.57	1.18
12	Mussel	15(0)	783.87		215.24	27.46	602.67	1.65
Spike 1	Mussel	15(0)	223.07	74.4	52.46	23.52	146.89	1.17
Spike 2	Mussel	15(0)	447.00	74.5	112.49	25.17	314.97	1.39
Spike 3	Mussel	15(0)	51.27	93.2	16.96	33.08	47.49	1.32
Spike 4	Mussel	15(0)	169.00	76.8	43.00	25.44	120.40	1.22
Spike 5	Mussel	11(0)	13.18		8.65	65.63	24.22	2.14

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06B. Interlaboratory study results for the determination of STX in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	S_R	RSD_R, %	R	HorRat
Practice sample	Mussel	15(0)	451.80		121.68	26.93	340.70	1.49
1	Scallop	15(0)	955.93		187.77	19.64	525.76	1.22
2	Clams	14(0)	294.57		82.80	28.11	231.84	1.46
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	15(0)	509.53		85.78	16.84	240.18	0.95
5D	Clams	15(0)	474.40		82.56	17.40	231.17	0.97
6	Clams	14	ND					
7	Mussel	12(0)	21.33		6.80	31.88	19.04	1.12
8	Mussel	15(0)	270.13		68.83	25.48	192.72	1.31
8D	Mussel	13(0)	269.08		49.81	18.51	139.47	0.95
9	Mussel	14(0)	119.64		38.16	31.90	106.85	1.45
10	Oyster	14(0)	140.00		45.24	32.31	126.67	1.50
10D	Oyster	15(0)	123.67		26.92	21.77	75.38	0.99
11	Oyster	15(0)	232.87		37.89	16.27	106.09	0.82
12	Mussel	14(0)	644.00		78.89	12.25	220.89	0.72
Spike 1	Mussel	13(0)	191.08	63.7	39.19	20.51	109.73	1.00
Spike 2	Mussel	15(0)	409.27	68.2	122.65	29.97	343.42	1.64
Spike 3	Mussel	11(0)	43.55	79.2	11.36	26.08	31.81	1.02
Spike 4	Mussel	15(0)	147.93	67.2	31.42	21.24	87.98	1.00
Spike 5	Mussel	9(0)	10.44		4.48	42.91	12.54	1.35

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

Figure **2005.06A** shows expected chromatographic responses for the toxins after periodate and peroxide oxidation. The chromatographic system must be able to separate with baseline resolution, the main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B-1 and STX, within a 15 min chromatographic run as shown in Figure **2005.06B**. A chromatography data processing workstation is recommended to analyze chromatograms and generate reports.

(**b**) *Hot plate.*

(**c**) *Vortex mixer.*

(**d**) *pH meter.*

(**e**) *Benchtop centrifuge.*—3600 × *g*.

(**f**) *Centrifuge tubes.*—Polypropylene, 50 mL.

(**g**) *Test tubes.*–Glass, graduated 5 and 15 mL.

(**h**) *SPE cartridges.*—Ensure that SPE cartridges used give minimum 80% recovery with standards and the correct elution patterns according to the directions in **F** and **G**, below.

(**i**) *C18 cartridges.*—Supelclean LC-18 cartridges (500 mg/3 mL volume; Supelco, Bellefonte, PA, USA; www.supelco.com).

(**j**) *SPE-COOH ion exchange cartridges.*—Bakerbond Carboxylic Acidsilane (COOH) bonded to silica gel (500 mg/3 mL volume; J.T. Baker, Phillipsburg, NJ, USA). Phenomenex (Torrance, CA, USA) or Isolute SPE-COOH cartridges are also suitable with the procedure.

C. Reagents

(**a**) *Analytical standards of STX, NEO, GTX1,4, GTX2,3, C1,2, B-1, and dcSTX.*—Separate solutions in 0.1 M acetic acid with concentrations ranging from 100 to 2000 µg/mL; available from National Research Council of Canada (Halifax, Canada). Dilute with water to obtain working stock solutions with concentrations ranging from 8 to 100 µg/mL. These are stable for at least a month.

Note: For convenience, standards can be combined into 3 mixtures by appropriate dilution of stock solutions in water. Those solutions must be adjusted to about pH 4 with 0.1 M acetic acid. All further dilutions should be made using 0.1 mM acetic acid, so that the solutions are kept at about pH 4. The mixtures are as follows: *Mix I.—For periodate oxidation.*—GTX1,4 and NEO; *Mix II.*—For peroxide oxidation—GTX2,3, STX, B-1, and dcSTX; *Mix III.—For periodate and peroxide oxidation.*—C1,2 and C3,4.

Figure **2005.06B** shows typical chromatograms of such mixtures.

Make dilutions with 0.1 mM acetic acid as described above. Store dilute standard solutions in plastic vials in glass containers deactivated by soaking the vials overnight in sodium hydroxide rinsed with water followed by methanol, and dried.

The oxidation products are not affected by the type of the vials; therefore, the oxidation reactions can be carried out in glass autosampler vials without deactivation.

(**b**) *Matrix modifier for periodate oxidation.*—Prepare by extracting from PSP-free oysters as in **E** and **F**. Adjust extract to pH 6.5 with 1 M NaOH and let stand 2–3 days to precipate coextracted material. Decant the supernate or filter it through a 0.45 µm Acrodisk filter and store in refrigerator when not in use. The initial PSP-free oyster extract can be stored frozen for 2 months at –20°C, but the matrix modifier must be prepared from it every

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Table 2005.06C. Interlaboraatory study results for the determination of NEO in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	10(2)	565.50		113.31	20.04	317.27	1.15
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	7(1)	33.14		12.75	38.47	35.70	1.44
5D	Clams	9(1)	40.00		17.41	43.53	48.75	1.68
6	Clams	15	ND					
8	Mussel	14(0)	283.86		91.76	32.33	256.93	1.67
8D	Mussel	14(0)	278.29		70.58	25.36	197.62	1.31
9	Mussel	13(0)	140.08		45.88	32.75	128.46	1.52
12	Mussel	15(0)	871.07		227.63	26.13	637.36	1.60
Spike 1	Mussel	14(0)	222.64	55.7	88.03	39.54	246.48	1.97
Spike 2	Mussel	15(0)	455.40	56.9	116.94	25.68	327.43	1.43
Spike 3	Mussel	14(0)	158.29[d]		39.36	24.87	110.21	1.18
Spike 4	Mussel	12(0)	213.33[e]		85.77	40.21	240.16	1.99
Spike 5	Mussel	12(0)	344.08[e]		163.71	47.58	458.39	2.53

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] NEO together with dcSTX but calculated as NEO.
[e] No NEO present in these samples. Peaks due to dcSTX but calculated as NEO.

2 weeks. Analyze the matrix modifier for PSP toxins by the periodate and peroxide oxidation to ensure absence of toxins before use.

(c) *Doubly deionized water.*

(d) *Acetic acid.*—Glacial.

(e) *Methanol.*—Analytical grade.

(f) *Acetonitrile.*—Analytical grade.

(g) *Ammonium formate.*—0.3 and 0.1 M aqueous solutions.

(h) *Ammonium acetate.*—0.01 M aqueous solution.

(i) *Sodium chloride.*—3 and 0.05 M aqueous solutions.

(j) *Sodium hydroxide.*—1 M aqueous solution.

(k) *Hydrogen peroxide solution.*—10% aqueous solution (store in refrigerator).

(l) *Disodium hydrogen phosphate (Na_2HPO_4).*—0.3 M aqueous solution.

(m) *Periodic acid solution.*—0.03 M aqueous solution (store solution in refrigerator).

(n) *Periodate oxidant.*—Prepare daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4, and adjust pH to 8.2 with 0.2 M NaOH using pH meter.

(o) *Mobile phase.*—A: 0.1 M ammonium formate; B: 0.1 M ammonium formate in 5% acetonitrile, both adjusted to pH 6 by adding 6 mL 0.1 M acetic acid.

Table 2005.06D. Interlaboratory study results for the determination of NEO in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	15(0)	642.80		147.95	23.02	414.26	1.35
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	10(0)	38.80		13.02	33.56	36.46	1.29
5D	Clams	10(0)	42.20		18.27	43.29	51.16	1.68
6	Clams	14	ND					
8	Mussel	13(1)	254.62		89.85	35.29	251.58	1.80
8D	Mussel	15(0)	266.20		92.93	34.91	260.20	1.79
9	Mussel	14(0)	97.64		46.37	47.49	129.84	2.09
12	Mussel	14(1)	842.93		199.36	23.65	558.21	1.44
Spike 1	Mussel	14(0)	212.29	53.1	68.05	32.06	190.54	1.59
Spike 2	Mussel	15(0)	492.43	61.6	159.58	32.41	446.82	1.82

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06E. Interlaboratory study results for the determination of NEO in the presence of dcSTX in spiked shellfish after SPE-COOH cleanup—Spike 3

Lab No.	NEO, µg/kg	Rec., %
2	107	53.5
6[a]	137	68.5
12[a]	154	77.2
15	86	43.0
18	75	37.5
19[a]	92	46.0
22	112	56.0
23	153	76.5
Mean	114.48	57.28
s_R		30.49
RSD_R	26.63	
R		85.37
HorRat	1.20	

[a] Calculation carried out in the coordinator's laboratory using results provided by the collaborators.

D. Preparation of Shellfish Tissue

As with the mouse bioassay [*see* 959.08 (*see* 49.10.01)], thoroughly clean outside of shellfish with fresh water, open shellfish, remove tissue, and transfer it to a homogenizer. Blend until homogeneous.

E. Extraction

In a 50 mL polypropylene centrifuge tube, mix 5.00 ± 0.10 g homogenized shellfish with 3.0 mL 1% acetic acid solution on a Vortex mixer, cap loosely (to avoid pressure buildup during heating), and place in a boiling water bath (100°C) so that contents of tube are below water line. Heat for 5 min. Do not place too many tubes in bath at once that the water bath stops boiling or temperature drops for more than 30 s. Remove from water bath, remix on a Vortex mixer, and cool by placing in a refrigerator or a beaker of cold water for 5 min. Centrifuge for 10 min at 4500 rpm (3600 × *g*) and decant the supernatant solution into a 15 mL graduated conical test tube. Add 3 mL 1% acetic acid solution to the centrifuge tube containing the solid residue, mix well on a Vortex mixer, and centrifuge again for 10 min at 4500 rpm (3600 × *g*). Collect the supernatant solution into the same graduated conical test tube that contains the first portion of extract and dilute to 10.0 mL with water.

F. SPE C18 Cleanup

Condition a 3 mL (500 mg sorbent) SPE C18 cartridge with 6 mL methanol followed by 6 mL water. Add 1 mL (0.5 g shellfish equivalent) of the crude extract to the cartridge. Keep flow rate between 2 and 3 mL/min for all elutions. Collect the effluent into a 5 mL graduated conical test tube. Do not let cartridge run dry. Wash cartridge with 2 mL water and combine washings with the effluent.

Adjust the extract to pH 6.5 with 1M NaOH using pH indicator paper (calibrated against a pH meter) and then dilute to exactly 4.0 mL with water. Use aliquots of this extract for oxidation with periodate and peroxide as described below. Also, analyze an aliquot of the extract without oxidation to verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP, and not to naturally fluorescent coextractives, by mixing a volume of extract and matrix modifier with water instead of periodate oxidant before injecting an aliquot into the LC system. The resulting chromatogram will permit identification of peaks arising from naturally fluorescent coextractives. PSP toxins do not produce peaks under these conditions.

G. SPE-COOH Cleanup

SPE-COOH ion exchange cleanup is used only for extracts that are found to contain *N*-1-hydroxylated PSP toxins after C18 cleanup. Condition a 3 mL SPE-COOH cartridge by passing 10 mL 0.01 M ammonium acetate solution through it. Keep flow rate

Table 2005.06F. Interlaboratory study results for the determination of dcSTX in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	9(0)	18.78		21.61	115.07	60.51	3.95
1	Scallop	13(0)	12.31		4.33	35.17	12.12	1.13
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	14(0)	8.14		2.71	33.29	7.59	1.01
5D	Clams	12(1)	7.42		1.83	24.66	5.12	0.74
6	Clams	15	ND					
7	Mussel	11(1)	10.55		2.02	19.15	5.66	0.60
11	Oyster	11(0)	3.45		1.63	47.25	4.56	1.26
12[d]	Mussel							
Spike 3	Mussel	15(0)	83.73	83.7	41.17	49.17	115.28	2.12
Spike 4	Mussel	15(0)	293.87	73.5	63.35	21.56	177.38	1.12
Spike 5	Mussel	14(1)	385.64	64.27	40.60	10.53	113.68	0.57

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] Not calculated. Only 7 laboratories reported numerical data; the remainder reported as not detected.

