Proposal for Task Force Consideration at the 2009 Biennial Meeting Interstate Shellfish Sanitation Conference	☒ **Growing Area** ☐ **Harvesting/Handling/Distribution** ☐ **Administrative**
Name of Submitter:	Jeffrey van de Riet
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Proposal Subject:	Method for the Determination of Paralytic Shellfish Toxins (PST) in Shellfish
Specific NSSP Guide Reference:	Section IV Guidance Documents, Chapter II Growing Areas, .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods.
Text of Proposal/ Requested Action	I am submitting for your review and consideration a method for the determination of Paralytic Shellfish Toxins (PST) in shellfish. This method provides an alternative to the current AOAC methods of analysis for the determination of PST in shellfish that is sensitive, robust and accurate. This post-column oxidation (PCOX) method was developed to provide a rapid, high throughput chemical assay for PST which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection. The shellfish tissues are blended with dilute acid, heated, and the supernatant is purified. The PST are separated chromatographically using ion pair chromatography and oxidized to a fluorescent derivative post column using a periodic acid, phosphate oxidant. The derivatized toxins are monitored using fluorescence detection. The method has been validated following guidelines recommended by the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods. Results were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06). The method development and single laboratory validation studies have been peer reviewed and accepted for publication in the Journal of the AOAC International. The PCOX method is simple, robust and provides repeatable precise and accurate results. I would like the Laboratory Methods Review Committee to approve the PCOX method as a suitable National Shellfish Sanitation Program laboratory test for the analysis of Paralytic Shellfish Toxins in shellfish.
Public Health Significance:	The method was developed to provide a rapid, high throughput chemical assay for Paralytic Shellfish Toxins (PST) which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection. There is a worldwide move to replace assays which use live animals as test subjects.
Cost Information (if available):	Total consumable costs for the analysis is estimated at $10/sample. A chemistry laboratory will usually be equipped with an LC system and will only require a post column system to be equipped to carry out the analysis at a cost of approximately $20,000. Total capital costs for the instrumentation required for the analysis is approximately $100.000.

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June 12, 2009

Laboratory Methods Review Committee
INTERSTATE SHELLFISH SANITATION CONFERENCE
209-2 Dawson Road
Columbia, SC 29223

Dear Colleagues;

I am submitting for your review and consideration a method for the determination of Paralytic Shellfish Toxins (PST) in shellfish. This method provides an alternative to the current AOAC methods of analysis for the determination of PST in shellfish that is sensitive, robust and accurate.

This post-column oxidation (PCOX) method was developed to provide a rapid, high throughput chemical assay for PST which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection. The shellfish tissues are blended with dilute acid, heated, and the supernatant is purified. The PST are separated chromatographically using ion pair chromatography and oxidized to a fluorescent derivative post column using a periodic acid, phosphate oxidant. The derivatized toxins are monitored using fluorescence detection. The method has been validated following guidelines recommended by the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods. Results were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06). The method development and single laboratory validation studies have been peer reviewed and accepted for publication in the Journal of the AOAC International.

The PCOX method is simple, robust and provides repeatable precise and accurate results. I would like the Laboratory Methods Review Committee to approve the PCOX method as a suitable National Shellfish Sanitation Program laboratory test for the analysis of Paralytic Shellfish Toxins in shellfish. If you require further information or have questions please contact me, my contact information is included below;

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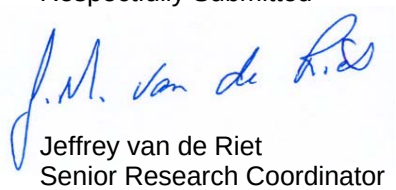
Respectfully Submitted



Jeffrey van de Riet
Senior Research Coordinator

Canada

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

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

Name of the New Method	Rapid Post-column Oxidation Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters and Scallops.
Name of the Method Developer	Jeffrey van de Riet- Senior Research Coordinator, Dartmouth Laboratory
Developer Contact Information	1992 Agency Drive Dartmouth, Nova Scotia Canada B3B 1Y9

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	The method was developed to provide a rapid, high throughput chemical assay for Paralytic Shellfish Toxins (PST) which would eliminate the need to sacrifice animals, using the AOAC mouse bioassay (MBA), for toxin detection.
2. What is the intended purpose of the method?	Y	This method is validated for the determination of PST in mussels, clams, oysters and scallops. The method provides an alternative methodology to the AOAC MBA for the analysis of PST in shellfish.
3. Is there an acknowledged need for this method in the NSSP?	Y	There is a worldwide move to replace assays which use live animals as test subjects.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Chemical. The PST are separated chromatographically using ion pair chromatography. The separated toxins are then oxidized to a fluorescent derivative post column using a periodic acid, phosphate oxidant. The derivatized toxins are monitored using fluorescence detection.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Rapid Post-Column Oxidation Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters and Scallops.
Method Scope	Y	This method is validated for the determination of Paralytic Shellfish toxins (PST) in mussels, clams, oysters and scallops.
References	Y	Rourke, W.A., Murphy, C.J., Pitcher, G., van de Riet, J.M., Burns, B.G., Thomas, K.M., Quilliam, M.A. (2008) J.AOAC Int 91(3), 589-597. van de Riet, J.M., Gibbs, R.S., Chou, F.W., Muggah, P.M., Rourke, W.A., Burns, B.G., Thomas, K. and Quilliam, M.A. (2009) J.AOAC Int, In Press. Additional references are included with the SOP in Appendix II
Principle	Y	The PST are extracted from the edible portion of molluscs by heating with dilute acid for 5 minutes in a boiling water bath. The deproteinized supernatant is adjusted to pH-4. The toxins are separated using ion pair chromatography and are oxidized post column to produce purines by breakage of a C4-C12 bond in a complex 3-ring structure characteristic of PSP toxins. The resulting products monitored with fluorescent detection.
Any Proprietary Aspects	N	None

Equipment Required	Y	- Liquid Chromatograph with a solvent selection valve, column switching valve, and fluorescence detector - Two post column pumps and a heater capable of maintaining 85 C - 1 mL reaction coil and miscellaneous PEEK tubing - General laboratory apparatus A detailed list of the required equipment can be found in the attached SOP
Reagents Required	Y	A detailed list of the required reagents can be found in the attached SOP, Appendix II.
Sample Collection, Preservation and Storage Requirements	Y	A detailed SOP, Appendix II is attached and includes all steps on the sample collection, preservation and storage requirements..
Safety Requirements	Y	All safety precautions are laid out in the method protocol.
Clear and Easy to Follow Step-by-Step Procedure	Y	A detailed SOP is attached and includes all steps on the sample analysis procedure. See Appendix II
Quality Control Steps Specific for this Method	Y	-Full Instrument calibration curve is analysed weekly -Calibration checks are run within each batch of injections after every 20 injections. - QC and recovery sample is analysed with each batch of extracts
C. Validation Criteria		
1. Accuracy / Trueness	Y	Accuracy/Trueness was assessed by recovery experiments, as recommended in Section A4.3.4 of the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods. Results were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06).
2. Measurement Uncertainty	Y	- The combined Measurement Uncertainty for the four matrices was determined to be 0.16 at the regulatory limit
3. Precision Characteristics (repeatability and reproducibility)	Y	- Repeatability and Reproducibility (Intermediate Precision) results are summarized in Appendix I Tables 1-3
4. Recovery	Y	- Recovery for the method ranged from 94 to 106 % over the three levels and 4 matrices. The data are summarized in Appendix I Table 4
5. Specificity	Y	- Specificity of the LC method is increased due to a number of characteristics of the method over the MBA. Summary of the specificity comparison to the AOAC MBA is found in Appendix I Table 8.
6. Working and Linear Ranges	Y	- The method has been validated at 0.4., 0.8 and 1.6 mg STX•diHCl eq/ kg (40, 80 and 160 ug STX•diHCl eq/100g). The linear range of the method is greater with an upper limit in excess of 2000 ug STX•diHCl eq/100g. A summary of the estimated linear range of the individual toxins is shown in Appendix I Table 7.
7. Limit of Detection	Y	Appendix I Table 5 summarizes the estimated limits of detection and quantitation for the individual PST according to the validated species.
8. Limit of Quantitation / Sensitivity	Y	Appendix I Table 5 summarizes the estimated limits of detection and quantitation for the individual PST according to the validated species.
9. Ruggedness	Y	A ruggedness study was conducted and the factors investigated had no observable effect. The studied factors are shown in Appendix I Table 6
10 Matrix Effects	Y	The validation data have demonstrated that the method is 'blind' to the matrix.
11 Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	Comparison of the PCOX method to the AOAC MBA, Lawrence and Oshima methods of analysis are shown in Appendix I Figure 1 to 4.
D. Other Information		
1. Cost of the Method	Y	The cost of consumables in the method is less than $10 per sample
2. Special Technical Skills Required to Perform the Method	Y	Competence in the operation and maintenance of a basic Liquid Chromatographic system.
3. Special Equipment Required and Associated Cost	Y	- Liquid Chromatograph- Isocratic LC with a solvent selection valve or binary or quaternary system with a fluorescence detector- $50000-100,000 CAN - Post-column derivitization system- $25000 CAN
4. Abbreviations and Acronyms Defined	Y	A detailed SOP is attached and includes all various abbreviations and acronyms used in the procedure..
5. Details of Turn Around Times (time involved to complete the method)	Y	A single LC system has the capacity to analyse 24 samples/24 hour period. If the analysis of C-toxins is not required capacity is

		50 samples/24 hour period.
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	CFIA laboratories are accredited to ISO 17025 by the Standards Council of Canada and maintain an internal QA system consistent with the IUPAC Harmonized Guidelines for Internal Quality Control in Analytical Laboratories (Pure & Applied Chemistry, **67**: 649-666 (1995).
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:

13. 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.
14. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.

15. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Analytical Methods for Trace-Level Concentrations of Organic Chemicals.
16. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
17. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
18. 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 I Validation Data.