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Table 2005.06G. Interlaboratory study results for the determination of dcSTX in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	10(0)	12.60		12.38	98.25	34.66	3.18
1	Scallop	12(1)	9.88		3.89	39.37	10.89	1.23
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	11(0)	8.36		4.86	58.13	13.61	1.77
5D	Clams	10(0)	6.80		3.33	48.97	9.32	1.44
6	Clams	14	ND					
7	Mussel	13(0)	11.62		3.04	26.16	8.51	0.84
11[d]	Oyster							
12	Mussel	10(0)	7.80		3.22	41.28	9.02	1.24
Spike 3	Mussel	13(0)	69.08	69.1	17.09	24.74	47.85	1.03
Spike 4	Mussel	15(0)	276.00	69.0	52.83	19.14	147.92	0.99
Spike 5	Mussel	15(0)	396.27	66.04	93.72	23.65	262.42	1.29

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] Not calculated. Only 6 laboratories reported numerical data; the remainder reported as not detected.

between 2 and 3 mL/min for all elutions. Do not let cartridge run dry. Discard the effluent. Pass a 2 mL aliquot (0.250 g shellfish tissue equivalent) of shellfish extract from the SPE C18 cleanup through the cartridge and collect the effluent in a 15 mL graduated conical test tube marked as Fraction No. 1. Then pass 4.0 mL water through the cartridge and collect into the same tube. Adjust final volume to 6.0 mL in total. This fraction contains the C toxins. Then pass 4.0 mL 0.05 M NaCl solution through the same cartridge, and collect in a 5 mL graduated conical test tube marked as Fraction No. 2. Ensure that the final volume is 4.0 mL. This fraction contains the toxins GTX1,4, GTX2,3, B-1, B-2, and dcGTX2,3. Next, pass 5.0 mL 0.3 M NaCl solution through the cartridge and collect in a 5 mL

Table 2005.06H. Interlaboratory study results for the determination of GTX1,4 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	11(0)	1437.55		649.76	45.20	1819.33	2.98
1	Scallop	10(0)	199.30		170.10	85.35	476.28	4.19
2	Clams	11(1)	285.27		60.08	21.06	168.22	1.09
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	11(0)	61.36		28.46	46.38	79.69	1.91
5D	Clams	11(0)	62.09		17.73	28.56	49.64	1.17
6	Clams	16	ND					
7	Mussel	15(0)	1489.13[d]		417.08	28.01	1167.82	1.86
8	Mussel	12(0)	572.67		156.87	27.39	439.24	1.57
8D	Mussel	13(0)	672.23		164.05	24.40	459.34	1.44
9	Mussel	13(0)	254.46		69.57	27.34	194.80	1.39
11	Oyster	12(0)	81.00		54.86	67.73	153.61	2.90
12	Mussel	10(0)	1815.70[d]		427.69	23.56	1197.53	1.61
Spike 1	Mussel	10(0)	240.10	60.0	115.87	48.26	324.44	2.43
Spike 2	Mussel	11(0)	1440.45[d]		274.83	19.08	769.52	1.26
Spike 5	Mussel	12(1)	2236.25[d]		571.98	25.58	1601.54	1.80

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] GTX1,4 together with C3,4 but calculated as GTX1,4.

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Table 2005.06I. Interlaboratory study results for the determination of GTX1,4 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	16(0)	1279.38		316.92	24.77	887.38	1.61
1[c]	Scallop							
2	Clams	13(1)	364.23		141.21	38.77	395.39	2.08
3	Oyster	15	ND[d]					
4	Mussel	15	ND					
5	Clams	13(0)	73.54		17.18	23.36	48.10	0.99
5D	Clams	13(0)	71.46		22.60	31.63	63.28	1.33
6	Clams	16	ND					
7	Mussel	14(1)	240.07		64.50	26.87	180.60	1.35
8	Mussel	15(1)	645.00		147.94	22.94	414.23	1.34
8D	Mussel	15(1)	674.73		120.99	17.93	338.77	1.06
9	Mussel	14(0)	235.57		44.94	19.08	125.83	0.96
11	Oyster	8(1)	48.88		13.03	26.66	36.48	1.06
12	Mussel	16(0)	2079.31		394.95	18.99	1105.86	1.33
Spike 1	Mussel	14(0)	267.50	66.9	86.80	32.45	243.04	1.66
Spike 2	Mussel	15(0)	628.07	78.5	167.26	26.63	468.33	1.55
Spike 5	Mussel	14(0)	265.21[e]		68.76	25.93	192.53	1.33

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.

[c] Not calculated. Half of the laboratories reported not detected.

[d] ND = Not determined.

[e] No GTX1,4 present in this sample. Peaks due to dcGTX2,3 (confirmed by peroxide oxidation) but calculated as GTX1,4.

Table 2005.06J. Interlaboratory study results for the determination of GTX2,3 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	12(1)	3291.17		523.82	15.92	1466.70	1.19
1	Scallop	16(0)	2526.00		652.30	25.82	1826.44	1.86
2	Clams	15(1)	2537.73		479.37	18.89	1342.24	1.36
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	16(0)	119.75		21.78	18.19	60.98	0.83
5D	Clams	16(0)	113.75		24.44	21.49	68.43	0.97
6	Clams	16	ND					
7	Mussel	14(0)	348.64		92.95	26.66	260.26	1.42
8	Mussel	16(0)	813.13		252.98	31.11	708.34	1.89
8D	Mussel	16(0)	757.31		180.55	23.84	505.54	1.43
9	Mussel	16(0)	316.44		65.95	20.84	184.66	1.10
10	Oyster	16(0)	367.25		88.99	24.23	249.17	1.30
10D	Oyster	15(1)	336.20		78.60	23.38	220.08	1.24
11	Oyster	15(1)	673.00		182.03	27.05	509.68	1.59
12	Mussel	15(1)	1855.93		505.27	27.22	1414.76	1.87
Spike 1	Mussel	14(1)	227.36	75.8	42.71	18.79	119.59	0.94
Spike 2	Mussel	16(0)	529.31	88.2	167.47	31.64	468.92	1.80
Spike 5	Mussel	16(0)	954.31	79.5	293.49	30.75	821.77	1.91

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.

[c] ND = Not determined.

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Table 2005.06K. Interlaboratory study results for the determination of GTX2,3 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	15(1)	3314.33		594.49	17.94	1664.57	1.34
1	Scallop	16(0)	2347.94		522.01	22.23	1461.63	1.58
2	Clams	16(0)	2697.50		506.20	18.77	1417.36	1.36
3	Oyster	15	ND[c]					
4	Mussel	14	ND					
5	Clams	15(1)	132.00		25.33	19.19	70.92	0.88
5D	Clams	16(0)	115.94		28.43	24.52	79.60	1.11
6	Clams	15	ND					
7	Mussel	15(0)	342.80		80.23	23.40	224.64	1.25
8	Mussel	16(0)	804.63		164.42	20.43	460.38	1.24
8D	Mussel	16(0)	745.94		164.78	22.09	461.38	1.32
9	Mussel	16(0)	320.88		58.22	18.14	163.02	0.96
10	Oyster	15(1)	369.47		71.38	19.32	199.86	1.04
10D	Oyster	15(0)	341.60		86.11	25.21	241.11	1.34
11	Oyster	16(0)	701.31		203.93	29.08	571.00	1.72
12	Mussel	16(0)	1873.94		345.02	18.41	966.06	1.27
Spike 1	Mussel	15(0)	261.27	87.1	42.72	16.35	119.62	0.84
Spike 2	Mussel	16(0)	563.56	93.9	116.81	20.73	327.07	1.19
Spike 5	Mussel	16(0)	1024.88	85.4	174.72	17.05	489.22	1.07

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

Table 2005.06L. Interlaboratory study results for the determination of B-1 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	11(0)	435.36		47.84	10.99	133.95	0.61
1	Scallop	13(0)	91.08		25.12	27.58	70.34	1.20
2	Clams	11(0)	31.00		7.36	23.74	20.61	0.88
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	13(1)	42.77		8.36	19.55	23.41	0.76
5D	Clams	13(1)	40.54		7.22	17.81	20.22	0.69
6	Clams	16	ND					
7	Mussel	15(0)	145.07		27.11	18.69	75.91	0.87
8	Mussel	15(0)	328.00		47.09	14.36	131.85	0.76
8D	Mussel	15(0)	334.13		51.52	15.42	144.26	0.82
9	Mussel	15(0)	119.80		25.34	21.15	70.95	0.96
10	Oyster	12(0)	37.67		9.26	24.58	25.93	0.94
10D	Oyster	14(0)	39.57		12.97	32.78	36.32	1.26
11	Oyster	14(0)	73.14		16.47	22.52	46.12	0.95
12	Mussel	15(0)	946.87		263.88	27.87	738.86	1.73
Spike 2	Mussel	14(0)	53.36		16.01	30.00	44.83	1.21
Spike 3	Mussel	15(0)	311.13	77.8	69.98	22.49	195.94	1.18
Spike 4	Mussel	15(0)	1212.53	75.8	239.77	19.77	671.36	1.27
Spike 5	Mussel	14(1)	691.00	86.4	223.40	32.33	625.52	1.91

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06M. Interlaboratory study results for the determination of B-1 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	14(0)	349.71		58.75	16.80	164.50	0.90
1	Scallop	12(1)	71.75		13.02	18.15	36.46	0.76
2	Clams	9(0)	27.11		4.43	16.34	12.40	0.59
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	12(0)	42.00		11.07	26.36	31.00	1.02
5D	Clams	12(0)	40.75		9.73	23.88	27.24	0.92
6	Clams	14	ND					
7	Mussel	13(0)	120.85		17.71	14.65	49.59	0.67
8	Mussel	14(0)	285.79		43.73	15.30	122.44	0.79
8D	Mussel	14(0)	274.14		52.47	19.14	146.92	0.98
9	Mussel	14(0)	123.71		29.11	23.53	81.51	1.07
10	Oyster	10(0)	30.60		5.99	19.58	16.77	0.72
10D	Oyster	12(0)	37.58		10.93	29.08	30.60	1.11
11	Oyster	12(0)	63.00		11.09	17.60	31.05	0.73
12	Mussel	14(0)	784.86		73.41	9.35	205.55	0.56
Spike 2	Mussel	13(0)	46.00		8.78	19.09	24.58	0.75
Spike 3	Mussel	13(0)	292.54	73.1	43.03	14.71	120.48	0.76
Spike 4	Mussel	14(0)	1064.71	66.5	147.33	13.84	412.52	0.87
Spike 5	Mussel	15(0)	619.67	77.5	85.39	13.78	239.09	0.80

[a,b] Number of laboratories where a=number of laboratories retained after Dixon statistical outliers removed and b = number of outlier laboratories.
[c] ND = Not determined.

Table 2005.06N. Interlaboratory study results for the determination of C1,2 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	12(0)	299.00		50.81	16.99	142.27	0.89
2	Clams	13(1)	253.62		56.83	22.41	159.12	1.14
3	Oyster	16	ND[c]					
4	Mussel	15	ND					
5	Clams	16(0)	256.50		74.66	29.11	209.05	1.48
5D	Clams	15(1)	239.13		50.81	21.25	142.27	1.07
6	Clams	16	ND					
7	Mussel	15(0)	912.40		301.90	33.09	845.32	2.04
8	Mussel	12(0)	118.08		63.81	54.04	178.67	2.45
8D	Mussel	10(1)	96.30		24.10	25.03	67.48	1.10
9	Mussel	9(0)	76.56		80.34	104.9	224.95	4.46
10	Oyster	11(0)	161.91		43.73	27.01	122.44	1.28
10D	Oyster	15(0)	189.73		58.51	30.84	163.83	1.50
11	Oyster	16(0)	357.69		58.20	16.27	162.96	0.87
12	Mussel	16(0)	256.50		67.38	26.27	188.66	1.34
Spike 2	Mussel	13(1)	734.85	73.5	108.97	14.83	305.12	0.88
Spike 5	Mussel	14(1)	1566.50	78.3	227.74	14.54	637.67	0.97

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06O. Interlaboratory study results for the determination of C1,2 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	14(0)	300.93		77.77	25.84	217.76	1.35
2	Clams	14(1)	261.07		66.24	25.37	185.47	1.30
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	14(1)	237.07		64.30	27.12	180.04	1.37
5D	Clams	15(0)	238.07		80.34	33.75	224.95	1.70
6	Clams	15	ND					
7	Mussel	15(0)	983.40		255.06	25.94	714.17	1.62
8	Mussel	9(0)	108.56		40.27	37.09	112.76	1.66
8D	Mussel	10(0)	93.40		35.75	38.28	100.10	1.67
9[d]	Mussel							
10	Oyster	14(0)	204.36		57.38	28.08	160.66	1.38
10D	Oyster	14(0)	200.71		48.51	24.17	135.83	1.19
11	Oyster	15(0)	349.07		81.27	23.28	227.56	1.24
12	Mussel	12(1)	257.75		39.23	15.22	109.84	0.78
Spike 2	Mussel	14(0)	859.64	86.0	204.53	23.79	572.68	1.45
Spike 5	Mussel	15(0)	1687.13	84.4	342.42	20.30	958.78	1.37

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] Not calculated. Only 3 laboratories reported numerical data; the remainder reported as not detected.

graduated conical test tube marked as Fraction No. 3. Ensure that the final volume is 5.0 mL. This fraction contains STX, NEO, and dcSTX. Proceed with oxidation and HPLC analyses.

If problems with detector sensitivity are encountered, concentrate each fraction from the ion exchange column in 50 mL round-bottom flasks instead of graduated conical test tubes and evaporate to ca 1 mL on a rotary evaporator with a water bath set at 45°C. Transfer the solution into a 5 mL graduated conical test tube using a Pasteur pipet. Rinse the 50 mL round-bottom flask 3 times with about 0.2–0.3 mL water each time, transferring the rinse into the graduated tube so that the final volume of extract is 2.0 mL. *Note*: A graduated 2 mL volumetric flask can also be used. Analyze Fraction Nos. 1–3 by LC after periodate and peroxide oxidations as described below.

H. Oxidation Reactions

(a) *Periodate oxidation.*—Dispense all reagents and solutions used in the oxidation reactions with autopipets (Eppendorf or equivalent) with disposable plastic tips. Add a 100 µL standard solution or test extract after SPE C18 or ion exchange cleanup to 100 µL of matrix modifier solution in a 1.5 mL microcentrifuge tube (or autosampler vial, if automated chromatographic analysis will be performed). Then add 500 µL periodate oxidant and mix well on a Vortex mixer. Let solution react at room temperature for 1 min; then add 5 µL concentrated acetic acid, and mix. Let mixture stand for at least 10 min at room temperature before injecting 50–100 µL into the LC system. This solution is stable for about 1 day for the nonhydroxylated toxins. However, for NEO, B-2, and GTX1,4, a slow degradation of the oxidation products occurs (about 30% over 8 h). Quantification can still be carried out after this if the standards

Table 2005.06P. Interlaboratory study results for the determination of C3,4 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
6	Clams	15	ND					
7	Mussel	13(0)	837.15		227.24	27.14	636.27	1.65
12	Mussel	10(1)	237.50		106.35	44.78	297.78	2.25
Spike 2	Mussel	9(0)	724.78	80.5	180.88	24.96	506.46	1.49
Spike 5	Mussel	15(0)	1425.07	79.2	301.97	21.19	845.52	1.40

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06Q. Interlaboratory study results for the determination of PSP in shellfish after SPE C18 cleanup—blind duplicates statistical treatment

PSP	Youden pairs	Matrix	Labs[a,b]	Mean, µg/kg	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HorRat
STX	5 and 5D	Clams	15(0)	520.03	31.38	6.03	71.46	13.74	87.85	200.10	0.78
	8 and 8D	Mussel	15 (0)	312.57	67.80	21.69	73.23	23.43	189.84	205.05	1.23
	10 and 10D	Oyster	15(0)	140.30	24.96	17.79	43.91	31.29	69.87	122.94	1.46
NEO	8 and 8D	Mussel	13(0)	280.00	43.14	15.41	83.82	29.94	120.79	234.70	1.55
dcSTX	5 and 5D	Clams	12(0)	7.46	0.68	9.08	2.02	27.04	1.90	5.65	0.81
GTX1,4	5 and 5D	Clams	9(0)	64.61	12.85	19.89	24.13	37.35	35.98	67.57	1.55
	8 and 8D	Mussel	11(0)	601.45	119.96	19.95	155.53	25.86	335.89	435.48	1.50
GTX2,3	5 and 5D	Clams	16(0)	116.75	15.77	13.51	23.15	19.83	44.15	64.82	0.90
	8 and 8D	Mussel	16(0)	785.22	135.94	17.31	220.26	28.05	380.64	616.72	1.69
	10 and 10D	Oyster	15(0)	347.13	66.64	19.20	81.10	23.36	186.59	227.09	1.25
B-1	5 and 5D	Clams	13(0)	41.65	5.26	12.62	7.82	18.78	14.72	21.90	0.73
	8 and 8D	Mussel	15(0)	331.07	27.86	8.42	49.17	14.87	78.01	137.68	0.79
	10 and 10D	Oyster	12(0)	38.58	8.35	21.63	11.69	30.30	23.37	32.73	1.16
C1,2	5 and 5D	Clams	15(0)	241.47	37.53	15.54	53.39	22.11	105.09	149.49	1.12
	8 and 8D	Mussel	8(0)	101.00	28.62	28.34	38.38	38.00	80.14	107.46	1.68
	10 and 10D	Oyster	11(0)	168.77	53.48	31.69	53.48	31.69	149.75	149.75	1.52

[a,b] Number of laboratories: a = number of laboratories retained after Cochran statistical outliers removed; b = number of outlier laboratories.

Table 2005.06R. Interlaboratory study results for the determination of PSP in shellfish after SPE-COOH cleanup—blind duplicates statistical treatment

PSP	Youden pairs	Matrix	Labs[a,b]	Mean, µg/kg	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HorRat
STX	5 and 5D	Clams	15 (0)	491.97	43.99	8.94	85.73	17.43	123.16	240.04	0.98
	8 and 8D	Mussel	13 (0)	273.31	36.19	13.24	60.05	21.97	101.32	168.15	1.13
	10 and 10D	Oyster	14(0)	132.14	32.87	24.88	37.88	28.67	92.05	106.08	1.32
NEO	8 and 8D	Mussel	13(0)	262.65	67.73	25.79	89.95	34.24	189.65	251.79	1.75
	5 and 5D	Clams	10 (0)	40.50	7.82	19.30	15.86	39.15	21.89	44.40	1.51
dcSTX	5 and 5D	Clams	9(0)	6.89	0.88	12.80	3.15	45.76	2.47	8.83	1.35
GTX1,4	5 and 5D	Clams	12 (0)	73.92	11.10	15.02	19.36	26.19	31.09	54.20	1.11
	8 and 8D	Mussel	15 (0)	659.87	85.84	13.01	135.04	20.47	240.36	378.12	1.20
GTX2,3	5 and 5D	Clams	15(0)	124.47	19.88	15.97	28.14	22.61	55.65	78.80	1.03
	8 and 8D	Mussel	16(0)	775.28	91.07	11.75	166.54	21.48	380.64	616.72	1.69
	10 and 10D	Oyster	14(0)	357.21	84.30	23.60	84.30	23.60	236.03	236.03	1.26
B-1	5 and 5D	Clams	11(0)	40.68	5.04	12.39	10.37	25.48	14.11	29.03	0.98
	8 and 8D	Mussel	14(0)	279.96	40.58	14.49	48.02	17.15	113.62	134.44	0.89
	10 and 10D	Oyster	10(0)	35.25	8.49	24.10	9.56	27.11	23.78	26.76	1.02
C1,2	5 and 5D	Clams	14(0)	230.43	46.06	19.99	62.14	26.97	128.97	174.01	1.35
	8 and 8D	Mussel	8(0)	100.94	19.99	19.80	40.52	40.14	55.96	113.45	1.78
	10 and 10D	Oyster	14(0)	202.54	37.12	18.33	52.66	26.00	103.93	147.46	1.28

[a,b] Number of laboratories: a = number of laboratories retained after Cochran statistical outliers removed; b = number of outlier laboratories.

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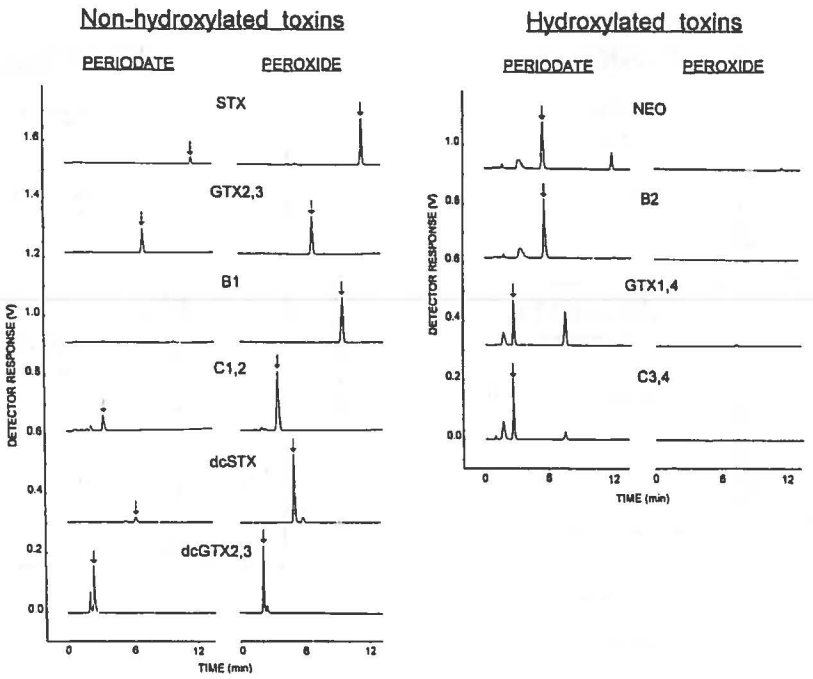


Figure 2005.06A. Chromatographic patterns showing oxidation products formed after periodate and peroxide oxidations of toxins included in this study. The same quantity of each toxin was used for each oxidation reaction. Arrows indicate peaks used for quantitation.

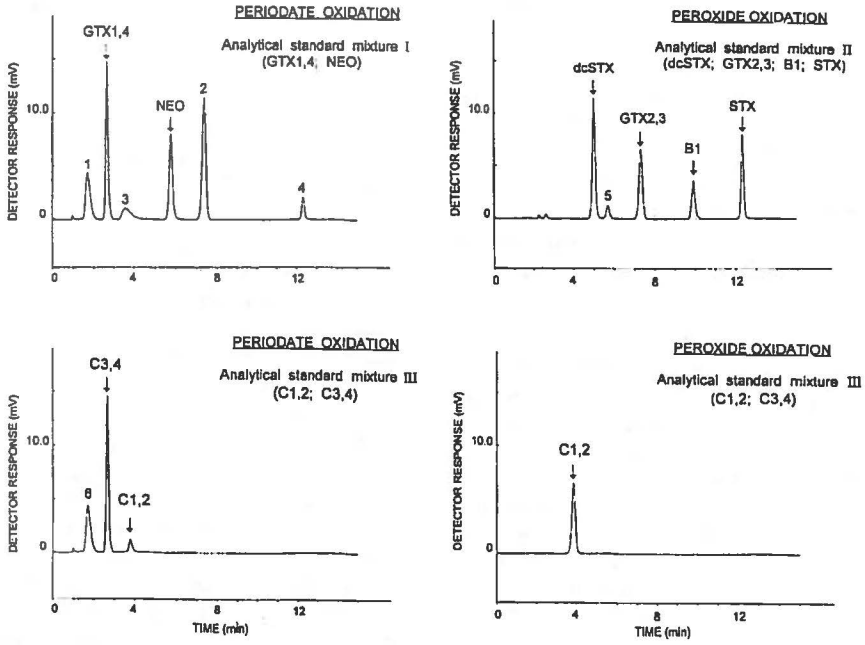


Figure 2005.06B. Typical chromatographic patterns obtained with 3 mixtures of analytical standards of PSP toxins. The hydroxylated toxins are oxidized with periodate and the nonhydroxylated toxins with peroxide. Arrows indicate peaks used for quantitation. Peaks 1 and 2 are secondary oxidation products of GTX1,4; peaks 3 and 4 are secondary oxidation products of NEO; peak 5 is a secondary oxidation product of dcSTX; and peak 6 is a secondary oxidation product of C3,4.

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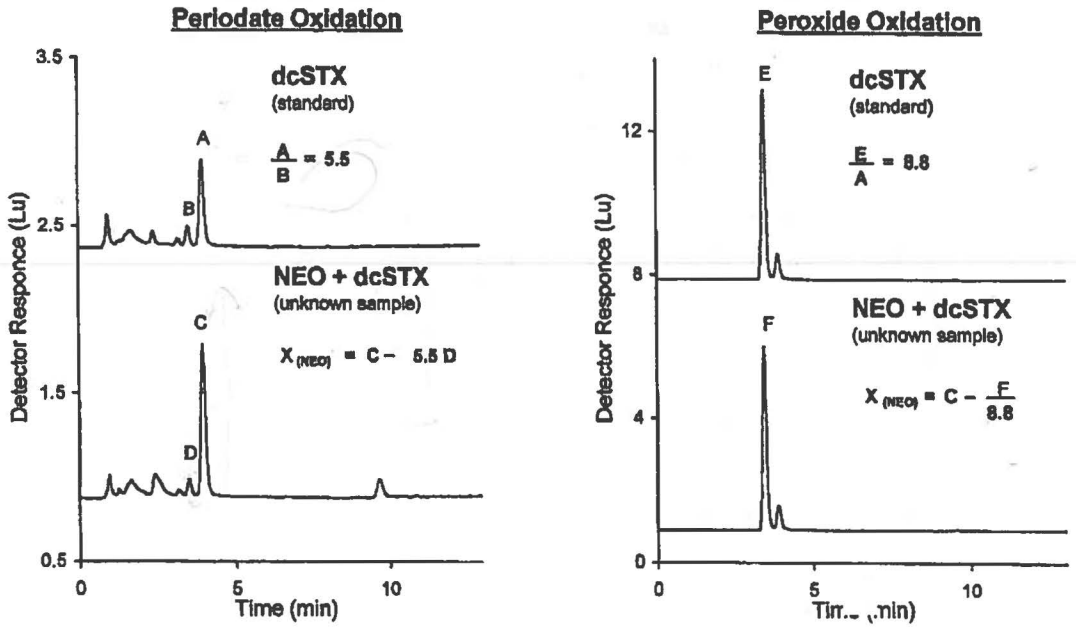


Figure 2005.06C. Chromatograms explaining the calculation of NEO in the presence of dcSTX. A, B, and E are the peak areas of dcSTX standard after periodate and peroxide oxidation; C, D, and F are the peak areas of an unknown sample contaminated with dcSTX and NEO after periodate and peroxide oxidation.

and test solutions are stored under the same conditions and analyzed together.

(**b**) *Peroxide oxidation.*—Add 25 µL 10% (w/v) aqueous H_2O_2 to 250 µL of 1M NaOH in a 1.5 mL plastic microcentrifuge tube (or autosampler vial, if automated chromatographic analysis will be performed), and mix. Then add 100 µL standard solution or test extract after SPE C18 or ion exchange cleanup. Mix and allow to react for 2 min at room temperature. Add 20 µL concentrated acetic acid and mix the solution. Inject 25–50 µL of this solution into the LC system. (*Note*: Injecting more than 50 µL may cause peak broadening.) The solution is stable for at least 8 h.

I. Calculations

Each toxin is quantitatively determined in shellfish tissue by direct comparison to analytical standards at similar concentrations as anticipated in the test portion [*see* below, section (**a**)]. For convenience, 3 analytical standard mixtures can be used for quantitating the toxins as described above.

STX, GTX2,3, B-1, and C1,2 produce single oxidation products with both oxidation reactions, while dcSTX produces 2 oxidation products with both reactions. However, NEO, B-2, GTX1,4, and C3,4 each produce 3 peaks after periodate oxidation but only the second eluting peaks are used for quantitation (Figure **2005.06B**). Because some PSP toxins (NEO and B-2; GTX1,4, and C3,4) give the same oxidation products, their quantitative determination can be made only after separation by COOH ion exchange chromatography as described above. If present in a high enough concentration, dcSTX will interfere with NEO quantification after periodate oxidation. NEO can be quantified by using mathematical ratios of peaks after periodate and peroxide oxidation as described below.