Accuracy and Trueness

Currently there are no materials available that are considered as Certified Reference Materials for PST. Analytical standards were obtained from NRCC, with supporting documentation. Accuracy/Trueness was assessed by recovery experiments, as recommended in Section A4.3.4 of the IUPAC Harmonized Guidelines for Single-laboratory Validation of Analytical Methods, Pure & Applied Chemistry, **74**: 835-855 (2002). The recoveries obtained by this methodology are shown in Table 4. Results from samples analysed by this method were also compared to those obtained using the AOAC MBA Method and those obtained using the AOAC pre-column oxidation method (AOAC Official Method 2005.06). Comparison to a reference method is also recommended in the IUPAC guideline cited above. The comparisons of the PCOX method to the AOAC and other methods of analysis are shown in Figures 1 to 4.

Repeatability and Intermediate Precision

Materials for the repeatability and intermediate precision were prepared by blending of blank materials (mussels, clams, scallops or oysters, respectively) with a highly contaminated mussel material. The materials were blended using a ratio of 1 part contaminated mussel to 100, 50 or 25 parts each of the respective blank study matrices to achieve concentrations that result in a total toxicity equivalent to ½ MRL, MRL and 2 MRL (0.40, 0.80, 1.60 µg STX•diHCl eq/kg) for each of the four matrix materials, as described above. The materials were extracted and analyzed according to the method, as described. The concentration of each detected toxin was determined and corrected for the method dilution. The repeatability is determined by conducting 5 replicate analyses, repeated over three days, for a total of 15 determinations for each matrix at each concentration.

The intermediate precision (repeatability) was determined on the same materials as were used for the repeatability. Sufficient material was preserved to allow for a second analyst to extract and analyse the same tissues on a second instrument. The second analyst reproduced the work conducted previously by the first analyst, by conducting analyses of 5 replicates for each matrix and concentration on each of 3 days, for a total of 15 determinations for each test material by each analyst. The data is summarized in Tables 1-3.

Table 1. Repeatability for Gonyautoxins and Saxitoxins

	Matrix	Average Concentration of PST (3 days, 5 replicates/day n=15 mg STX eq/kg ± %RSD)						
		Total	**GTX4**	**GTX1**	**GTX3**	**GTX2**	**NEO**	**STX**
0.40 mg/kg	Clams	0.42 ± 2.2%	**0.016 ± 17%**	**0.051 ± 7.7%**	0.050 ± 6.6%	0.067 ± 3.9%	0.065 ± 9%	0.17 ± 3.2%
	Mussels	0.41 ± 6%	**0.019 ± 16%**	**0.049 ± 24%**	0.051 ± 2.2%	0.061 ± 15%	0.063 ± 8.4%	0.17 ± 5.5%
	Scallops	0.45 ± 3.5%	**0.021 ± 16%**	**0.048 ± 10%**	0.060 ± 2.7%	0.081 ± 3.7%	0.061 ± 8.6%	0.18 ± 4.5%
	Oysters	0.38 ± 7.2%	**0.017 ± 48%**	**0.072 ± 35%**	0.047 ± 2.8%	0.066 ± 2.7%	0.050 ± 8.8%	0.13 ± 11%
0.80 mg/kg	Clams	0.83 ± 2.2%	**0.032 ± 4.6%**	0.099 ± 6.9%	0.099 ± 2%	0.13 ± 2.9%	0.13 ± 7.4%	0.35 ± 3.1%
	Mussels	0.79 ± 3.9%	**0.040 ± 5.8%**	0.097 ± 3.7%	0.096 ± 1.7%	0.12 ± 8.3%	0.12 ± 4.9%	0.32 ± 4.2%
	Scallops	0.84 ± 1.9%	**0.032 ± 10%**	0.090 ± 5.5%	0.11 ± 1.3%	0.14 ± 2.3%	0.11 ± 5.7%	0.35 ± 2.9%
	Oysters	0.67 ± 3.7%	**0.029 ± 34%**	0.11 ± 17%	0.089 ± 3.9%	0.12 ± 4.1%	0.082 ± 8.7%	0.24 ± 17%
1.60 mg/kg	Clams	1.660 ± 2%	0.065 ± 6%	0.20 ± 6.2%	0.20 ± 1.1%	0.26 ± 1.5%	0.24 ± 2.9%	0.69 ± 3.4%
	Mussels	1.650 ± 3.1%	0.064 ± 4.4%	0.20 ± 2%	0.20 ± 1.7%	0.26 ± 4.8%	0.25 ± 6.5%	0.68 ± 3.6%
	Scallops	1.670 ± 2%	0.063 ± 6.4%	0.19 ± 3.5%	0.21 ± 2%	0.26 ± 3.4%	0.22 ± 9%	0.71 ± 2.6%
	Oysters	1.380 ± 6.2%	0.063 ± 25%	0.20 ± 11%	0.18 ± 3.9%	0.24 ± 6.3%	0.18 ± 11%	0.51 ± 13%

Values in **BOLD and Underlined** are below the LOQ for one of the matricies tested
Materials were pooled tissues, analysed in replicate (5 reps/day), repeated on three days (n=15)

The repeatability of the method for the N-sulfocarbamoyl-gonyautoxin C1 and C2 was a challenge as these toxins are not prevalent in materials that were available for use in this study. For this reason the repeatability for the C1 and C2 toxins was only determined in a single material at a single concentration. The analysis of this material was replicated (5 times) each day and repeated over 3 three days. The concentrations of the toxins were calculated and the results are summarized in Table 2.

Table 2. Repeatability for C-toxins

Day	Replicate	C-1	C-2	Total
1	1	0.021	0.17	0.21
	2	0.022	0.17	0.21
	3	0.022	0.17	0.21
	4	0.022	0.18	0.22
	5	0.022	0.17	0.21
	Average	0.020	0.170	0.210
	STD Dev	0.0004	0.0016	0.0033
	RSD	1.8%	0.9%	1.6%
2	1	0.023	0.17	0.21
	2	0.023	0.17	0.22
	3	0.023	0.17	0.21
	4	0.024	0.17	0.23
	5	0.023	0.17	0.22
	Average	0.020	0.170	0.220
	STD Dev	0.0005	0.0030	0.0058
	RSD	2.3%	1.8%	2.7%
3	1	0.021	0.16	0.22
	2	0.022	0.16	0.22
	3	0.023	0.16	0.22
	4	0.022	0.16	0.22
	5	0.022	0.16	0.22
	Average	0.020	0.160	0.220
	STD Dev	0.0004	0.0010	0.0030
	RSD	2.2%	0.6%	1.4%
Combined	Average	0.02	0.17	0.22
	SD	0.0	0.0	0.0
	%RSD	3.1%	2.7%	2.4%

The relative standard deviation under repeatability conditions for the toxins that were present in the samples above the limit of quantitation was below 13% in all cases. This is within the acceptable range as indicated by AOAC International. The relative standard deviation for the C-toxins as determined is below 5% and is within the acceptable range as indicated by the AOAC. The repeatability for all toxins in all matrices were relatively consistent. The one exception was STX in oysters, which was observed to show the greatest variation but is within acceptable ranges.

Table 3. Intermediate Precision (within-lab reproducibility) of the PCOX method for the analysis of PST in shellfish.

	Matrix	Analyst	3-day Average Concentration of PST (n=15, mg STX eq/kg ± %RSD)						
			Total	**GTX4**	**GTX1**	**GTX3**	**GTX2**	**NEO**	**STX**
0.40 mg/kg	Clams	1	0.42 ± 2.2%	**0.016 ± 17%**	**0.051 ± 7.7%**	0.050 ± 6.6%	0.067 ± 3.9%	0.065 ± 9.0%	0.17 ± 3.2%
		2	0.44 ± 3.0%	**0.022 ± 32%**	**0.075 ± 9.6%**	0.050 ± 3.9%	0.055 ± 9.6%	0.058 ± 10%	0.17 ± 4.0%
		Avg.	0.43 ± 3.0%	**0.019 ± 32%**	**0.063 ± 22%**	0.050 ± 5.3%	0.061 ± 12%	0.062 ± 11%	0.17 ± 3.6%
		HorRat	**0.17**	**1.12**	**0.90**	**0.21**	**0.51**	**0.45**	**0.18**
	Mussels	1	0.41 ± 6.0%	**0.019 ± 16%**	**0.049 ± 24%**	0.051 ± 2.2%	0.061 ± 15%	0.063 ± 8.4%	0.17 ± 5.5%
		2	0.38 ± 0.1%	**0.03 ± 0.3%**	**0.052 ± 0.2%**	0.052 ± 0.03%	0.040 ± 0.1%	0.063 ± 0.2%	0.15 ± 0.04%
		Avg.	0.39 ± 7.5%	**0.22 ± 27%**	**0.049 ± 22%**	0.050 ± 4.0%	0.052 ± 22%	0.061 ± 13%	0.16 ± 8.2%
		HorRat	**0.41**	**0.95**	**0.87**	**0.16**	**0.90**	**0.55**	**0.39**
	Scallops	1	0.45 ± 3.5%	**0.021 ± 16%**	**0.048 ± 10%**	0.060 ± 2.7%	0.081 ± 3.7%	0.061 ± 8.6%	0.18 ± 4.5%
		2	0.48 ± 5.5%	**0.14 ± 98%**	**0.064 ± 16%**	0.067 ± 11%	0.084 ± 5.9%	0.063 ± 19%	0.19 ± 3.6%
		Avg.	0.46 ± 5.7%	**0.015 ± 60%**	**0.056 ± 20%**	0.064 ± 10%	0.083 ± 5.3%	0.062 ± 15%	0.18 ± 5.3%
		HorRat	**0.32**	**2.00**	**0.81**	**0.41**	**0.23**	**0.61**	**0.26**
	Oysters	1	0.38 ± 7.2%	**0.017 ± 48\%**	**0.072 ± 35%**	0.047 ± 2.8%	0.066 ± 2.7%	0.050 ± 8.8%	0.13 ± 11%
		2	0.37 ± 3.9%	**0.015 ± 47%**	**0.049 ± 9.8%**	0.047 ± 4.4%	0.064 ± 9.4%	0.051 ± 5.5%	0.15 ± 3.1%
		Avg.	0.38 ± 5.8%	**0.016 ± 48%**	**0.060 ± 35%**	0.047 ± 3.8%	0.065 ± 7.0%	0.050 ± 7.2%	0.14 ± 10%
		HorRat	**0.32**	**1.62**	**1.47**	**0.15**	**0.29**	**0.29**	**0.48**
0.80 mg/kg	Clams	1	0.83 ± 2.2%	**0.032 ± 4.6%**	0.099 ± 6.9%	0.099 ± 2.0%	0.13 ± 2.9%	0.13 ± 7.4%	0.35 ± 3.1%
		2	0.84 ± 2.8%	**0.038 ± 12%**	0.13 ± 7.4%	0.10 ± 3.8%	0.12 ± 7.6%	0.11 ± 4.3%	0.34 ± 4.6%
		Avg.	0.84 ± 2.5%	**0.03.5 ± 13%**	0.11 ± 15%	0.10 ± 3.3%	0.12 ± 7.3%	0.12 ± 9.7%	0.34 ± 4.0%
		HorRat	**0.15**	**0.50**	**0.69**	**0.15**	**0.34**	**0.44**	**0.21**
	Mussels	1	0.79 ± 3.9%	**0.040 ± 58%**	0.097 ± 3.7%	0.096 ± 1.7%	0.12 ± 8.3%	0.12 ± 4.9%	0.32 ± 4.2%
		2	0.70 ± 3.8%	**0.039 ± 17%**	0.089 ± 8.3%	0.094 ± 4.4%	0.093 ± 3.3%	0.099 ± 9.0%	0.28 ± 2.9%
		Avg.	0.74 ± 6.9%	**0.40 ± 43%**	0.093 ± 7.3%	0.095 ± 3.5%	0.11 ± 15%	0.11 ± 11%	0.30 ± 7.0%
		HorRat	**0.41**	**1.66**	**0.32**	**0.15**	**0.67**	**0.51**	**0.37**
	Scallops	1	0.84 ± 1.9%	**0.032 ± 10%**	0.090 ± 5.5%	0.11 ± 1.3%	0.14 ± 2.3%	0.11 ± 5.7%	0.35 ± 2.9%
		2	0.93 ± 1.7%	**0.031 ± 26%**	0.11 ± 12%	0.13 ± 8.8%	0.16 ± 2.2%	0.12 ± 4.6%	0.39 ± 2.5%
		Avg.	0.88 ± 5.7%	**0.031 ± 20%**	0.10 ± 14%	0.12 ± 9.8%	0.15 ± 5.6%	0.11± 5.5%	0.37 ± 5.6%
		HorRat	**0.35**	**0.73**	**0.64**	**0.45**	**0.26**	**0.25**	**0.31**
	Oysters	1	0.67 ± 3.7%	**0.029 ± 33%**	0.11 ± 17%	0.089 ± 3.9%	0.12 ± 4.1%	0.082 ± 8.7%	0.24 ± 17%
		2	0.68 ± 2.6%	**0.029 ± 40%**	0.087 ± 3.6%	0.086 ± 2.5%	0.12 ± 3.9%	0.10± 15%	0.27 ± 2.0%
		Avg.	0.68 ± 3.2%	**0.029 ± 37%**	0.099 ± 19%	0.087 ± 3.6%	0.12 ± 4.0%	0.092 ± 16%	0.26 ± 12%
		HorRat	**0.19**	**1.35**	**0.83**	**0.16**	**0.18**	**0.73**	**0.61**
1.60 mg/kg	Clams	1	1.66 ± 2.0%	0.065 ± 6.0%	0.20 ± 6.2%	0.20 ± 1.1%	0.26 ± 1.5%	0.24 ± 2.9%	0.69 ± 3.4%
		2	1.69 ± 1.9%	0.069 ± 5.9%	0.24 ± 5.9%	0.20 ± 3.0%	0.26 ± 6.1%	0.22± 3.4%	0.69 ± 3.2%
		Avg.	1.67 ± 2.0%	0.067 ± 6.7%	0.22 ± 12%	0.20 ± 2.5%	0.26 ± 4.5%	0.23 ± 6.2%	69 ± 3.3%
		HorRat	**0.14**	**0.28**	**0.61**	**0.13**	**0.23**	**0.31**	**0.19**
	Mussels	1	1.65 ± 3.1%	0.064 ± 4.4%	0.20 ± 2.0%	0.20 ± 1.7%	0.26 ± 4.8%	0.25 ± 6.5%	68 ± 3.6%
		2	1.56 ± 2.4%	0.076 ± 14%	0.21 ± 7.1%	0.20 ± 3.5%	0.22 ± 1.9%	0.22 ± 6.1%	61 ± 2.0%
		Avg.	1.60 ± 4.1%	0.070 ± 13%	0.21 ± 6.4%	0.20 ± 2.7%	0.24 ± 8.1%	0.24 ± 8.8%	0.64 ± 6.0%
		HorRat	**0.28**	**0.57**	**0.32**	**0.13**	**0.41**	**0.45**	**0.35**
	Scallops	1	1.67 ± 2.0%	0.063 ± 6.4%	0.19 ± 3.5%	0.21 ± 2.0%	0.26 ± 3.4%	0.22± 9.0%	0.71 ± 2.6%
		2	1.81 ± 2.9%	0.060 ± 21%	0.226 ± 12%	0.24 ± 7.1%	0.30 ± 2.4%	0.22 ± 7.5%	0.76 ± 3.2%
		Avg.	1.74 ± 4.8%	0.061 ± 15%	0.20 ± 12%	0.23 ± 7.7%	0.28 ± 7.4%	0.22 ± 8.3%	0.74 ± 4.6%
		HorRat	**0.33**	**0.64**	**0.59**	**0.39**	**0.38**	**0.42**	**0.28**
	Oysters	1	1.38 ± 6.2%	0.063 ± 25%	0.20 ± 12%	0.18 ± 3.9%	0.24 ± 6.3%	0.18 ± 11%	0.51 ± 13%
		2	1.42 ± 1.4%	0.058 ± 18%	0.18 ± 3.2%	0.18 ± 1.3%	0.25 ± 3.6%	0.19 ± 2.5%	0.56 ± 1.7%
		Avg.	1.40 ± 4.6%	0.061 ± 22%	0.19 ± 10%	0.18 ± 3.0%	0.24 ± 5.3%	0.18 ± 8.0%	0.53 ± 9.6%
		HorRat	**0.30**	**0.92**	**0.51**	**0.15**	**0.27**	**0.39**	**0.55**

Values in **Bold and Underlined** are below the LOQ for one of the matricies tested
Materials were pooled tissues, analysed in replicate (5 reps/day), repeated on three days (n=15) by each analyst

As this is a report on single laboratory validation, true between-lab reproducibility can not be determined; however, within-lab intermediate precision was determined for this method. A second analyst analysed the same materials that were employed for the repeatability study on a separate instrument. These data were compiled and a Horwitz ratio (HorRat) was determined for each analyte concentration studied in each matrix. These data are summarized in Table 3. Ideal values for HorRat are between 0.3 and 1.2, and for this method all values are all below 1.0. A number of results are below 0.3, which is an indication of analyst bias. The analysts in question had some indication of the expected levels of the toxins in the tissues and this knowledge may have lead to results that were unexpectedly close in agreement resulting in lower than ideal HorRat values. In addition, both analysts, trained by the method developers, have a consistent approach to integration and interpretation of the chromatograms. These two factors may be expected to lead to analyst bias regardless of the proper intentions of each analyst and will therefore result in artificially low HorRat values. A true indication of the method repeatability will be determined by an inter-laboratory or full collaborative study at a later date.

Recovery

The method recovery, defined as the fraction or percentage of an analyte recovered following extraction and analysis of a blank sample to which the analyte has been added at a known concentration, was determined in mussels, clams, scallops and oysters, at concentrations designed to result in a total toxicity equivalent to ½ MRL, MRL and 2 MRL (0.40, 0.80, 1.60 mg STX•diHCl eq/kg). Recovery experiments were carried out at three levels for each species of shellfish, with the determinations being replicated (5 times) on each day and repeated over 3 three days, for a total of 15 determinations for each matrix at each concentration. Pre-weighed portions of blank tissue were fortified with aliquots (125, 250 or 500 µL) of a mixture of PST to achieve the desired concentrations of analytes in tissues. The fortification solution by the extraction and dilution of highly contaminated tissue and subsequent fortification with other PST standards to obtain a solution containing the most common and most toxic PST analogues available. The materials were extracted and analyzed according to the method, as described. The concentration of each detected toxin was determined and converted to STX equivalents, the total toxicity value was calculated by combining the STX equivalents of the individual toxins. The fortification solution was calibrated against the matrix fortified analytical standard and the concentration of each toxin was determined. The recovery was calculated by evaluating the amount of each toxin found as well as the total toxins recovered from the sample against the amount of toxins added. Table 4 summarizes the results obtained from the recovery experiments.

Table 4. Recovery of PST using the CFIA PCOX methodology.