(**a**) *Calculate the concentration of PSP (in µg/kg).*—

$$\text{Toxin concentration, } \mu g/kg = \frac{Ax\ Cs\ Vx\ D\ 1000}{As\ M}$$

where Ax = peak area of the toxin in the extract analyzed; As = peak area of the nearest standard; Cs = concentration of standard (µg/mL); Vx = final volume of extract analyzed (mL); D = dilution factor [for those extracts where PSP toxins are above the most concentrated standard mixture, dilute with water after SPE cleanup, so that the toxin concentrations fall in the range of the standards, and reanalyze beginning at step **H** above (note the dilution factor)]; M = amount of test portion (g) carried through cleanup procedure, for example, 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup; 1000 = factor to convert results from µg/g to µg/kg.

(**b**) *Calculating NEO in the presence of dcSTX.*—The peak area of NEO in a test portion containing both NEO and dcSTX can be calculated in 2 ways. Figure **2005.06C** shows chromatograms after periodate and peroxide oxidation of a dcSTX standard and an unknown test portion containing a mixture of NEO and dcSTX.

Mark the peak areas with letters as follows: *dcSTX standard.*—A = second (larger) peak, periodate oxidation; B = first (smaller) peak, periodate oxidation; E = first (larger) peak, peroxide oxidation. *Unknown sample.*—C = second (larger) peak, periodate oxidation; D = first (smaller) peak, periodate oxidation; F = first (larger) peak, peroxide oxidation.

Method 1.—If the concentration of dcSTX in the test portion containing a mixture of NEO and dcSTX is significant to produce a well-integrated first peak (peak D, Figure **2005.06C**) with periodate

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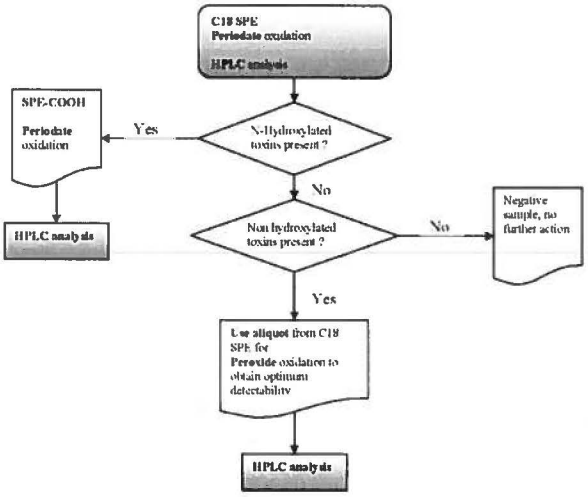


Figure 2005.06D. Schematic diagram of procedure.

oxidation, then calculate the peak area corresponding to NEO (peak $X_{(NEO)}$) from the ratio of the 2 dcSTX peak areas produced with periodate oxidation of the standard.

For the dcSTX standard analyzed with periodate oxidation, the ratio of the 2 peak areas is calculated as

$$\frac{A}{B} = \frac{X_{(dcSTX)}}{D} \Rightarrow X_{(dcSTX)} = \frac{D\,A}{B}$$

where $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - \frac{D\,A}{B}$$

Method 2.—If the concentration of dcSTX in the test portion containing NEO and dcSTX is not significant enough to produce a well-integrated first peak (peak D, Figure **2005.06C**) with periodate oxidation, then calculate the peak area corresponding to NEO (peak $X_{(NEO)}$) from the ratio of the dcSTX peak produced with periodate oxidation (peak A, Figure **2005.06C**) and the dcSTX peak area produced with peroxide oxidation (peak E, Figure **2005.06C**) in the standard.

For a dcSTX standard analyzed with periodate and peroxide oxidations, the ratio of the 2 peak areas is calculated as

$$\frac{E}{A} = \frac{F}{X_{(dcSTX)}} \Rightarrow X_{(dcSTX)} = \frac{F\,A}{E}$$

where $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - \frac{F\,A}{E}$$

J. Application of the Method for Routine Analysis

To efficiently apply this method to routine analysis where large numbers of test samples need to be analyzed, the following procedure is recommended (*see* Figure **2005.06D**). (*1*) Analyze test extracts after C18 SPE cleanup and periodate oxidation. If no peaks correspond to any of the PSP standards, the test sample is negative and no further analyses are required. (*2*) If any of the nonhydroxylated toxins, STX, dcSTX, GTX2,3, B-1, or C1,2, are present but the *N*-hydroxylated toxins, NEO, GTX1,4, and C3,4, are absent, then the nonhydroxylated toxins can be quantified by direct comparison to known standards carried through the periodate oxidation. However, the peroxide oxidation reaction is much more sensitive for B-1, C1,2, and dcSTX and would be preferred if optimum detectability is required. The peroxide oxidation reaction may be used to confirm findings of all toxins obtained by the periodate oxidation. (*3*) If both *N*-hydroxylated and nonhydroxylated toxins are present, the nonhydroxylated toxins are quantified after another aliquot of the test extract is oxidized using hydrogen peroxide, and the peaks are compared to standards similarly oxidized. The reasons for this are that with periodate oxidation some of the hydroxylated toxins produce peaks that elute with their nonhydroxylated analogues and that for some toxins (especially B-1, C1,2, and dcSTX) peroxide oxidation yields much better detectability. (*4*) For quantification of any *N*-hydroxylated toxins, another portion of the test extract is cleaned up using the SPE-COOH cartridge step, and the appropriate fraction is oxidized after periodate oxidation. This step separates C3,4 from GTX1,4 and B-2 from NEO, enabling all toxins to be quantified by direct comparison to standards carried through the same periodate oxidation procedure.

Reference: J. AOAC Int. 88, 1714(2005).

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ISSC Method Application and Single Lab Validation Summary of Required Elements 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 summary of required elements 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 in this document have been defined and accompany the summary of required elements 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 Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish; Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection
Name of the Method Developer/Submitter	Developed by James Lawrence, Barbara Niedwiadek, and Cathie Menard, Health Canada. Submitted by Leanne J. Flewelling, Florida Fish and Wildlife Conservation Commission
Developer Contact Information	100 8th Avenue SE, St. Petersburg, FL 33701 727-386-3437 leanne.flewelling@myfwc.com
Required Elements	**Brief Description**
A. Need for the New Method	
1. Clearly define the need for which the method has been developed.	Paralytic Shellfish Poisoning (PSP) occurs worldwide and poses a serious threat to shellfish consumers. AOAC Official Method 2005.06 (OMA 2005.06) was developed to provide a quantitative chemical method for determination of PSP toxins in shellfish. Currently approved methods in the NSSP include the APHA Mouse Bioassay (for all matrices covered by the NSSP), the Receptor Binding Assay (for mussels only), and the PCOX HPLC method (for mussels, clams, oysters, and scallops). Each method has its advantages and drawbacks, and NSSP laboratories benefit from choices that may be driven by access to equipment, the matrices of interest, or local PSP profiles.
2. What is the intended purpose of the method?	The intended purpose of the method is as an Approved Method of PSP testing in mussels, clams, oysters, and scallops.
3. Is there an acknowledged need for this method in the NSSP?	Yes. PSP toxin testing is required to support shellfish safety decisions under the NSSP. AOAC Official Method 2005.06 provides a validated chemical method that may be used to support regulatory monitoring and offers advantages over the MBA and RBA, including toxin-specific quantitation and reduced reliance on animal testing.
4. What type of method? i.e. chemical, molecular, culture, etc.	Chemical analytical method: High performance liquid chromatography with fluorescence detection (HPLC-FLD) following prechromatographic oxidation of PSP toxins.
B. Method Documentation	
1. Method documentation includes the following information:	

Method Title	AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish; Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection (OMA 2005.06)
Method Scope	The published AOAC method is applicable to determination of STX; NEO; GTX2,3; GTX1,4; dcSTX; B-1/GTX5; C1,2; and C3,4 in mussels, clams, oysters, and scallops.
References	AOAC International (2005). Official Method 2005.06, Paralytic Shellfish Poisoning Toxins in Shellfish; Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. Official Methods of Analysis of AOAC International. Appendix D. Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005). Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Collaborative study. Journal of AOAC International, 88, 1714-1732. Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009). Refinement and extension of AOAC Method 2005.06 to include additional toxins in mussels: Single-laboratory validation. Journal of AOAC International, 92(1), 190-207. Turner, A.D., Hatfield, R.G., Rapkova-Dhanji, M., Norton, D.M., Algoet, M., & Lees, D.N. (2010). Single-laboratory validation of a refined AOAC HPLC Method 2005.06 for oysters, cockles, and clams in UK shellfish. Journal of AOAC International, 93(5), 1482-1493.
Principle	Shellfish tissues are extracted with acetic acid. Extracts are cleaned up using SPE-C18 cartridges, with SPE-COOH fractionation applied when required for N-hydroxylated toxins. Toxins are oxidized with periodate and/or peroxide to fluorescent derivatives and analyzed by reversed-phase HPLC with fluorescence detection. Concentrations are calculated by comparison with toxin standards and summed as saxitoxin equivalents.
Any Proprietary Aspects	No proprietary method components are required.
Equipment Required	Required equipment: HPLC system with fluorescence detection, centrifuge, vortex mixer, pH meter, boiling water bath or hot plate, SPE manifold, and standard laboratory equipment. Consumables: Reversed-phase C18 column, SPE cartridges
Reagents Required	Reagents: acetic acid, ammonium formate, acetonitrile, methanol, sodium hydroxide, hydrogen peroxide, periodic acid/periodate oxidant components, sodium chloride solutions, matrix modifier, and certified PSP toxin standards.
Sample Collection, Preservation and Storage Requirements	At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection,

	shellfish should be placed in dry storage between 0- and 10-degrees C. Shellfish not shucked on the day of collection should be refrigerated. Refrigeration must not exceed 48 hours. If shellfish are refrigerated, only live animals are used in the analysis. The outside of shellfish are cleaned with fresh water. Adductor muscles are cut and the shell is opened. The inside of the shellfish is rinsed with fresh water to remove sand and other foreign material. Meats are shucked from the shells being careful not to cut or damage the body of the mollusk. Approximately 100-120 grams of meat are collected, in a single layer, on a number 10 sieve, and the sample is drained for 5 minutes. Any pieces of shell are discarded. Drained meats are blended at high speed until homogenous (60-120 seconds) and extracted for PSP toxins (see protocol in Appendix B). Samples must be processed within 24 hours of shucking.
Safety Requirements	General chemical safety practices (e.g. personal protective equipment including gloves, safety glasses and laboratory coat) must be followed.
Clear and Easy to Follow Step-by-Step Procedure	See protocol in Appendix B.
Quality Control Steps Specific for this Method	A multipoint calibration (at least 6 points) of each of the PSP analogs or standard mix solutions is performed during initial method verification and at least annually thereafter to confirm the established analytical working range and detector response for each analog. Calibration linearity R^2 must be ≥ 0.98. More frequent calibrations are performed as warranted by instrument performance, following significant service or changes to the analytical system or procedure that may affect detector response or quantitation, and with a new standard lot. Routine sample quantitation is performed using appropriate analytical standards in accordance with AOAC OMA 2005.06. The standards are analyzed at the beginning of the batch and periodically thereafter at a frequency established by the laboratory to verify stability of detector response and retention time. Additional standards are analyzed at the end of the batch. Peaks must have S/N > 10 and be above the lowest calibration curve point for quantitation. The main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B1, and STX must be separated with baseline resolution. Peak asymmetry is routinely monitored to evaluate the performance of the column. The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3. Identification retention time of sample vs standard must be within ±0.2 min.
Laboratory Evaluation Checklist for Use During Evaluations of Proper Method Implementation	See Appendix C.

C. Validation Criteria	
1. Accuracy / Trueness	Accuracy/trueness is supported by data in the AOAC collaborative study on fortified mussels (summarized in Appendix A Table A1). Published data were handled as directed in ISSC SOP VII.1 and calculated accuracies were STX 75.8%; NEO 58.5%; dcSTX 69.4%; GTX1,4 74.6%; GTX2,3 81.5%; B-1/GTX5 79.1%; C1,2 76.7%; C3,4 79.6%.
2. Measurement Uncertainty	Measurement uncertainty was not reported as a primary endpoint of the AOAC collaborative study. However, the study provides strong uncertainty-related evidence through the blind duplicates, including repeatability, reproducibility, repeatability limits, reproducibility limits, and HorRat values (results reported in Lawrence et al. 2005 Tables 2005.06Q and 2005.06R). These data are directly relevant to the precision component of measurement uncertainty. The spike recovery tables provide additional information relevant to recovery bias and interlaboratory variability in recovery (Appendix A Table A1). Additional supportive data for measurement uncertainty is provided in Turner et al. 2009 (Table 16 and Appendix A Table A3). Here method uncertainty for PSP toxins in mussel tissue was calculated using standardized uncertainty components derived from precision, recovery, and reproducibility/long-term performance. Combined expanded uncertainty (k = 2) for individual toxins ranged from 0.34 to 1.03. Turner et al. 2010 applied the same approach to oysters and hard clams. Combined expanded uncertainty (k = 2) for individual toxins ranged from 0.22 to 0.70 (Table 13 and Appendix A Table A5).
3. Precision Characteristics (repeatability and reproducibility)	The AOAC collaborative study provides interlaboratory reproducibility statistics, including RSDR and HorRat values, across multiple PSP toxins and matrices. Repeatability and reproducibility of three blind duplicate treatments are reported in Tables 2005.06Q and 2005.06R, with all HorRat values <2.0. Repeatability is further supported by Turner et al. 2009 (Tables 11 and 12 and Appendix A Table A3) for mussels and Turner et al. 2010 selected OMA-relevant data for oysters and hard clams (Appendix A Table A5).
4. Recovery	The AOAC collaborative study included fortified mussel samples and published recoveries for individual toxin analogs in the collaborative tables. Recoveries for non-hydroxylated PSP toxins using SPE C18 cleanup were STX 74.4%–93.2%; dcSTX 64.3%–83.7%; GTX2,3 75.8%–88.2%; B-1/GTX5 75.8%–86.4%; and C1,2 73.5%–78.3%. Recoveries for N-1-hydroxylated PSP toxins using SPE-COOH ion exchange cleanup were NEO 53.1%–61.6%; GTX1,4 66.9%–78.5%; and C3,4 79.2%–80.5%.
5. Specificity	Specificity was not systematically assessed in the AOAC collaborative study; however, the method uses toxin-specific oxidation chemistry, chromatographic retention time, SPE cleanup/fractionation, and comparison with standards. The method also includes analysis of unoxidized extracts to identify naturally fluorescent coextractives.

6. Working and Linear Ranges	Working and linear ranges are not defined in the AOAC collaborative study or the OMA. Turner et al. 2009 and 2010 reported that PSP toxin calibration curves were linear over the working calibration range of 0–96 µg STX eq/100 g (0 to 9.6 µg STX eq/100 g for B-1/GTX5) and 0–120 µg STX eq/100 g (0 to 12 µg STX eq/100 g for B-1/GTX5), respectively.
7. Limit of Detection	In the collaborative study, Lawrence et al. noted that the limits of detection and quantitation varied for different PSP toxins and depended on the sensitivity of the fluorescence detectors used by the participating laboratories. LOD is described by the AOAC method applicability thresholds: "The method is applicable to quantifying saxitoxin, >2.2 µg/kg; GTX 2,3 together, >12.5 µg/kg (lowest concentration tested); B-1, >2.7 µg/kg; dcSTX, >0.8 µg/100 g; NEO, >4.0 µg/100 g; GTX 1,4 together, >5.0 µg/100 g; C1,2 together, >9.3 µg/100 g; and C3,4 together, >72.5 µg/100 g." Additional information on LOD from studies by Turner et al. 2009 and 2010 is provided in Appendix A Tables A3 and A5.
8. Limit of Quantitation / Sensitivity	Limits of quantitation (LOQ) are not stated in the OMA. Information on LOQ from studies by Turner et al. 2009 and 2010 is provided in Appendix A Tables A3 and A5.
9. Ruggedness	Ruggedness was not reported in the AOAC collaborative study; however, indirect support is provided through successful multi-lab implementation. Turner et al. 2009 and 2010 evaluated ruggedness using Plackett-Burman experimental designs for multiple PSP toxins. None of the tested parameters produced a statistically significant effect on method stability, as all calculated t-test values were below the critical value. See Turner et al. 2009 (Tables 14 and 15) and 2010 (Table 3).
10. Matrix Effects	Matrix effects were not evaluated as a separate validation parameter in the AOAC collaborative study. However, the study included multiple shellfish matrices — mussels, clams, oysters, and scallops — from geographically diverse sources and with varied PSP toxin profiles. The resulting recovery, reproducibility, and HorRat data therefore provide indirect evidence that the method performed acceptably across the shellfish matrices included in the official method scope. The authors reported that collaborators did not have difficulty with the shellfish types analyzed and that the method performed satisfactorily for all sample types studied.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Collaborative study samples were analyzed by mouse bioassay. These results were in very good agreement with HPLC-FLD results over the full concentration range studied. (Lawrence et al., 2005, Table 1) Additional comparative data in support of the OMA matrices are reported in Turner et al 2009 and 2010 and by the submitting laboratory (Appendix A, Table A7 and Figure A1).

D. Other Information	
1. Cost of the Method	The cost of chemicals and consumables including certified toxin standards, C18 columns, SPE cartridges, solvents/reagents, and plastic and glass consumables to run the method is estimated at $14-$25 per sample, depending on the toxin present. Other method cost categories include equipment and equipment maintenance costs (see D3) and labor.
2. Special Technical Skills Required to Perform the Method	Analysts must be trained in marine biotoxin sample handling, shellfish tissue extraction, SPE cleanup/fractionation, oxidation chemistry, HPLC-FLD operation, chromatographic integration, toxin calibration, toxicity equivalency calculations, and quality system documentation.
3. Special Equipment Required and Associated Cost	The estimated cost for an HPLC system with fluorescence detector and autosampler is $90,000-$130,000 depending on configuration and service options. Refurbished systems may be available for less. The estimated cost for an SPE vacuum manifold system, including vacuum pump, tubing, trap, fittings, and accessories is $4,000. An automated SPE system greatly reduces the method time/labor and is estimated at $70,000-$110,000. Additional standard lab equipment needs include a centrifuge, pH meter, vortex mixer, boiling water bath, and freezer/refrigerator storage for samples and standards.
4. Abbreviations and Acronyms Defined	AOAC = AOAC INTERNATIONAL; CRM = certified reference material; dcNEO = decarbamoyl neosaxitoxin; dcSTX = decarbamoyl saxitoxin; dcGTX2,3 = decarbamoyl gonyautoxins 2 and 3; FLD = fluorescence detection; GTX = gonyautoxin; HPLC = high-performance liquid chromatography; FLD = fluorescence detection; LOD = limit of detection; LOQ = limit of quantitation; LRM = laboratory reference material; MBA = mouse bioassay; NEO = neosaxitoxin; NSSP = National Shellfish Sanitation Program; PSP = paralytic shellfish poisoning; SPE = solid-phase extraction; STX = saxitoxin; TEF = toxicity equivalency factor.
5. Details of Turn Around Times (time involved to complete the method)	The method includes tissue homogenization, acetic acid extraction, SPE cleanup. SPE fractionation as needed, oxidation, HPLC-FLD analysis, chromatographic review, quantitation, quality control review, and reporting. Turnaround time for a batch of 10-20 samples is 24-48 hours depending on the profile present and number of injections required.
6. Provide Brief Overview of the Quality Systems Used in the Lab	This information is unavailable for the collaborative study, which is the primary reference for OMA 2005.06. For supporting comparative data generated by the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute's, the HAB Biotoxin Laboratory maintains and follows a Quality Assurance Program to ensure the precision, accuracy and reliability of all toxin analyses and for the production of scientifically sound, legally defensible data. Thorough documentation and standardization of laboratory processes, procedures and activities are required. Key practices include

	the use of Standard Operating Procedures, standard methods, training, quality control, QC acceptance criteria, corrective actions, sample integrity and chain of custody, and database record keeping and tracking. All QA practices are consistent with Good Laboratory Practices and all applicable safety, environmental and legal regulations and guidelines.
Submitters Signature	Date:
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.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
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5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
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.

Appendix A: PSP AOAC OMA 2005.06 Proposal

AOAC Official Method 2005.06: Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection

AOAC Official Method (OMA) 2005.06 is a High Performance Liquid Chromatography (HPLC) with fluorescence detection (FLD) method for the quantitative determination of paralytic shellfish poisoning (PSP) toxins in shellfish following prechromatographic oxidation with periodate and peroxide. The method was validated through an AOAC collaborative study and adopted as AOAC Official Method 2005.06, First Action, in 2005. The published collaborative study included 21 shellfish test samples representing mussels, clams, oysters, and scallops, with results obtained from 18 laboratories in 14 countries. The method scope includes STX, NEO, GTX2,3, GTX1,4, dcSTX, B-1/GTX5, C1,2, and C3,4. (B-2/GTX6 was included qualitatively in the original collaborative study, and Turner et al. 2009 validated the method for additional toxins dcNEO and dcGTX2,3. These additional forms are not included in the scope of this proposal, but will be the topic of a future method extension.)

In the ISSC Constitution Bylaws and Procedures, updated July 22, 2024, Procedure XV. Procedure for the approval of analytical methods for the NSSP, section vii(a) states that: ***AOAC approved methods that have undergone the AOAC Official Methods of Analysis (OMA) or FDA Office of Foods Level 3 or 4 validations may be accepted as an NSSP method without Single Lab Validation providing the AOAC or FDA multi-laboratory validation was performed in the raw molluscan shellfish matrix for which the Conference intends it to be used and is deemed by ISSC as fit for purpose.***

The European Union (EU) integrated OMA 2005.06 into its regulatory monitoring in 2006, and in 2019, officially adopted OMA 2005.06 as the EU Reference Method for the detection of PSP toxins in shellfish. This method has been rigorously vetted and validated and has been deemed to be fit for purpose across the EU.

The validation of this HPLC-FLD method is detailed in the 2005 Journal of AOAC INTERNATIONAL article by Lawrence et al., with the foundational development published in 2001 by Lawrence and Niedzwiadek. Since 2005, numerous publications have documented method refinements, modifications to improve recovery and detection of specific toxins, and validation of additional matrices. For the purposes of this ISSC application, however, the method specifics and the requested matrix scope are limited to those presented in the AOAC Official Method.

Supportive evidence: Selected statistics and information from some subsequent studies are referenced here only as additional supportive evidence. Turner et al. (2009) provides detailed single-laboratory performance statistics for mussels and additional toxins. Turner et al. (2010) provides supplemental validation evidence for OMA-relevant oyster and hard-clam matrices from a broader non-mussel bivalve validation study. The supplemental Turner statistics support method performance characteristics but do not expand the requested scope beyond the OMA matrices. Turner et al. (2012) is referenced as supplemental matrix-effects and comparability evidence for oysters. That study investigated why LC-FLD results in oysters were higher than mouse bioassay (MB) results and concluded that the discrepancy was attributable to suppression of the MB by oyster-associated metals, especially zinc and manganese, rather than enhancement of LC-FLD fluorescence. Paired HPLC/MB data generated by the submitting lab (Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute [FWRI]) are added as laboratory-specific supplemental comparative evidence].

Published Summary Statistics Supporting ISSC Acceptance

The tables below provide published summary statistics taken from Lawrence et al. (2005), Turner et al. (2009), Turner et al. (2010), and Turner et al. (2012) sources. Supplemental statistics are summarized only where relevant to the OMA-as-published application.

Table A1. Summary of results of spiked mussels presented in Lawrence et al. (2005). For N-1-hydroxylated toxins (NEO, GTX1,4) data from SPE-COOH cleanup is presented. For non-hydroxylated toxins (all others) data from SPE C18 cleanup is presented.

Analyte	Inferred Spike Level	number of labs	Mean Recovery (%)	Recovery RSD_R (%)	HorRat
NEO	200	8	57.3	26.6	1.20
	400	14	53.1	32.1	1.59
	800	14	61.6	32.4	1.82
GTX1,4	400	14	66.9	32.4	1.66
	800	15	78.5	26.6	1.55
STX	55	15	93.2	33.1	1.32
	220	15	76.8	25.4	1.22
	300	15	74.4	23.5	1.17
	600	15	74.5	25.2	1.33
dcSTX	100	15	83.7	49.2	2.12
	400	15	73.5	21.6	1.12
	600	14	64.3	10.5	0.57
GTX2,3	300	14	75.8	18.8	0.94
	600	16	88.2	31.6	1.80
	1200	16	79.5	30.8	1.91
B1	400	15	77.8	22.5	1.18
	800	15	86.4	32.3	1.91
	1600	15	75.8	19.8	1.27
C1,2	1000	13	73.5	14.8	0.89
	2000	14	78.3	14.5	0.97

Table A2. Lawrence et al. (2005) collaborative precision from blind duplicate treatments.

Cleanup / Table	Matrices represented	RSDr range (%)	RSDR range (%)	HorRat range
SPE-C18, Table 2005.06Q	Clams, mussels, oysters	6.03-31.69	13.74-38.00	0.73-1.69
SPE-COOH, Table 2005.06R	Clams, mussels, oysters	8.94-25.79	17.15-45.76	0.89-1.78

Table A3. Turner et al. 2009 mussel single-laboratory validation statistics

Criterion	Published statistic	Source table / section
Linearity	Working calibration range 0-96 µg STX eq/100 g for most toxins; r^2 values 0.902-0.996; F-test values all below F-critical = 1.98.	Table 4
LOD/LOQ	LODs ranged from 0.3 +/- 0.1 to 16.0 +/- 4.0 µg STX eq/100 g; LOQs ranged from 0.6 +/- 0.1 to 38 +/- 9 µg STX eq/100 g.	Table 7
Recovery	Triplicate mussel homogenate spikes at 40 and 16 µg STX eq/100 g showed recoveries by toxin listed in Table A4 below.	Table 10
Repeatability, naturally contaminated mussels	Total PSP intrabatch mean RSD 8% (range 2-12%; HorRat 0.82); total PSP interbatch mean RSD 14% (range 10-22%; HorRat 0.95).	Table 12
Measurement uncertainty	Combined expanded uncertainty (k = 2) for individual toxins ranged from 0.34 to 1.03.	Table 16
Comparability	HPLC versus MB comparison for mussels: n = 40, correlation coefficient 0.93, mean HPLC-FLD/MB result 101%, ratio RSD 30%, no significant statistical difference by two-tailed t-test (t = 0.76; t-critical = 2.02).	Parallel testing / Figure 5

Table A4. Turner et al. 2009 mussel recovery by toxin

Toxin	Recovery at 40 µg STX eq/100 g, % (RSD)	Recovery at 16 µg STX eq/100 g, % (RSD)	AOAC 2005.06 interlaboratory recovery range (both SPEs), %
GTX1,4[a]	81 (4) [a]	112 (4) [a]	67-79
dcNEO	53 (4) [a]	29 (4) [a]	NA
NEO	107 (6) [a]	136 (2) [a]	53-62
dcSTX	68 (8)	85 (1)	64-84
GTX2,3	66 (9)	94 (7)	76-88
GTX5	76 (7)	82 (1)	76-86
STX	68 (5)	122 (0.2)	74-93
dcGTX2,3	70 (18)	82 (1)	NA
C1,2	63 (10)	79 (6)	74-78

[a]Using modified SPE method.