Spike Level	Matrix		Percent Recovery (3 days, 5 replicates/day n=15)										
			Total	**GTX4**	**GTX1**	**dcGTX3**	**dcGTX2**	**GTX3**	**GTX2**	**NEO**	**STX**	**C-1**	**C-2**
Toxin Concentration mg/kg			**0.44**	**0.013**	**0.058**	**0.000**	**0.000**	**0.039**	**0.076**	**0.069**	**0.181**	**0.000**	**0.000**
0.4 mg/kg	Clams	Avg.	99	100	101	ND	ND	103	105	103	102	ND	ND
		SD	7	13	10			9	13	13	6		
	Mussels	Avg.	102	113	110	ND	ND	114	106	104	113	ND	ND
		SD	1	22	11			15	5	9	6		
	Scallops	Avg.	94	132	105	ND	ND	105	103	76	101	ND	ND
		SD	9	63	13			7	10	7	8		
	Oysters	Avg.	97	104	137	ND	ND	103	104	97	88	ND	ND
		SD	4	15	38			4	7	5	11		
Toxin Concentration mg/kg			**0.88**	**0.030**	**0.014**	**0.020**	**0.006**	**0.079**	**0.17**	**0.12**	**0.38**	**0.0002**	**0.0010**
0.8 mg/kg	Clams	Avg.	104	109	102	ND	ND	106	109	101	106	ND	ND
		SD	12	12	6			7	13	13	9		
	Mussels	Avg.	106	100	107	107	121	115	107	103	111	101	91
		SD	2	10	6	13	20	15	3	4	4	11	13
	Scallops	Avg.	97	138	107	131	70	108	107	75	105	ND	ND
		SD	8	14	9	10	29	7	9	5	9		
	Oysters	Avg.	106	90	126	ND	ND	110	116	99	92	127	105
		SD	5	21	16			4	10	3	9	40	34
Toxin Concentration mg/kg			**1.76**	**0.047**	**0.020**	**0.004**	**0.009**	**0.149**	**0.30**	**0.22**	**0.68**	**0.0003**	**0.0002**
1.6 mg/kg	Clams	Avg.	102	103	99	128	159	103	107	98	104	88	ND
		SD	7	9	5	6	47	5	10	11	7	20	
	Mussels	Avg.	106	103	104	105	119	115	108	105	110	98	90
		SD	2	14	3	9	20	16	3	3	4	13	8
	Scallops	Avg.	99	132	105	117	82	106	104	75	104	147	103
		SD	7	13	5	9	17	6	8	4	8	34	23
	Oysters	Avg.	104	104	114	151	186	107	109	99	89	126	98
		SD	3	18	7	14	26	3	3	5	7	42	27

Values in **Bold and Underlined** are below the LOQ for one of the matricies tested
Materials were pooled tissues, analysed in replicate (5 reps/day), repeated on three days (n=15)

At the concentrations evaluated in this study, an IAEA/FAO/IUPAC/AOAC expert consultation report on single laboratory validation recommended that an acceptable recovery range was 70-100%. The average total toxicity recoveries ranged from 94 to 106 % for the three levels studied. Individual toxin recoveries were between 90-110 %. The recovery of NEO in Scallops was determined to be approximately 75% which was significantly lower than the recovery of any of the other toxins. While this recovery is lower than expected, it still falls within the acceptable range specified by the expert consultation report and has been shown to be consistent between various analysts. The fortification levels for some of the toxins were below the LOQ for those compounds at one or more of the fortification levels. For this reason the recoveries for these toxins are higher than would be ideal (greater than 110%) and the RSDs are large. For toxins fortified at levels above the LOQ the RSDs were generally below 15%. Statistical analysis shows that although the toxin recovery from the various matrices differs, the recoveries are within acceptable values.

Limit of Determination and Quantification

The limit of determination (LOD), the lowest concentration of analyte that can be detected and limit of quantification (LOQ), the lowest concentration of analyte that can be quantified, are determined for each matrix by analysis of five replicate extracts of blank matrix, repeated over 6 days (n=30). The baseline signal to noise ratio was determined at the approximate retention time for all toxins. This noiise response (height units) was multiplied by a factor of 3 and converted to µmoles of toxin using the response from the working standard. The amount of toxin was corrected for method dilution and toxicity (relative to STX) to result in an LOD expressed as; mg STX•diHCl eg/kg for each toxin. The LOQ for the method was calculated by multiplying the LOD by a factor of 3.

Table 5. Estimated Limits of Detection and Quantitation for the individual PST in the validated matrices.

Toxin	Clams		Mussels		Scallops		Oysters	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
GTX4	0.0120	0.036	0.0160	0.048	0.016	0.048	0.026	0.078
GTX1	0.0210	0.063	0.0240	0.072	0.024	0.072	0.037	0.111
dcGTX3	0.0025	0.008	0.0008	0.002	0.018	0.054	0.003	0.008
GTX5	0.0060	0.018	0.0032	0.010	0.007	0.021	0.008	0.024
dcGTX2	0.0070	0.021	0.0021	0.006	0.005	0.014	0.007	0.021
GTX3	0.0025	0.008	0.0012	0.004	0.003	0.008	0.003	0.009
GTX2	0.0310	0.093	0.0220	0.066	0.024	0.072	0.029	0.087
NEO	0.0250	0.075	0.0240	0.007	0.024	0.072	0.026	0.078
dcSTX	0.0096	0.029	0.0077	0.023	0.008	0.023	0.010	0.029
STX	0.0170	0.051	0.0130	0.039	0.013	0.039	0.014	0.042
C-1	0.0004	0.001	0.0002	0.001	0.001	0.003	0.000	0.001
C-2	0.0008	0.002	0.0008	0.002	0.001	0.004	0.009	0.028
Total	0.135	0.404	0.115	0.345	0.143	0.430	0.172	0.515

LOD = 3 xS/N
LOQ= 3 x LOD

While the total toxicity LOD and LOQ are calculated by summing the LOD or LOQ for each toxin, this assumes the presence of all toxins are at the respective limit. In reality, as the toxicity increases from zero, one will see one or two of the more predominant toxins at the low concentrations. As the toxicity increases, the abundance of the predominant toxins increases and the less predominant congeners begin to appear. The realistic LOD and LOQ are approximately 0.03 and 0.1 mg STX•diHCl eq/kg of tissue, respectively. This is a 4-fold improvement in the detection capability of the method over the conventional AOAC MBA. These improved limits provide a better early warning system from monitoring programs as well as better information about the toxin profiles in the concentration range of interest.

Ruggedness

The ruggedness of an analytical method is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure. The ruggedness approach used in this validation was Youden's factorial approach, where seven variables were combined in a specific manner to determine the effects of all seven variables using eight combinations in a single experiment. Seven variables were tested using a partial factorial approach followed by statistical evaluation of significance using a two-sample t-test assuming equal variance. The experiment was carried out in its entirety twice on separate days, with mean values being used for statistical evaluation. The material used was an incurred mussel tissue that was established to contain 1.72 mg STX•diHCl eq/kg. The seven variables tested are listed in Table 6.

Ruggedness of the technique was studied and statistical analysis was carried out using a two sample t-test. The statistical analysis indicated that the single factor that showed a significant affect on the results was the type of filter membrane used in the analysis. The Teflon membrane showed a significantly higher result for the total toxicity as well as several individual toxins over the nylon membrane. When this factor was then further studied independently it was found not to have a significant impact on the results.

Table 6. Factors evaluated in ruggedness experiments

Variable #	Description	Original Condition		Alternate Condition	
1	Concentration of HCl for initial extraction	A-	0.1 M	a-	0.12 M
2	Delay after acid addition before boiling	B-	no delay	b-	10 min delay
3	Time in boiling water bath	C-	5 min	c-	10 min
4	Final pH	D-	3	d-	2.5
5	Volume of TCA added	E-	25 µL	e-	20 µL
6	Volume of NaOH added	F-	35 µL	f-	40 µL
7	Filter material	G-	nylon	g-	teflon

Sample #	Factor Combinations	Measurement
1	A B C D E F G	s
2	A B c D e f g	t
3	A b C d E f g	u
4	A b c d e F G	v
5	a B C d e F g	w
6	a B c d E f G	x
7	a b C D e f G	y
8	a b c D E F g	z

Other factors were investigated as part of the optimization of method performance, but were not part of the statistical design to determine ruggedness/robustness. Specifically, various post-column reactors and coils were evaluated. The reactor used in this study required modification from the original manufacturer's design. As purchased, the reactor system has a large amount of heat exchanger tubing included as part of the system. This plumbing results in peak broadening and loss of resolution for the various toxins. Once this tubing is excluded from the flow path, the peak resolution is restored. Other post column reactor systems have been evaluated and found to be suitable. Some systems such as the Pickering Pinnacle PCX have incorporated the pumps and reactor system. The Pinnacle system was evaluated and provided better sensitivity which was attributed to less baseline noise from the post column pumps.

Linearity and Analytical range

The matrix fortified calibration curves of the toxins are linear at all ranges examined in this study. The concentration of toxins chosen for study are close to or at the limits of detection ranging up to 5.00 mg STX•diHCl eq/kg. The equations for typical curves and correlations are shown in Table 7. All the correlation coefficients are greater than 0.99. The ranges examined effectively encompass the regulatory limit of 0.80 mg STX•diHCl eq/kg for a typical toxin profile.

Table 7. Linearity and Linear Range of the calibrations curves as determined by serial dilutions of the working standards.