Table A5. Turner et al. 2010 selected OMA-relevant oyster and hard-clam statistics

Criterion	Pacific/native oysters	Hard clams	Source table / section
Linearity	r^2 values across listed toxins generally 0.949-0.995; F-test values below F-critical = 2.08.	r^2 values 0.984-0.994 for listed toxins; F-test values below F-critical = 2.08.	Table 4
LOD	Oysters: approximately 0.2-16 µg STX eq/100 g across toxins and oyster species.	Hard clams: predicted values <16 µg STX eq/100 g; reported estimates approximately 0.2-14.9 µg STX eq/100 g for applicable toxins.	Table 6
LOQ	Oysters: approximately 0.6-40.0 µg STX eq/100 g across toxins and oyster species.	Hard clams: many values <16 µg STX eq/100 g; highest listed values 32 µg STX eq/100 g for GTX1,4 and NEO.	Table 7
Recovery (excluding N-1-hydroxylated toxins due to SPE method modification)	Mean recoveries averaged 92% in Pacific oysters and 81% in native oysters.	Mean recovery averaged 83%.	Recovery discussion and Table 8
Short-term repeatability	Mean short-term RSD across non-mussel validation was 5.3%, with most values <10%; exceptions were mainly N-hydroxylated toxins in oysters and hard clams.	Same study-wide interpretation; hard-clam exceptions mainly N-hydroxylated toxins.	Precision discussion / Table 10
Measurement uncertainty	Standardized uncertainty values: Pacific oyster 0.14-0.34; native oyster 0.11-0.33. Expanded uncertainty (k = 2) values: Pacific oyster 0.28-0.68; native oyster 0.22-0.66.	Standardized uncertainty values: 0.11-0.35. Expanded uncertainty (k = 2) values: 0.22-0.70.	Table 13

Table A6. Turner et al. 2012 oyster matrix-effects investigation supports that oyster LC-FLD/MB discrepancies reflect MB suppression by metals, not LC-FLD fluorescence enhancement.

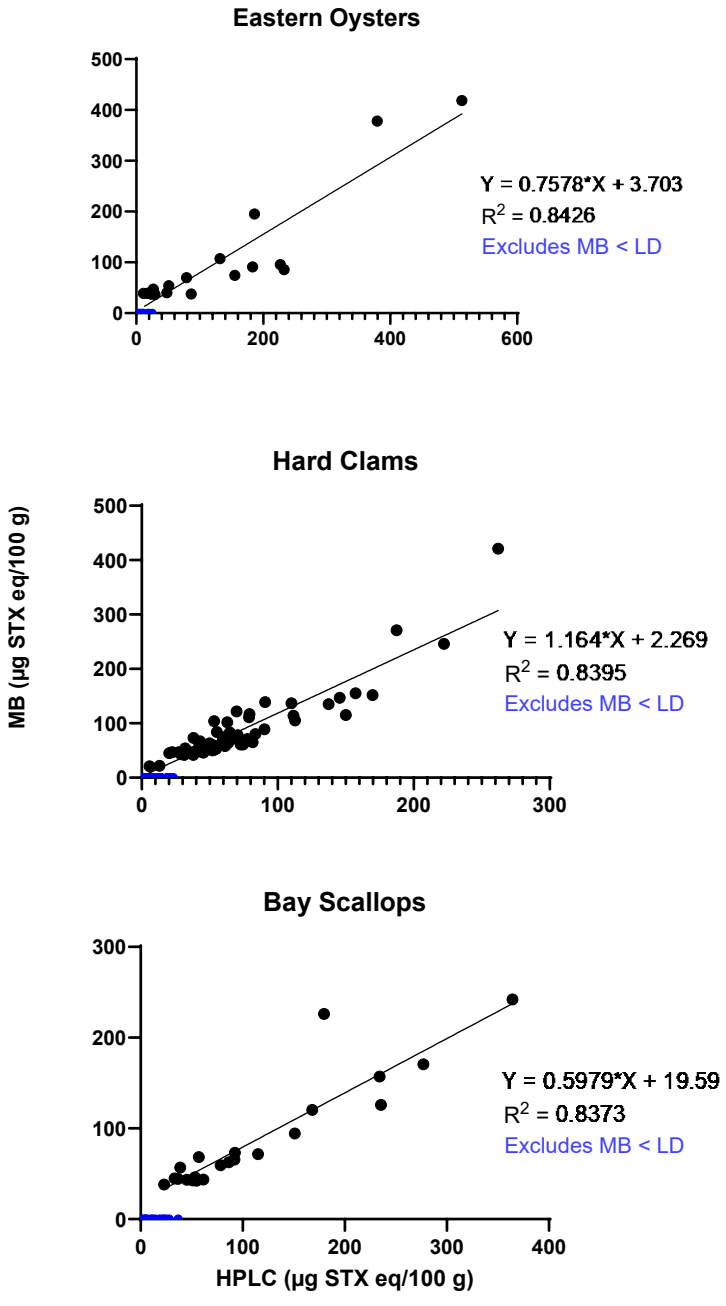
Study element	Result / interpretation
Purpose	Investigated why AOAC 2005.06 LC-FLD results in oysters were often 2-3 times higher than MB results.
Initial comparison	PSP-contaminated oyster, cockle, and mussel HCl extracts were analyzed by MB and LC-FLD; oyster samples showed the largest LC-FLD/MB differences.
Matrix component testing	Nutritional components and conductivity did not explain the discrepancy. Zinc and manganese identified as the strongest correlates with LC-FLD/MB toxicity ratio.
Correlation with metals	Correlation of LC-FLD/MB ratio with zinc was r = 0.81 and with manganese was r = 0.77; copper and cadmium correlations were lower.
Demetallation	Removing metals from oyster extracts reduced the mean LC-FLD/MB ratio from 277% in untreated extracts to 178% after pH 12 precipitation and 124% after PSA SPE demetallation.
Zinc fortification	Adding zinc to mussel and cockle extracts did not enhance LC-FLD results (LC-FLD untreated vs fortified correlation r = 0.94) but suppressed MB response; samples fortified above 180 microg/mL zinc had a mean LC-FLD/MB ratio of 306%.

Table A7. FWRI supplemental HPLC/MB comparative data

FWRI paired HPLC/MB results are included as supplemental laboratory-specific evidence. These data are not intended to replace the published validation statistics for AOAC Official Method 2005.06. Action-level agreement evaluated using 80 µg STX eq/100 g.

Matrix	n	Pearson r	Action-level agreement	HPLC >=80 / MB <80	MB >=80 / HPLC <80
Bay scallop	43	0.94	90.7%	4	0
Eastern oyster	28	0.87	92.9%	2	0
Hard clam	98	0.93	90.8%	2	7
All matrices	169	0.88	91.1%	8	7

Figure A1. FWRI supplemental HPLC/MB comparative data. Regressions exclude results where mouse bioassay was below the level of detection.



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Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection

After: AOAC International. (2005). AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish: Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection.

Principle

This method is applicable to determination of STX, NEO, GTX2,3, GTX1,4, dcSTX, B-1/GTX5, C1,2, and C3,4 in mussels, clams, oysters, and scallops. In this procedure, homogenized shellfish are extracted by heating with 1% acetic acid. Extracts are cleaned up using solid-phase extraction (SPE) C18 cartridges. Pre-chromatographic oxidation of the toxins present in extracts to fluorescent derivatives enables their detection by liquid chromatography. Extracts containing the toxins NEO, GTX1,4, and C3,4 must be further purified by using SPE-COOH cleanup/separation. PSP toxin concentrations are calculated by direct comparison with analytical standards.

Equipment and consumables required

- Electric blender
- Stainless steel No. 10 sieve
- Shucking knives
- Analytical balance
- Vortex mixer
- 2 mL volumetric flasks or graduated cylinders
- Polypropylene centrifuge tubes (graduated) with screw caps (50 and 15 mL)
- 5-10 mL graduated conical test tubes or equivalent
- Graduated cylinders (50 mL and 1-2 L)
- pH meter
- pH strips
- Boiling water bath capable of maintaining 100 °C
- Centrifuge capable of 3600 ×*g* and compatible with 50 mL tubes
- Glass beakers
- 5 mL cryovials
- Adjustable micropipettes and tips covering 5–500 µL
- Pipettes or dispensers covering 1–10 mL
- Timer
- Spatula
- Weigh boats
- Autosampler vials and inserts

- SPE C18 cartridges: Supelclean LC-18 cartridges (500 mg/3 mL volume) (Supelco) or equivalent.
- SPE COOH ion exchange cartridges: Bakerbond Carboxylic Acid silane (COOH) bonded to silica gel (500 mg/3 mL volume) (J.T. Baker) or equivalent.
- SPE manifold or automated SPE system capable of holding 3 mL cartridges.
- A high performance liquid chromatography system (HPLC) equipped with the following:
    - mobile phase system delivering a pulse-free flow of 2.0 mL/min
    - solvent degasser (recommended, not specified in OMA)
    - autosampler (preferably refrigerated to 6 °C) (recommended, not required in OMA)
    - injection loop suitable for 25-100 µL injections
    - temperature controlled column compartment capable of controlling temperature at 35 °C (recommended, not specified in OMA)
    - fluorescence detector able to achieve the required sensitivity with excitation set at 340 nm and emission at 395 nm
    - reversed phase C18 column (15 cm × 4.6 mm id, 5 µm particle size)

Optional for concentrating fractions:

- Rotary evaporator with water bath capable of maintaining 45 °C
- 50 mL round-bottom flasks
- Pasteur pipettes

Reagents

- Acetonitrile and methanol, HPLC grade.
- High-purity reagent water.
- Glacial acetic acid, analytical grade and its solutions: 1% (1 mL of glacial CH_3COOH to 100 mL with water) and 0.1 M (572 µL of glacial acetic acid to 100 mL with water).
- Sodium hydroxide (NaOH), 1 M (4 g to 100 mL with water); NaOH, 0.2 M (10 mL of 1 M NaOH diluted to 50 mL with water).
- Hydrogen peroxide (H_2O_2), 10% aqueous solution. Prepare fresh by mixing 1 volume of 30% H_2O_2 with 2 volumes of high-purity reagent water (1:2, v/v). Store the 30% H_2O_2 at 4 °C.
- Ammonium acetate, 0.01 M (0.77 g to 100 mL with water; mix to dissolve; dilute 10 mL of this solution to 100 mL with water to obtain 0.01 M).
- Sodium chloride (NaCl), 0.05 M (0.29 g to 100 mL with water) and 0.3 M (1.75 g to 100 mL with water).
- Ammonium formate, 0.3 M (4.73 g to 250 mL with water).
- Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), 0.3 M (20.1 g to 250 mL with water). For anhydrous Na_2HPO_4, use 10.65 g to 250 mL with water.
- Periodic acid, 0.03 M (1.71 g to 250 mL with water). Store at 4 °C, protected from light.
- Periodate oxidant: prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4 and adjusting the pH to 8.2 with 0.2 M NaOH using a pH meter.
- Mobile phase: Prepare as required. A: 0.1 M ammonium formate (6.31 g ammonium formate dissolved in 1 L of water) and B: 0.1 M ammonium formate in 5% acetonitrile:water (6.31 g ammonium formate dissolved in 950 mL of water and 50 mL of acetonitrile added), both adjusted to pH 6 with 0.1 M acetic acid using a pH meter.

- Analytical standards of STX, NEO, GTX1,4, GTX2,3, C1,2, B-1 (GTX5) and dcSTX can be purchased from National Research Council Canada (Store as directed).

  Recommended analytical standard working stocks: Transfer 0.5 mL from each vial into a tared 2-mL volumetric tube and record the weight. Bring final volume to 2 mL with high purity water and record the final weight. Calculate stock concentration: µM in vial x wt. of transferred solution/final weight. Record the standard lot, date diluted, and stock concentrations. Store these stock solutions in plastic or deactivated glass vials at -20 °C (or as specified in NRC documentation). These are stable for at least a month.

  For convenience, standards may be further diluted and combined into three mixtures by appropriate dilution of the working stock solutions. AOAC OMA 2005.06 does not prescribe specific concentrations for these mixed standards. Working standard concentrations of approximately 0.05–0.4 µg/mL, depending on the toxin analog and detector response, may be convenient for routine analysis. Those solutions must be adjusted to about pH 4 with 0.1 M acetic acid. All further dilutions should be made using 0.1 mM acetic acid, so that the solutions are kept at about pH 4.

  - Mixture I: GTX1,4 and NEO
  - Mixture II: GTX2,3 , B1 (GTX5), STX , and dcSTX
  - Mixture III: C1,2 and C3,4

  Individual laboratory concentration targets will vary based on detector sensitivity and can be adjusted to achieve desired range of quantitation. Select round, standard volumes to maximize pipet accuracy, and calculate true final concentrations of each solution.
- Matrix modifier: An extract prepared from blank (PSP free) oysters, which have been extracted and cleaned up on an SPE C18 cartridge as described below. The extract is adjusted to pH 6.5 with 1 M NaOH and filtered using a 0.45 µm polyvinylidene fluoride (PVDF) filter.

Shellfish preparation

1. Collect at least 12 animals and a total mass of 100-120 grams of meat per sample.
2. Immediately after collection, shuck shellfish or place in dry storage between 0 and 10 °C. Store samples in a clean, waterproof, puncture resistant container and ship overnight for processing. Time from collection to extraction initiation should not exceed 24 hours.
3. Clean the outsides of the shellfish with fresh water.
4. Cut adductor muscles and open the shell. Rinse the inside of the shellfish with fresh water to remove sand and other foreign material.
5. Collect meat, in a single layer, on a number 10 sieve, and allow the sample to drain for 5 minutes. Discard any pieces of shell.
6. Blend the drained meats at high speed until homogenous (60-120 seconds).

Sample extraction

1. In a 50 mL polypropylene centrifuge tube mix 5.00 g (± 0.10 g) of shellfish sample with 3.0 mL of 1% acetic acid solution on a Vortex mixer for 2 min (European Union Reference Laboratory for Marine Biotoxins [EURLMB], 2020). Cap it loosely (to prevent pressure build up during heating) and then place

it in a boiling water bath (100 °C) so that the contents of the tube are below the water line. Heat for 5 min. Do not place so many tubes in the bath at once that the water bath stops boiling or the temperature drops for more than 30 sec.

2. Remove sample from the water bath, remix on a Vortex mixer, and cool by placing it in a refrigerator or a beaker of cold water for 5 minutes.

3. Centrifuge for 10 minutes at 3600 ×*g* and decant the supernatant into a 15 mL graduated conical test tube.

4. Add 3 mL of 1% acetic acid solution to the centrifuge tube containing the solid residue, mix well on a Vortex mixer for 2 min, and centrifuge again for 10 minutes at 3600 x*g*. Collect the supernatant into the same graduated conical test tube that contains the first portion of extract and make up the volume to 10.0 mL with water.

SPE clean-up

Ensure that SPE cartridges used give minimum 80% recovery with standards and the correct elution pattern according to steps below.

1. Condition a 3 mL SPE C18 cartridge with 6 mL of methanol followed by 6 mL of water. Add 1 mL (0.5 g shellfish equivalent) of the above crude extract to the cartridge. Keep the flow rate between 2-3 mL/min for all elutions. Collect the effluent into a graduated conical test tube (or equivalent). Do not let the cartridge run dry. Wash the cartridge with 2 mL of water and combine the washings with the effluent.

2. Adjust the extract to pH 6.5 with 1 M NaOH using pH indicator paper (calibrated against a pH meter) and then adjust the volume to exactly 4.0 mL with water. Use aliquots of this extract for oxidation with periodate and peroxide as described below.

3. **SPE-COOH ion exchange cleanup is required only for extracts that were found to contain N-1 hydroxylated PSP analogs after C18 cleanup (but can also be used to analyze non-hydroxylated analogs with peroxide oxidation if additional clean-up is needed).** Condition a 3 mL SPE-COOH cartridge by passing 10 mL of 0.01 M ammonium acetate through it. Keep the flow rate between 2-3 mL/min for all elutions. Do not let the cartridge run dry. Discard the effluent.

4. Fraction #1: Pass a 2 mL aliquot (0.250 g shellfish tissue equivalent) of shellfish extract from the SPE C18 cleanup through the cartridge and collect the effluent in a graduated conical test tube (or equivalent) marked as Fraction #1. Then pass 4.0 mL water through the cartridge and collect into the same tube. Ensure final volume is 6.0 mL in total. This fraction contains the C toxins.

5. Fraction #2: Pass 4.0 mL of 0.05 M NaCl solution through the same cartridge, collect in a graduated conical test tube (or equivalent) marked as Fraction #2. Ensure that the final volume is 4.0 mL. This fraction contains the toxins GTX1,4, GTX2,3, B1, B2 and dcGTX2,3.

6. Fraction #3: Then pass 5.0 mL of 0.3 M NaCl solution through the cartridge and collect in a graduated conical test tube (or equivalent) marked as Fraction #3. Ensure that the final volume is 5.0 mL. This fraction contains STX, NEO, and dcSTX.

Note: If problems with detector sensitivity are encountered each fraction can be concentrated. Collect each fraction from the ion exchange column into 50 mL round bottom flasks instead of graduated conical test tubes and evaporate to approximately 1 mL on a rotary evaporator with a water bath set at 45 °C.

Transfer the solution into a 5 mL graduated conical test tube using a Pasteur pipet. Rinse the 50 mL round bottom flask 3 times with about 0.2-0.3 mL of water each time, transferring the rinse into the graduated tube just so the final volume of extract is 2.0 mL. Note: A graduated 2 mL volumetric flask can also be used. Analyze fractions #1, #2, and #3 by LC after periodate and peroxide oxidations as described below.

Pre-column oxidation

All reagents and solutions used in the oxidation reactions are dispensed using autopipettes with disposable plastic tips.

Periodate oxidation: Add a 100 µL volume of standard solution or sample extract after SPE C18 or ion exchange cleanup to 100 µL of matrix modifier solution in an autosampler vial. Add 500 µL of periodate oxidant and mix well on a Vortex mixer. Allow the solution to react at room temperature for 1 minute, then add 5 µL of concentrated acetic acid and mix. Allow the mixture to stand for 10 minutes at room temperature before injecting 50-100 µL into the LC system. *Maintain consistent injection volumes across samples and standards.*

Peroxide oxidation: Add 25 µL of 10% (w/v) aqueous H_2O_2 to 250 µL of 1 M NaOH in an autosampler vial and mix. Then add 100 µL of standard solution or sample extract after SPE C18 or ion exchange cleanup. Mix and allow to react for 2 minutes at room temperature. Add 20 µL of concentrated acetic acid and mix the solution. Inject 25-50 µL of this solution into the LC system. (Note: Injecting more than 50 µL may cause peak broadening). *Maintain consistent injection volumes across samples and standard.*

Note: Oxidation reactions may be performed manually or by an automated pre-injection procedure using an HPLC autosampler capable of programmed reagent addition, mixing, reaction timing, and injection. When automated oxidation is used, the reagent proportions, order of addition, reaction times, and quenching conditions shall be equivalent to those specified in AOAC OMA 2005.06. Standards and sample extracts shall be subjected to the same automated oxidation procedure. Performance of the automated procedure shall be demonstrated to be equivalent to the manual oxidation procedure before routine use.

Calibration Curves

Perform a multipoint calibration (at least 6 points) of each of the analogs or standard Mix solutions during initial method verification and at least annually thereafter to confirm the established analytical working range and detector response for each analog. More frequent calibration may be performed as warranted by instrument performance. A new multipoint calibration shall also be performed following significant service or changes to the analytical system or procedure that may affect detector response or quantitation or with a new standard lot.

Routine sample quantitation may be performed using appropriate analytical standards in accordance with AOAC OMA 2005.06. The standards should be analyzed at the beginning of the batch and periodically thereafter at a frequency established by the laboratory to verify stability of detector response and retention time. Additional standards should be analyzed at the end of the batch.

HPLC analysis

The column should be a reversed phase C18 with dimensions of 15 cm × 4.6 mm id, 5 µm particle size. If a column of different dimensions is used, the gradient and flow rate conditions may have to be altered to retain the necessary separation of the PSP oxidation products within an approximately 20-minute chromatographic run.

The gradient used to elute the PSP oxidation products consists of two mobile phases (see Reagents above) under the following conditions: 0 to 5% mobile phase B in the first 5 min, 5 to 70% B for the next 4 min, and back to 0% B over the last 2 min. The system is permitted to run for another 3 minutes with 100% mobile phase A before the next injection. The flow rate is 2 mL/min.

For monitoring the LC effluent, a dual monochromator fluorescence detector, with excitation set to 340 nm and emission to 395 nm, is required. The detector must be sensitive enough to produce a peak with 10:1 signal/noise ratio for an injection of 400 pg of STX standard carried through the peroxide oxidation procedure under the chromatographic conditions employed.

The chromatographic system must be able to separate with baseline resolution the main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B1, and STX within a 20-minute chromatographic run.

Column temperature is not mentioned in the OMA, but maintaining a stable column temperature ensures reproducible and reliable chromatographic separations. Recommended column temperature is 35° C (EURLMB, 2020).

Equilibrate the system for at least 20 minutes with 100% mobile phase A before a run.

Prior to injecting samples, inject Mix I (periodate oxidation) and Mix II (peroxide oxidation) in duplicate, and Mix III (both oxidations, 1 injection each) at a single concentration each to verify system performance including peak separation.

Inject the matrix modifier (carried through periodate oxidation) to identify possible interferences.

Inject C18-cleaned sample extracts (periodate oxidation). If no peaks are observed, samples are negative. If extracts are found to contain N-1 hydroxylated PSP analogs, perform SPE-COOH ion exchange cleanup as described above and inject appropriate fractions (periodate oxidation). If non-hydroxylated PSP analogs are observed or suspected, inject the C18-cleaned extracts after peroxide oxidation*.

> **If PSP profiles typically contain only non-hydroxylated analogs, peroxide oxidations may be carried out first, but all samples should be analyzed with periodate oxidation as well for documentation.*

Analyze in separate sequences extracts oxidized with periodate and peroxide and include a chemical blank (oxidize 1% acetic acid solution with peroxide and periodate) with each sequence.

Include a negative control matched matrix as a matrix blank (periodate oxidation) for each matrix analyzed.

Include a positive control analyzed as appropriate with each run.

To verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP and not to naturally fluorescent sample coextractives, a portion of each extract, after C18 cleanup, must be analyzed without oxidation. A volume of sample extract and matrix modifier are mixed with water instead of periodate oxidant before injecting it into the LC system. The resulting chromatogram will enable identification of peaks arising from naturally fluorescent coextractives. PSP toxins do not produce peaks under these conditions. These can be done at the end of the run.

Calculations

STX, GTX2,3, B-1, and C1,2 produce single oxidation products with both oxidation reactions, while dcSTX produces two oxidation products with both reactions. NEO, GTX1,4, and C3,4 each produce three peaks after periodate oxidation but only the second eluting peaks are used for quantitation (Figure 1). Because some PSP toxins (NEO and B-2; GTX1,4, and C3,4) give the same oxidation products, their quantitative determination can be made only after separation by COOH ion exchange chromatography as described above. If present in a high enough concentration, dcSTX will interfere with NEO quantification after periodate oxidation. NEO can be quantified by using mathematical ratios of peaks after periodate and peroxide oxidation as described below.

Quantitation requires a S/N >10 and is determined by direct comparison with an analytical standard at a concentration similar to that anticipated in the sample. If the toxin concentration is lower than the lowest concentration of the calibration curve for that analog, the sample is below detection limit for that analog. If it is higher than the highest concentration of the calibration curve for that analog, then do an appropriate dilution with water and rerun the sample to quantify that analog.

Concentration of individual toxin analogs in shellfish (µg/100 g) =

$$\frac{A_x \times C_s \times V_x \times D \times 100}{A_s \times M}$$

where:

A_x = peak area of the toxin in the extract analyzed

A_s = peak area of the nearest standard

C_s = concentration of standard (µg/mL)

V_x = final volume of extract analyzed (mL): 4 mL for C18 SPE; 6 mL for SPE-COOH Fraction 1; 4 mL for Fraction 2; and 5 mL for Fraction 3.

D = dilution factor (for those extracts where PSP toxins are above the highest standard and require dilution and reanalysis)

M = amount of test portion (g) carried through cleanup procedure: 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup

100 = factor to convert results from µg/g to µg/100 g

The total toxicity in saxitoxin equivalents (STX eq) is calculated by multiplying each toxin analog concentration by its toxicity equivalent factor (TEF) as determined by Oshima (1995), corrected for its molecular weight (Table 1), and summing them.

Total toxicity in shellfish (µg STX eq/100 g) =

$$\sum TEF_i \times C_{xi}$$

where:

TEF_i = toxicity equivalent factor for each analog detected:

STX = 1; NEO = 0.88; dcSTX = 0.6; GTX1,4 = 0.63; GTX2,3 = 0.38; B1 = 0.051; C1,2 = 0.032; C3,4 = 0.022

For epimeric pairs, the mean of the two TEFs is used.

C_{xi} = concentration of each toxin analog measured in the shellfish sample (µg/100 g)

Example of Calculating NEO in the Presence of dcSTX (Refer to Figure 2)

Since the second peak from dcSTX is the same oxidation product as obtained from NEO, and these toxins are both in Fraction 3 after SPE-COOH, NEO must be calculated by difference when these two toxins are present together.

The peak area of NEO in a sample containing both NEO and dcSTX can be calculated in two ways.

Figure 2 shows chromatograms after periodate and peroxide oxidation of a dcSTX standard at 0.05 µg/mL and an unknown sample containing a mixture of NEO and dcSTX.

The peak areas are as follows:

dcSTX standard	A = 5.1470
	B = 0.9335
	E = 45.230
unknown sample	C = 9.4006
	D = 0.8347
	F = 43.795

Method 1. If the concentration of dcSTX in the sample containing a mixture of NEO and dcSTX is significant enough to produce a sensitive, well-integrated first peak (Fig.2, peak D) with periodate oxidation, then you can calculate the peak area corresponding to NEO (Fig. 2, peak $X_{(NEO)}$) from the ratio of the two dcSTX peak areas produced with periodate oxidation of the standard.

For the dcSTX standard analyzed with periodate oxidation, the ratio of the two peak areas is calculated as:

$$A / B = 5.1470 / 0.9335 = 5.5 = X_{(dcSTX)} / D \quad \Rightarrow \quad X_{(dcSTX)} = 5.5D$$

where: $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - 5.5D = 9.4006 - 5.5 \times 0.8347 = 4.8098$$

Method 2. If the concentration of dcSTX in the sample containing NEO and dcSTX is not significant enough to produce a well-integrated first peak (Fig, 2, peak D) with periodate oxidation, then you can calculate the peak area corresponding to NEO (Fig. 2, peak $X_{(NEO)}$) from the ratio of the dcSTX peak produced with periodate oxidation (Fig.2, peak A) and the dcSTX peak area produced with peroxide oxidation (Fig.2, peak E) in the standard.

For a dcSTX standard analyzed with periodate and peroxide oxidations, the ratio of the two peak areas is calculated as:

$$E / A = 45.230 / 5.1470 = 8.8 = F / X_{(dcSTX)} \quad \Rightarrow \quad X_{(dcSTX)} = F / 8.8$$

where: $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - F / 8.8 = 9.4006 - 43.795 / 8.8 = 4.4239$$

Method 1 yields a peak area estimation of 4.81 for NEO while method 2 yields a value of 4.42. The results differ by about 10%, which is within the experimental error of the entire method. The regression equation of the NEO calibration curve can then be used to calculate the concentration of NEO in the oxidized sample or fraction.