	Range				Calibration Curve	
	ng injected		mg / kg		Equation	Correlation
	lower	upper	lower	upper		
GTX4	0.08	5.08	0.017	1.110	y = 92.341x - 0.09	R^2 =1.0
GTX1	0.31	21.1	0.068	4.590	y = 48.248x - 2.137	R^2 =0.9999
dcGTX3	0.02	1.11	0.36	24.200	y = 1096x - 13.021	R^2 =0.9987
GTX5	0.05	0.84	0.011	0.184	y = 238.59x - 0.5407	R^2 =0.9995
dcGTX2	0.05	1.61	0.011	0.350	y = 360.45x - 2.2149	R^2 =0.9998
GTX3	0.02	2.45	0.004	0.530	y = 622.69x - 14.544	R^2 = 0.9996
GTX2	0.13	4.16	0.021	0.910	y = 101.46x + 0.13	R^2 = 1.00
NEO	0.17	11.6	0.037	2.520	y = 62.793x - 6.2136	R^2 = 0.9994
dcSTX	0.16	2.56	0.034	0.560	y = 117.12x - 2.4566	R^2 = 0.9989
STX	0.18	5.88	0.039	1.280	y = 69.892x - 4.1551	R^2 = 0.999
C1	0.0026	0.162	0.0011	0.076	y = 2567.8x + 0.4421	R^2 = 0.9999
C2	0.0065	0.798	0.028	0.350	y = 1353.6x - 7.6822	R^2 = 0.999
C3	0.0015	0.483	0.00007	0.021	y = 2741.9x - 0.1585	R^2 = 0.9998
C4	0.0106	0.166	0.0046	0.072	y = 618.28x + 0.5843	R^2 = 0.9996

The method was developed as part of a project to compare results obtained from analytical methods of analysis to the results obtained from the AOAC MBA. A long term comparison study was carried out where extracts used for MBA analysis were also analyzed by the CFIA-PCOX method. The results of this long term study are presented in Figure 1 and show good correlation of the PCOX results to the results obtained by the MBA.

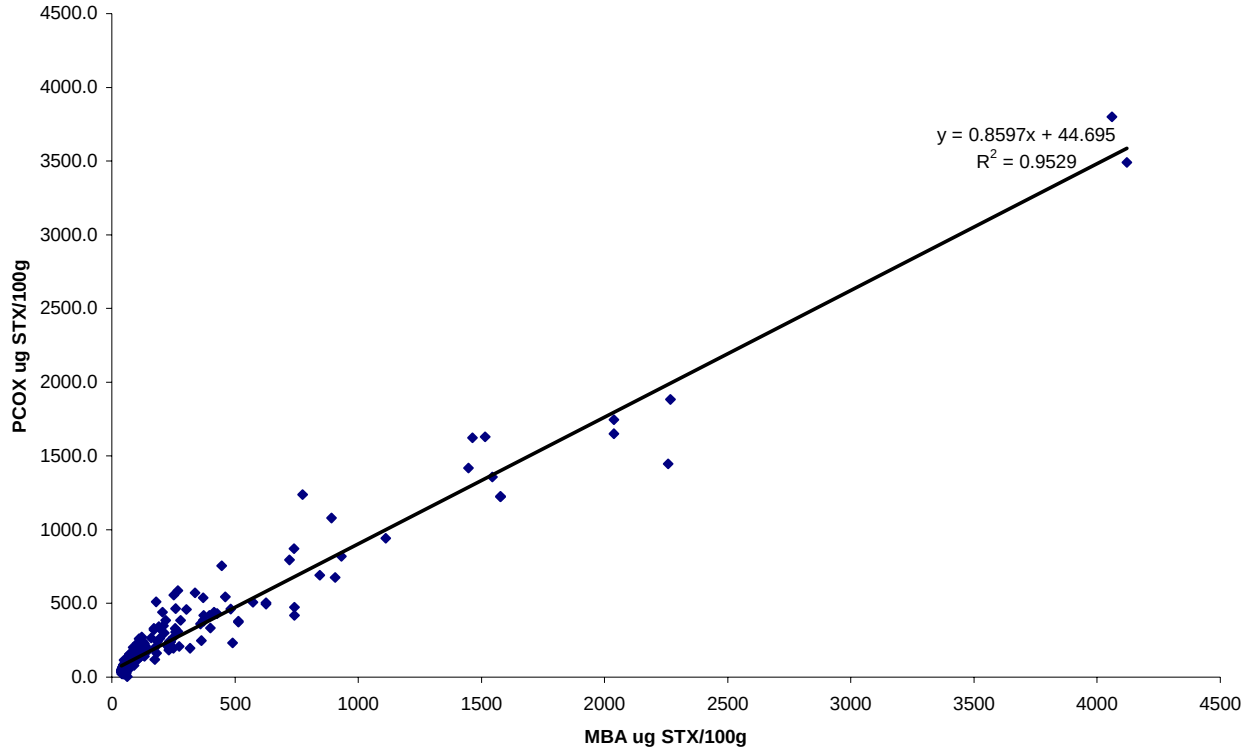


Figure 1. Comparison of the results obtained by the AOAC-MBA to the results obtained from the CFIA-PCOX methodology

This method has been employed to compare results obtained to those obtained in laboratories in other countries, which used other methods of analysis on samples collected in their monitoring programs. Samples or extracts were obtained, analysed by PCOX and results compared to those obtained from the method utilized in the source country; Norway {Oshima}, United Kingdom {AOAC Official Method 995.08 and AOAC Official Method 2005.06}, and New Zealand {AOAC Official Method 995.08}. The results obtained from those samples compared well to the results obtained from the source country using various methods (Figures 1 to 4). This indicates that the CFIA-PCOX method results are in agreement with other methods currently employed worldwide and that it therefore is a viable alternative to these various methods. The authors therefore have proposed that the method should be considered for further assessment by a full AOAC collaborative study.

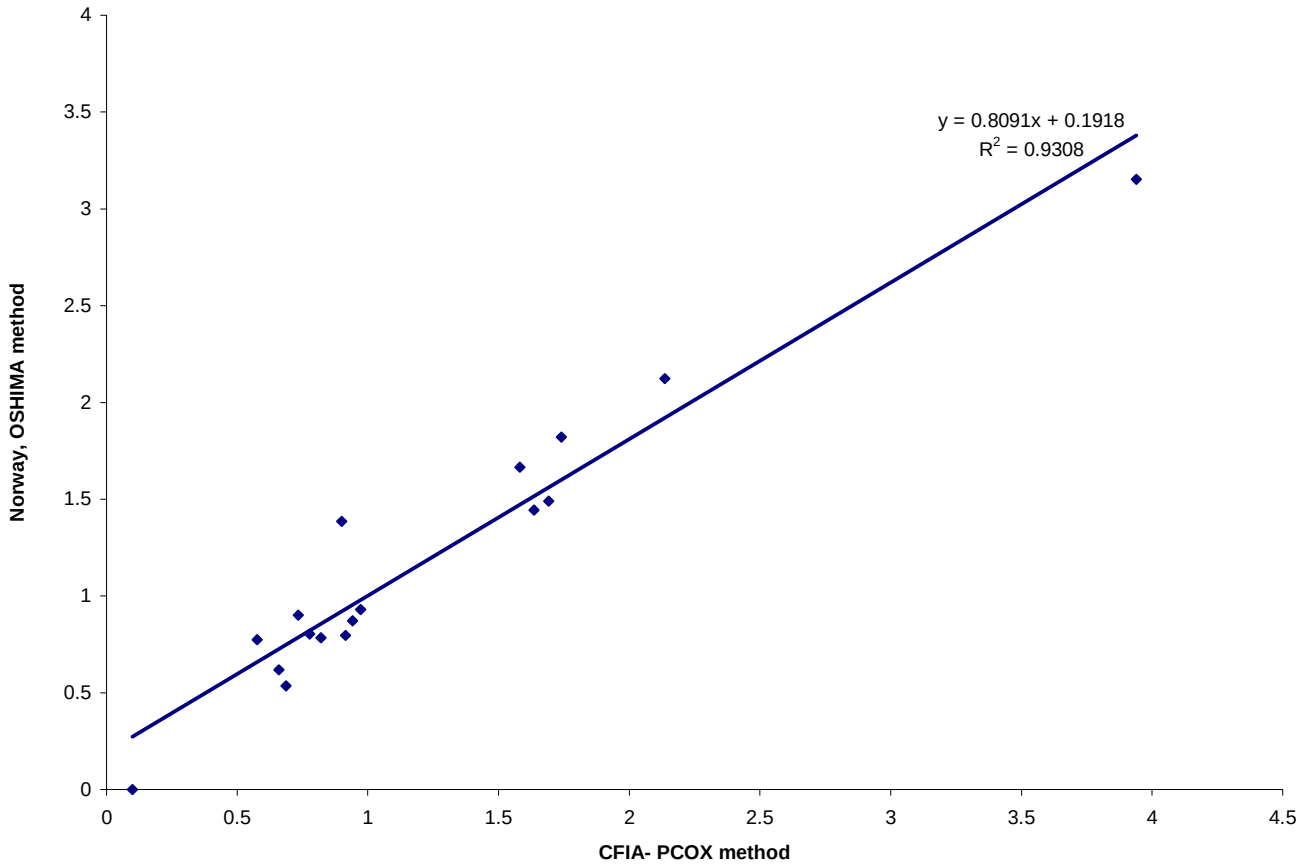
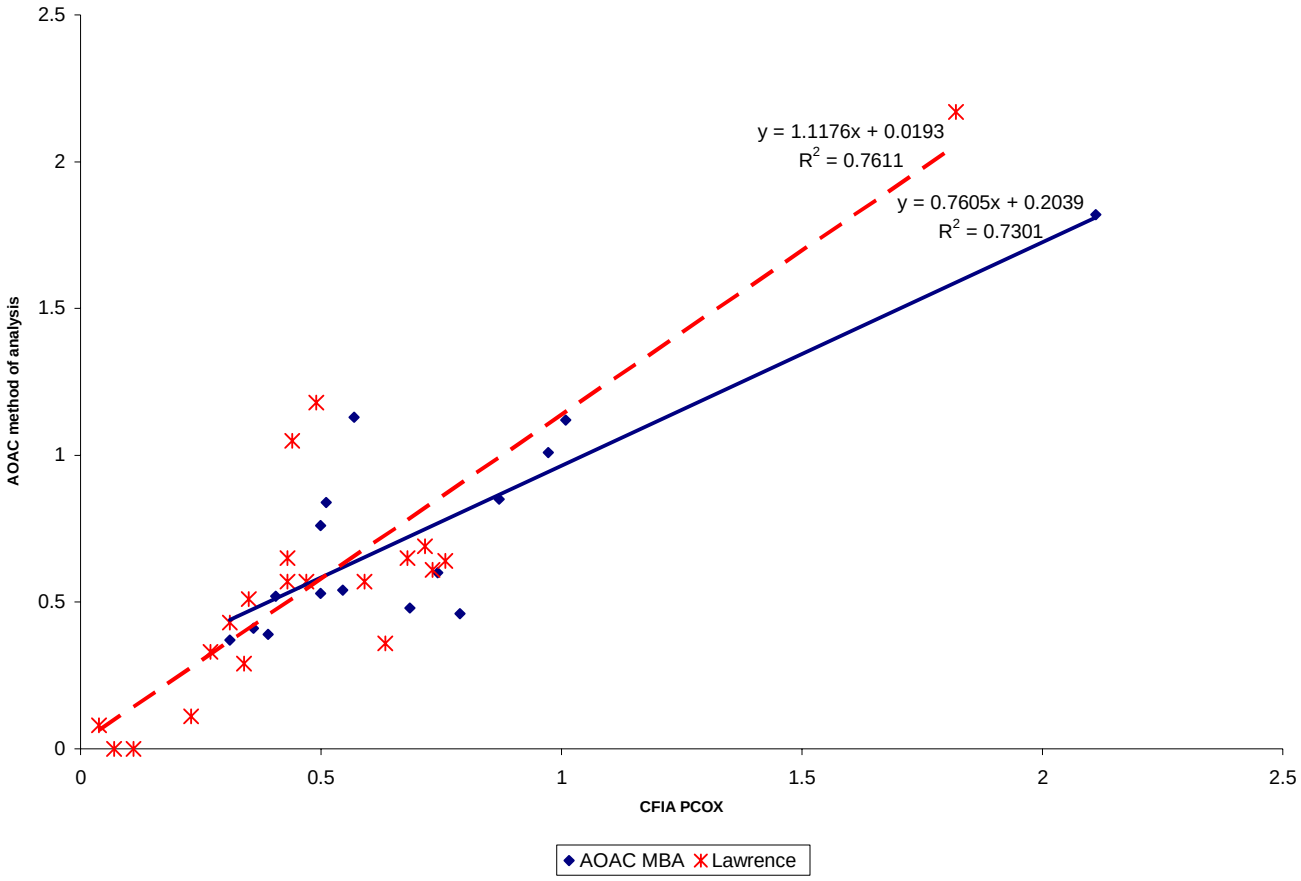


Figure 2. Comparison of PST results (mg STX•diHCl eq/kg) from tissues supplied and analysed by Norwegian School of Veterinary Science laboratory and the CFIA Dartmouth Laboratory



*Note: Twenty-four samples were provided however only the data from twenty were plotted as four of the samples were below the LOD of the MBA

Figure 3. Correlation of results obtained (mg STX•diHCl eq/kg) by CEFAS laboratories utilizing AOAC MBA and Lawrence methods of analysis to the CFIA-PCOX method for PST.

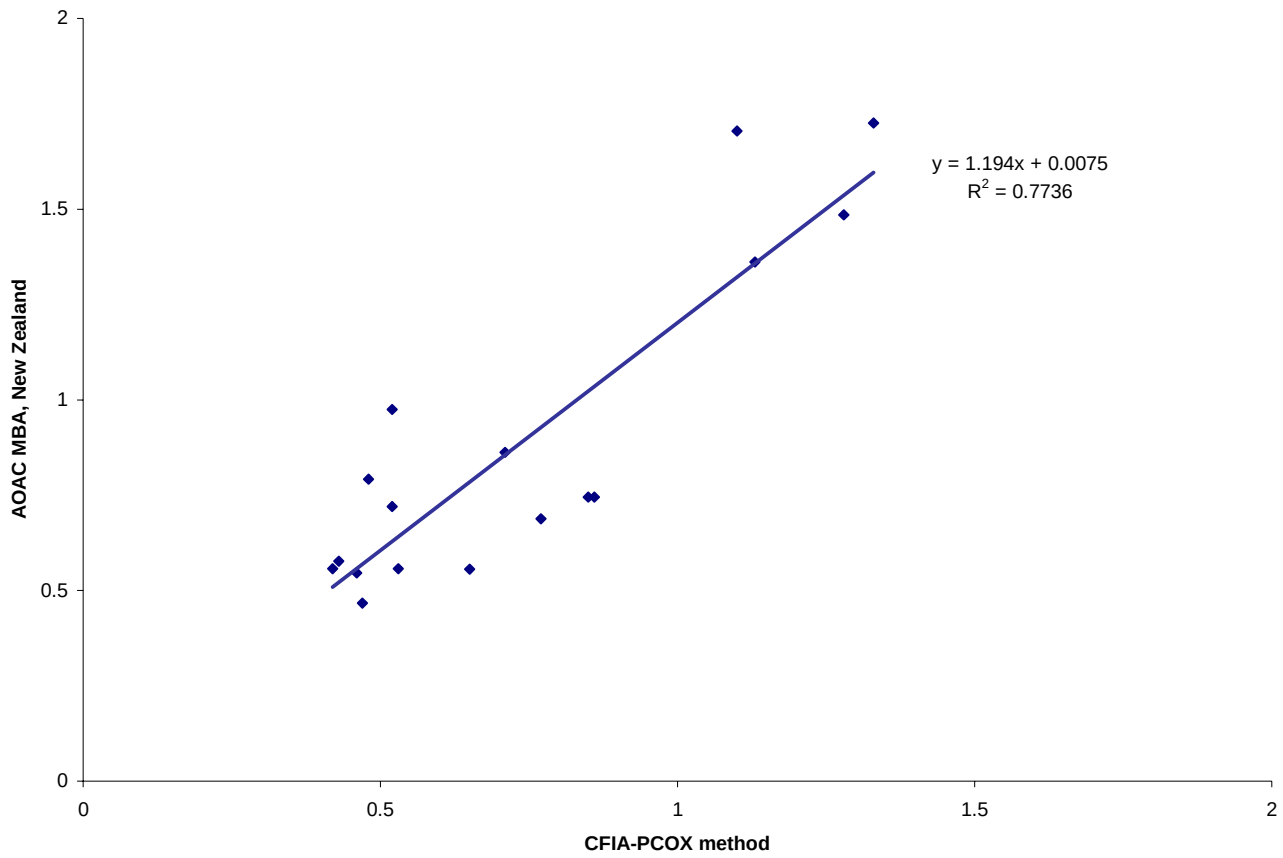


Figure 4. Correlation of PST toxicity (mg STX•diHCl eq/kg) in samples analysed by the MBA at the Cawthron Institute (NZ) and CFIA-PCOX.

Specificity

The current method of choice for the analysis of PST by most regulatory authorities is the AOAC mouse bioassay. This methodology employs and extraction of the toxins with a dilute acid and an injection in to the interperitoneal cavity of a mouse. The level of toxicity on the sample is inversely proportional to the time required for the mouse to die. This method is relatively non specific and has been shown to be subject to various interferences such as salts, high level of metals and pesticides. The CFIA-PCOX methodology utilizes a similar extraction technique as the MBA but a number of other steps are added into the process to provide greater specificity. Table 8 is a side by side comparison of the two methods (AOAC MAB and the CFIA-PCOX) highlighting the specificity created by the various steps in the procedure.

The table shows that the PCOX method adds specificity in to the analysis by building on the basis of the MBA extraction and adding steps such as protein precipitation, chromatographic separation of the toxins, oxidation of the toxins to a purine and detection of the purine by fluorescence at specific excitation and emission wavelengths

Table 8. Comparison of the specificity of the AOAC MAB and CFIA-PCOX methodologies

Method step	MBA Method	LC Method	Specificity
Data from the chemistry used in the extraction and clean-up procedure	1. Acid extraction: Acid extractables, <pH4	1. Acid extraction: Acid extractables, pH 2 to 4 2. De-proteination:-Proteins removed	1. Specificity is equivalent for both methods 2. Specificity of LC method is increased, due to removal of proteins (i.e., response in LC method cannot be caused by protein-based material)
Data from the subsequent chromatography	Not applicable	Substances must be initially retained on a reversed phase LC column and eluted by the mobile phase gradient conditions with characteristic retention times between 6 – 20 minutes	Specificity of LC method is increased by limiting the method to analytes retained and chromatographed on a LC column, additional selectivity is provided by the chromatographic separation and the characteristic retention times of the analytes which may be compared to reference standards.
Data from the detecting spectroscopy or electrochemistry	Not applicable	The LC method includes a post-column oxidation reaction which is specific to molecular structures which are oxidized by a phosphoric acid, periodic acid buffer solution to form purines by breakage of a C4-C12 bond in a complex 3-ring structure characteristic of PSP toxins, with formation of an aromatic ring structures which produces characteristic fluorescence.	The LC detection method is specific to compounds with complex 3-ring chemical structures which form fluorescent purines via the post-column reaction.
Detection	Mice respond to toxins, particularly neurotoxins.	Characteristic fluorescence (excitation: 330 nm, emission: 390 nm) associated with conversion of PSP toxins to purines.	MBA response is not specific to PSP, but is a general response to toxins, particularly neurotoxins; fluorescence associated with the LC method has been characterized by mass spectrometry to demonstrate it is from the reaction of PSP toxins with the periodic acid solution to form purines which exhibit native fluorescence [see Janacek, M., Quilliam, M.A. & Lawrence, J.F. *Journal of Chromatography*, **644 (**1993) 321-331]
data from the "blank" reagents	No positive response	No positive response	Methods have equivalent specificity in this regard.
data from the "blank" samples	Method has an expected "false positive" rate	No interfering co-extractives have been detected to date.	Response of LC method is "compound specific", as individual analytes are separated by LC prior to detection (selectivity of LC separation plus specificity of detection method)
data from library searches for potential interferences or matches	Other known toxins elicit a positive response (eg., neurotoxins such as carbamate and organophosphate insecticides)	None identified to date	Available data suggest LC method is more specific than mouse bioassay.
Data and arguments why potential interferences in practice do not or likely will not interfere	MBA is specific to "toxins", not to PSP toxins	LC combines selectivity of clean-up and separation with specificity of detection reaction.	LC method has greater selectivity and specificity than the MBA, plus analytes in extracts may be confirmed by LC/MS/MS.
Other data i.e choice of matrix, other quality control data			Sample source, collection, transport and storage are equivalent – no additional specificity is associated with these factors for either method.