Quality Control Criteria

1. Only certified reference materials are used for standard solutions.
2. Calibration linearity $R^2 \geq 0.98$
3. 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.
4. Peak must have S/N > 10 and be above the lowest calibration curve point for quantitation.
5. The main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B1, and STX must be separated with baseline resolution.
6. Peaks asymmetry is routinely monitored to evaluate the performance of the column.
7. The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.
8. Identification retention time of sample vs standard must be within ±0.2 min.

References

AOAC International. (2005). AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish: Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. In Official Methods of Analysis of AOAC International. Gaithersburg, MD: AOAC International.
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Figure 1 . PSP toxin peaks using periodate oxidation and peroxide oxidation. Arrows indicate peaks used for quantification.

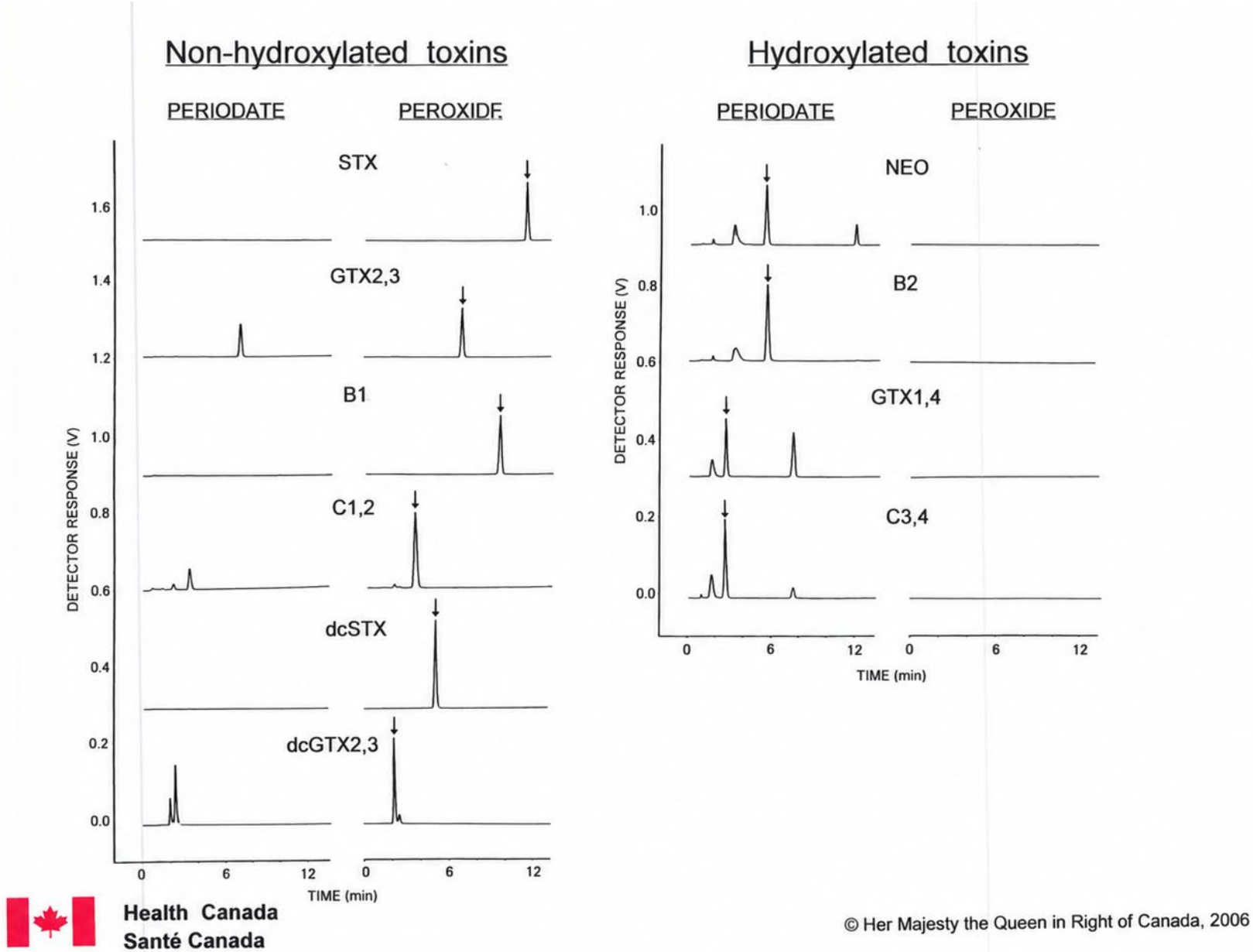
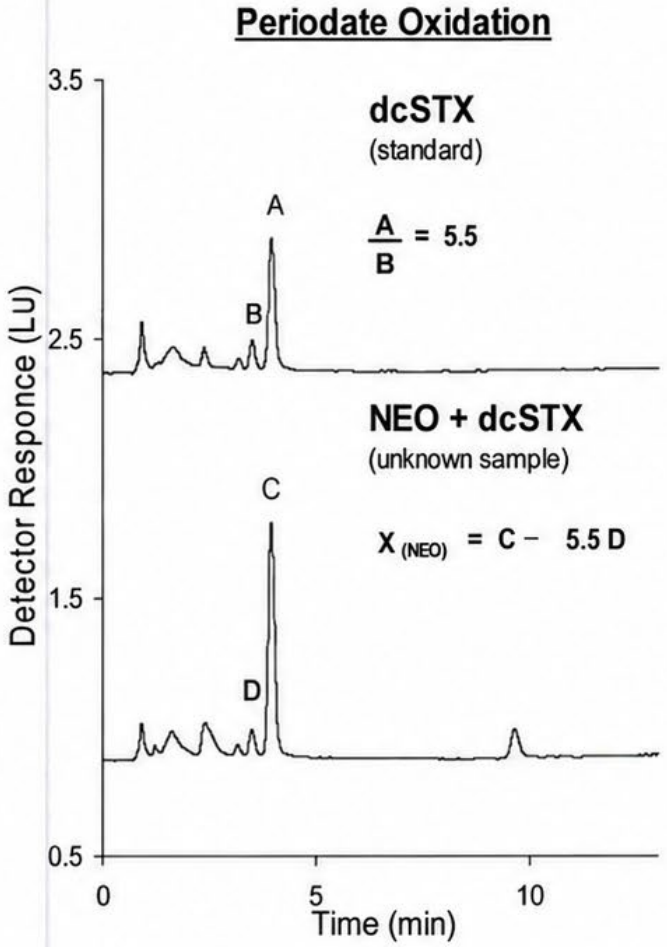
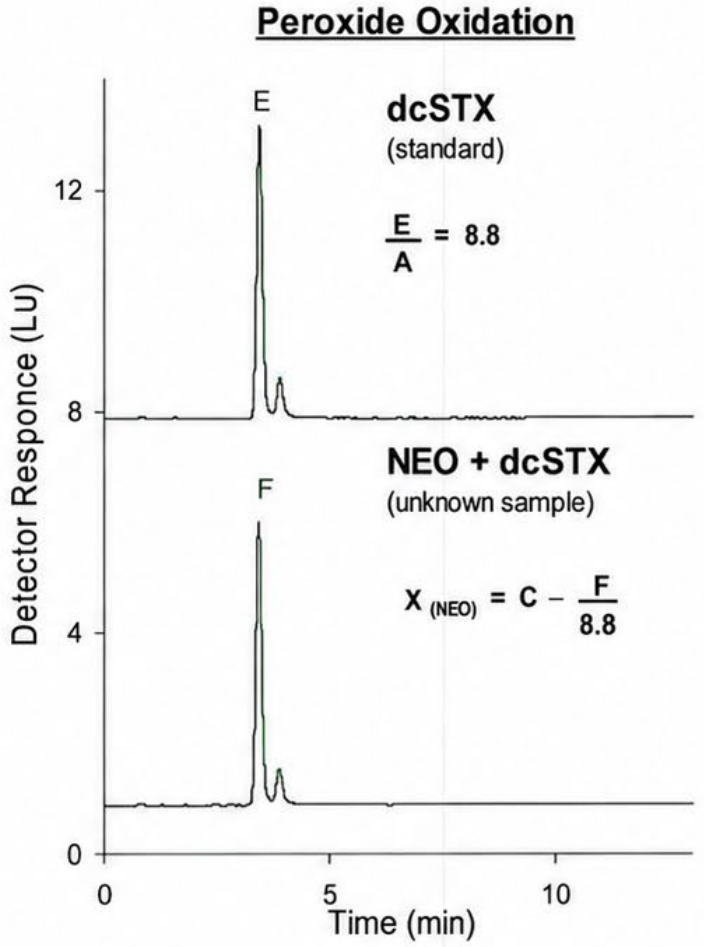


Figure 2: For use in example of calculating NEO in the presence of dcSTX.





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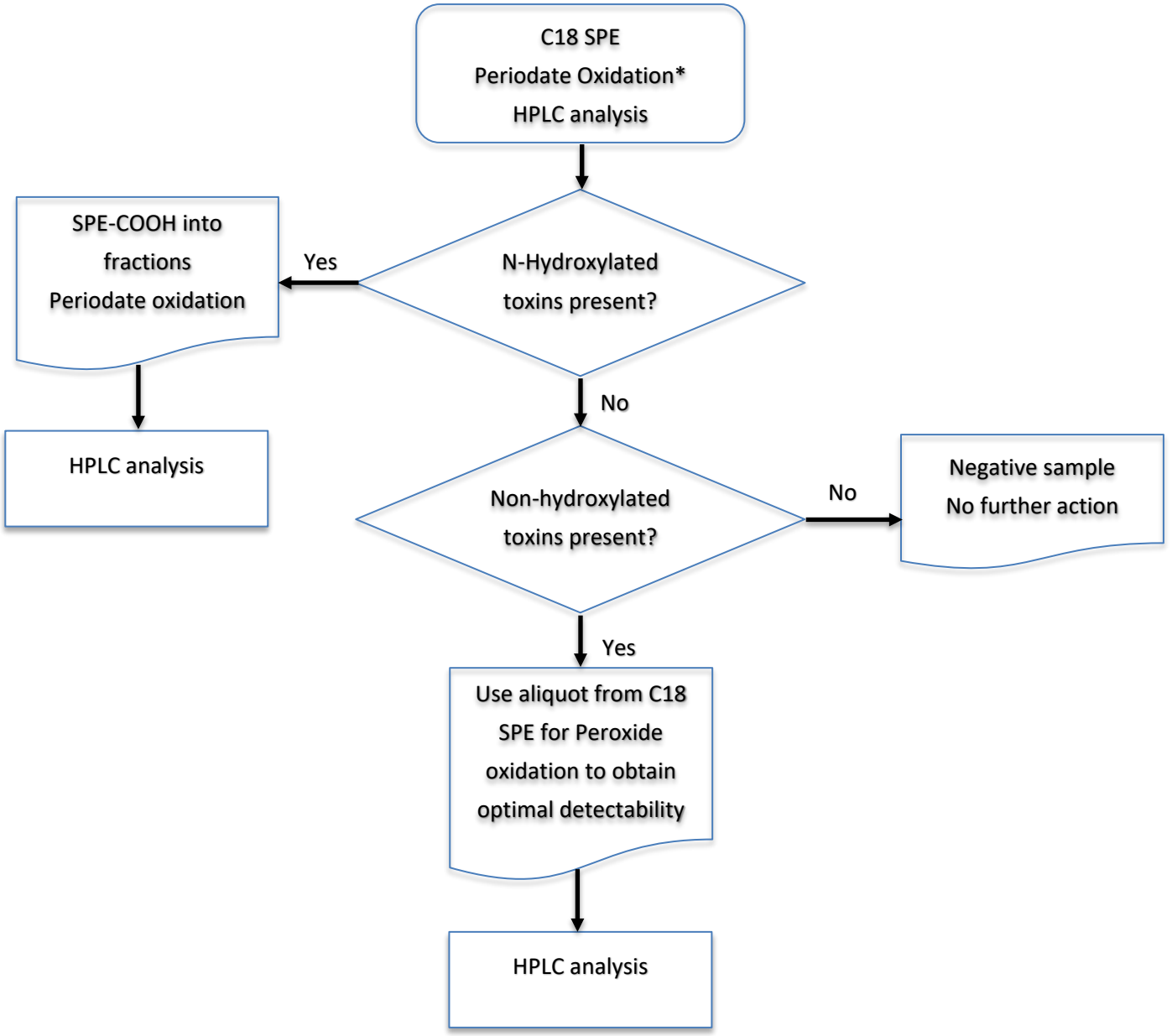
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Table 1. Toxicity of PSP toxin analogs relative to STX

	MU/µmol*	Relative toxicity	Molecular weight	MU/µg	Relative toxicity corrected for molecular weight
B1/GTX5	160	6.4%	379.4	0.42	5.08%
C1	15	0.6%	475.4	0.03	0.38%
C2	239	9.6%	475.4	0.50	6.06%
C3	33	1.3%	491.4	0.07	0.81%
C4	143	5.8%	491.4	0.29	3.51%
dcGTX2	1617	65.1%	352.3	4.59	55.3%
dcGTX3	1872	75.4%	352.3	5.31	64.1%
dcSTX	1274	51.3%	256.26	4.97	59.9%
GTX1	2468	99.4%	411.4	6.00	72.3%
GTX2	892	35.9%	395.35	2.26	27.2%
GTX3	1584	63.8%	395.35	4.01	48.3%
GTX4	1803	72.6%	411.4	4.38	52.8%
neoSTX	2295	92.4%	315.29	7.28	87.7%
STX	2483	100.0%	299.29	8.30	100%

*Oshima, Y. (1995). Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. Journal of AOAC International, 78(2), 528–532.

Recommended Method Procedure



**If PSP profiles typically contain only non-hydroxylated toxins, peroxide oxidations may be carried out first for efficiency, but all samples should be analyzed with periodate oxidation as well for documentation.*