Rapid Post-column Oxidation (PCOX) Method for the Determination of Paralytic Shellfish Toxins in Mussels, Clams, Oysters and Scallops.

1. PURPOSE

1.1. To give specific information required to carry out the method of analysis for the determination of paralytic shellfish toxins in shellfish by the CFIA Post-column Oxidation (PCOX) method.

2. REFERENCES

2.1. Rourke, W.A., Murphy, C.J., Pitcher, G., van de Riet, J.M., Burns, B.G., Thomas, K.M., Quilliam, M.A. (2008). Rapid Post-column Methodology for Determination of Paralytic Shellfish Toxins in Shellfish Tissue. J.AOAC Int 91(3), 589-597.

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2.3. AOAC. (1995b). Paralytic shellfish poison: Biological method. Sec. 35.1.37, Method 959.08. In Official Methods of Analysis of AOAC International, 16th ed., P.A. Cunniff (Ed.), p. 22-23. AOAC International, Gaithersburg, MD.

3. SCOPE

3.1. This method is validated for the determination of Paralytic Shellfish toxins (PST) in tissues of mussels, clams, oysters and scallops.

3.2. This method is an alternative to AOAC MBA methodology for the analysis of PST in molluscan shellfish

3.3. This method has been used to determine paralytic shellfish toxin concentrations in shellfish tissue ranging from 0 to 5000 ug STX diHCl/100 g.

4. DEFINITIONS

4.1. CRM = Certified reference material

4.2. PSP = Paralytic shellfish poisoning

4.3. C1 = N-sulfocarbamoylgonyautoxin-C1

4.4. C2 = N-sulfocarbamoylgonyautoxin-C2

4.5. C3 = N-sulfocarbamoylgonyautoxin-C3

4.6. C4 = N-sulfocarbamoylgonyautoxin-C4

4.7. dcGTX1 = decarbamoylgonyautoxin-1

4.8. dcGTX2 = decarbamoylgonyautoxin-2

4.9. dcGTX3 = decarbamoylgonyautoxin-3

4.10. dcGTX4 = decarbamoylgonyautoxin-4

4.11. dcSTX = decarbamoylsaxitoxin

4.12. GTX1 = gonyautoxin-1

4.13. GTX2 = gonyautoxin-2

4.14. GTX3 = gonyautoxin-3

4.15. GTX4 = gonyautoxin-4

4.16. GTX5 = gonyautoxin-5

4.17. GTX6 = gonyautoxin-6

4.18. NEO = neosaxitoxin

4.19. STX = saxitoxin

4.20. RCF = Rotor centrifugal force units

5. EQUIPMENT & MATERIALS REQUIRED

5.1. Equipment

5.1.1. Volumetric pipets: 1.0 mL, 2.0mL, 4.0 mL, 10.0 mL and 15.0 mL capacities

5.1.2. Volumetric flasks, various volumes

5.1.3. Analytical & top-load balances

5.1.4. Boiling water bath

5.1.5. Accurate timing device

5.1.6. Sieve, No. 10 mesh

5.1.7. Blender, small food processor or equivalent

5.1.8. 50 mL polypropylene tubes or equivalent

5.1.9. Dispenser capable of delivering 5 mL or equivalent

5.1.10. Centrifuge capable of holding 50 mL polypropylene tubes and of generating ~ 5000 RCF (g's)

5.1.11. Microcentrifuge tubes 1.5-2 mL

5.1.12. Pippettor(s) capable of delivering 20-1000 µL

5.1.13. 13 mm nylon syringe filters (0.2 µm) or equivalent

5.1.13.1. 3 mL disposable syringes if using syringe filters

5.1.14. Microcentrifuge capable of generating ~16000 RCF (g's)

5.1.15. High Recovery autosampler vials and caps

5.1.16. pH meter

5.1.17. Vortex mixer

5.2. Instrumentation:

5.2.1. LC pump system able to generate rapid, reliable binary gradients at flow rates of up to 1.5 mL/min and at pressures of at least 3000 psi

5.2.2. Autosampler system able to communicate with the pumps and data system and provide up to 100 µL injection volumes either in one injection or repeated smaller injections

5.2.3. Column oven able to maintain a column temperature of 50°C

5.2.4. LC fluorescence detector able to achieve the required sensitivity at excitation λ = 330 nm and emission λ = 390 nm

5.2.5. Two post-column pumps able to deliver acid and oxidant at flow rates up to 0.5 mL/min

5.2.6. Knitted reaction coil, 1 mL volume, 5 m x 0.5 mm or equivalent

5.2.7. Post-column reaction oven able to maintain a temperature of 85°C

5.2.8. LC columns

5.2.8.1. GTXs & STXs: Agilent Zorbax Bonus RP, 4.6 x150 mm, 3.5 µm particle size

5.2.8.2. C toxins: Thermo BetaBasic 8, 4.6 x 250 mm, 5 µm particle size

5.3. Reagents

5.3.1. Deionized water (DIW), 18Ω resistance or equivalent

5.3.2. Acetic acid (HOAc), glacial

5.3.2.1. 10% (v/v) HOAc: Pipet 10.0 mL of concentrated HOAc to a 100.0 mL volumetric flask containing 70 mL of DIW, dilute to the mark with DIW and mix well.

5.3.3. DIW (pH 5.00): Acidify DIW to pH 5.00 by dropwise addition of 10% HOAc.

5.3.4. Acetonitrile (MeCN), HPLC grade

5.3.5. Concentrated Hydrochloric acid (HCl), reagent grade

5.3.5.1. 5.0 M HCl: Add 413.2 mL of concentrated HCl to a 1.0 L volumetric flask containing 300 mL of DIW, dilute to the mark with DIW and mix well.

5.3.5.2. 0.1 M HCl: Add 40.0 mL of 5.0 M HCl to a 2.0 L volumetric flask containing 1.5 L of DIW, dilute to the mark with DIW and mix well.

5.3.5.3. 3 mM HCl: Pipet 15.0 mL of 0.1 M HCl to a 500.0 mL volumetric flask containing 300 mL of DIW, dilute to the mark with DIW and mix well.

5.3.6. Ammonium hydroxide (NH_4OH), reagent grade

5.3.6.1. 1% (v/v) NH_4OH: Pipet 1.0 mL of NH_4OH to a 100.0 mL volumetric flask containing 80 mL of DIW, dilute to the mark with DIW and mix well.

5.3.7. 1.0 M Tetrabutyl ammonium dihydrogen phosphate solution

5.3.8. Trichloroacetic acid (TCA), reagent grade

5.3.8.1. 30% (w/v) TCA: Dissolve 15.0 g of TCA in a 50.0 mL volumetric flask, dilute to the mark with DIW and mix well.

5.3.9. Sodium hydroxide (NaOH), reagent grade

5.3.9.1. 5.0 M NaOH: Weigh 200 g of NaOH, dissolve in 1.0 L DIW and mix well.

5.3.10. o-Phosphoric acid (H_3PO_4), reagent grade

5.3.10.1. 0.5 M H_3PO_4: Add 33.9 mL of H_3PO_4 to 800 mL of DIW in a 1.0 L volumetric flask, dilute to the mark with DIW and mix well.

5.3.11. 1-Heptane sulphonate sodium salt monohydrate

5.3.11.1. 0.5 M 1-Heptane sulphonate: Weigh 11.01 g of 1-heptane sulphonate sodium salt monohydrate for every 100.0 mL of DIW. Mix well, and store in fridge as solution degrades rapidly.

5.3.12. Periodic acid (H_5IO_6), reagent grade

5.3.12.1. 0.05 M H_5IO_6: Dissolve 11.4 g of H_5IO_6 in a 1.0 L volumetric flask, dilute to the mark with DIW and mix well.

5.3.13. Nitric acid (HNO_3), reagent grade

5.3.13.1. 0.75 M HNO_3: Add 101.2 mL of concentrated HNO_3 to 1.6 L of DIW. Make to volume (2.0 L), mix well, and then filter through 0.22 µm nylon membrane.

5.3.14. Post-Column Oxidant: Add 400 mL of 0.5 M H_3PO_4 to 1.2 L DIW and stir well. Add 200 mL of 0.05 M H_5IO_6, stir well, and check pH. If pH is approximately 4 then discard and start over. If pH is approximately 1.5 then adjust the pH to 7.8 with 5 M NaOH. Transfer to a 2.0 L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.15. GTX & STX mobile phase "A": Add 44.0 mL of 0.5 M heptane sulphonate to 1.8 L DIW and mix well. Add 22.0 mL of 0.5 M H_3PO_4 and mix well. Adjust the pH to 7.1 using concentrated NH_4OH. Transfer to a 2.0 L volumetric flask and dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.16. GTX & STX mobile phase "B": Add 22.0 mL of 0.5 M heptane sulphonate to 800mL DIW and mix well. Add 33.0 mL of 0.5 M H_3PO_4 and mix well. Adjust the pH to 7.1 using concentrated NH_4OH. Add 115 mL of MeCN and mix well. Transfer to a 1.0 L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.17. C toxin mobile phase "C": Add 4.0 mL of 1.0 M tetrabutyl ammonium dihydrogen phosphate solution to 1.8 L of DIW using a volumetric pipet. If pH is above 5.8 then adjust the pH to 5.8 by adding 10% (v/v) HOAc dropwise, but if the pH is below 5.8 then adjust the pH to 5.8 by adding 1% NH_4OH dropwise. Transfer to a 2.0 L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.3.18. C toxin mobile phase "D": Add 2.0 mL of 1.0M tetrabutyl ammonium dihydrogen phosphate solution to 900mL DIW using a volumetric pipet. If pH is above 5.8 then adjust the pH to 5.8 by adding 10% (v/v) HOAc dropwise, but if the pH is below 5.8 then adjust the pH to 5.8 by adding 1% NH_4OH dropwise. Add 40mL MeCN and mix well. Transfer to a 1.0L volumetric flask, dilute to the mark with DIW and mix well. Filter through 0.45 µm membrane.

5.4. Standards

5.4.1. Primary Standards- C1, C2, dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX (National Research Council Institute for Marine Biosciences, Halifax, NS). All

standards are obtained from the National Research Council's Certified Reference Material Program, and have certified values. These standards are then used to make stock and working solutions.

5.4.2. C3 & C4 are NRC in house reference materials that will become commercially available when certification is complete.

5.4.3. Stock Standards-

Stock solutions are approximately 4 fold dilutions of NRC CRM's (Various concentrations based on CRM concentration).

5.4.3.1. Remove CRM ampoules from fridge/freezer and allow to reach room temperature.

5.4.3.2. Weigh an empty 2.0 mL volumetric flask.

5.4.3.3. Open the ampoule of CRM by carefully cracking at the scored line. Transfer as much liquid as possible to the volumetric flask.

5.4.3.4. Weigh the volumetric flask that now contains the CRM and determine the mass of transferred CRM by difference.

5.4.3.5. Dilute to 2.0 mL using 0.003 M HCl for GTX & STX or DIW (pH 5.00) for C toxins and mix well.

5.4.3.6. Weigh the full flask and determine the final volume of the solution.

5.4.3.7. Calculate the concentration based on the CRM documentation.

5.4.4. Working Standards

Working solutions (Various concentrations based on stock concentration). Standard solutions are generally separated into two categories, C toxins and GTXs & STXs. One working standard includes C1, C2, C3 and C4 while another working standard includes dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO and STX.

5.4.4.1. GTX & STX neat mixed working solution

5.4.4.1.1. Weigh an empty 5.0mL volumetric flask after wiping the outside of the flask

5.4.4.1.2. Transfer volume of stock solution from table below to volumetric flask recording weight of flask after each addition.

Toxin	**Volume of Stock (uL)** (Solution from 5.2.20)
dcGTX2&3	200
GTX2&3	200
dcSTX	200
STX	200
GTX1&4	400
GTX5	400
NEO	400

5.4.4.1.3. Dilute to 5.0mL using 0.003 M HCl.

5.4.4.1.4. Weigh full flask to determine final volume of solution.

5.4.4.1.5. Calculate concentration based on dilution factor and stock solution concentrations.

5.4.4.2. C-toxin neat mixed working solution

5.4.4.2.1. Weigh an empty 5.0mL volumetric flask after wiping the outside of the flask.

5.4.4.2.2. Transfer volume of stock solution from table below to volumetric flask recording weight of flask after each addition.

Toxin	**Volume of Stock (uL)** (Solution from 5.2.20)
C1&2	400
C3&4	800

5.4.4.2.3. Dilute to 5.0mL using DIW (pH 5.00).

5.4.4.2.4. Weigh full flask to determine final volume of solution.

5.4.4.2.5. Calculate concentration based on dilution factor and stock solution concentrations.

5.4.4.3. Matrix fortified working standards

5.4.4.3.1. Follow instructions for making up neat working standard (5.4.4.1) using a toxin free, de-proteinated, mussel extract as the diluent.

6. SAFETY PRECAUTIONS

6.1. Follow normal laboratory practices for a safe and healthy working environment.

6.2. Always wear gloves when handling PSP standards and samples.

6.3. Always wear a mask when weighing 1-heptane sulphonate.

6.4. Always work in a fumehood when using NH_4OH.

7. POLICY

7.1. Only Trained and authorized analysts shall perform this analysis.

8. INSTRUCTIONS

8.1. Sampling Procedure

8.1.1. Take a representative sample of the shellfish that require testing, usually consisting of 12-18 market size shellfish. This number should ensure the selection of sound animals suitable for analysis. Ensure the shellfish yield approximately 100 g of meats and shell liquor.

8.1.2. Rinse samples to remove sand, dirt and mud and place in a clean plastic bag.

8.1.3. Mark or tag all samples using waterproof markers for identification purposes. Label the sample in such a way that the identity of the sample can not be lost during shipment.

8.1.4. Ensure that the integrity of the sample is maintained by proper storage. Maintain the state of the sample.

8.1.4.1. Refrigerate samples of shucked or live shellfish immediately after collection by packing in crushed ice and keeping them in ice until examined. The shellfish must not come into direct contact with ice.

8.1.4.2. Keep frozen samples frozen in a freezer or in a carton/cooler with ice packs and ship the sample as quickly as possible to ensure that the sample remains in the frozen state.

8.2. Sample Preparation

8.2.1. Live Bivalve Molluscan Shellfish -

8.2.1.1. Thoroughly clean the outside of the shellfish with running tap water. Open the shell by cutting the adductor muscles without cutting or damaging the viscera. Rinse the inside with tap water to remove sand or other foreign material.

8.2.1.2. Remove tissue of 12-15 animals from the shell, for most shellfish collect the entire shell contents. For scallops, separately collect the digestive gland, adductor muscles, gonad, etc. for analysis as stipulated by regulatory requirements.

8.2.1.3. Collect the tissue to be used for the analyses onto a number 10 sieve. Allow to drain for approximately 5 minutes. Remove any pieces of shell or other foreign matter. Discard draining.

8.2.1.4. Transfer meats to a suitable vessel and blend/grind until homogenous.

8.2.2. Frozen in the shell Bivalve Molluscan Shellfish

8.2.2.1. Allow frozen products to thaw under controlled conditions to prevent decomposition, preferably under refrigeration overnight. Thaw frozen product under controlled conditions to prevent decomposition (preferably refrigerated overnight). Do not drain

8.2.2.2. Remove tissue of 12-15 animals from the shell, for most shellfish collect the entire shell contents. For scallops, separately collect the digestive gland, adductor muscles, gonad, etc. for analysis as stipulated by regulatory requirements.

8.2.2.3. Homogenize as per 8.1.2.4

8.2.3. Refrigerated/Frozen shucked ProductsRefrigerated Shucked Products

8.2.3.1. Refrigerated shucked products, such as clams, mussels, oysters, or scallops, use the sample as provided. - do not drain. Homogenize as per 8.2.1.3

8.2.3.2. Frozen product must be allowed to thaw under controlled conditions to prevent decomposition, preferably under refrigeration overnight. Homogenize as per 8.2.1.4. Use refrigerated shucked products as provided. Do not drain.

8.2.4. Frozen Shucked Product

8.2.4.1. Thaw frozen product under controlled conditions to prevent decomposition (preferably refrigerated overnight). Do not drain

8.2.4.2. Homogenized as per 8.2.1.4

8.2.5. Frozen breaded product

8.2.5.1. If frozen and breaded, thaw, remove breading, and homogenize as per 8.2.1.4

8.3. Extraction

8.3.1. Include the appropriate QA samples with each analytical run (generally a blank sample, spiked sample and a check sample).

8.3.2. Accurately weigh 5 g of homogenized material into 50 mL polypropylene tube and record weight.

8.3.3. Add 5 mL 0.1 M HCl and mix on a vortex mixer.

8.3.4. Check pH and adjust pH to between 2 and 4 using 5M HCl or NaOH if necessary

8.3.5. Cap tubes tightly and place in a boiling water bath for 5 minutes.

8.3.6. Allow tubes to cool to room temperature and check to ensure that the pH is between 2.0 and 4.0. If the pH must be lowered, then add 5 M HCl dropwise while stirring until the pH is <4.0. If the pH is adjusted to below 2, discard sample and start extraction again. If pH must be raised, add 5 M NaOH dropwise while stirring until the pH is between 2.0 and 4.0.

8.3.7. Centrifuge tubes at ~5000 RCF for 5 minutes

8.3.8. Pipette 500.0 µL of supernatent into a microcentrifuge tube.

8.3.9. Add 25.0 µL of 30% (w/v) TCA and mix using a vortex mixer.

8.3.10. Centrifuge at ~16000 RCF for five minutes.

8.3.11. Add 30.0 µL 1.0 M NaOH and mix using a vortex mixer to neutralize TCA.

8.3.12. Centrifuge at ~16000 RCF for five minutes.

8.3.13. Filter through a 0.2 µm syringe filter into an LC autosampler vial. Divide sample into two LC autosampler vials if GTX & STX and C toxin analyses are being performed on separate instruments.

8.4. LC Conditions

8.4.1. GTX & STX Analysis Conditions

Mobile Phase
A - 11 mM Heptane sulphonate, 5.5 mM H_3PO_4, pH 7.1
B - 11 mm Heptane sulphonate, 16.5 mM H_3PO_4 in 11.5% MeCN, pH 7.1
Column Flow 0.8 mL/minute
Column oven temperature 40°C (see 8.9)
Detector Fluorescence
Excitation λ = 330nm
Emission λ = 390nm

8.4.2. C-Toxins Analysis Conditions

Mobile Phase
C - 2 mM tetrabutyl ammonium phosphate, pH 5.8
D - 2 mM tetrabutyl ammonium phosphate, pH 5.8 in 4% MeCN
Column Flow 0.8 mL/minute

Column oven temperature 15°C
Detector Fluorescence
Excitation λ = 330nm
Emission λ = 390nm

8.4.3. Post-Column Reaction Module Conditions

Oxidant Flow Rate	0.4mL/minute
Acid Flow Rate	0.4mL/minute
Reactor Temp.	85°C
Reaction Coil	1mL (5m x 0.5mm)

8.5. Equilibrate the system for at least 20 minutes with 100% solvent A.

8.6. The toxins are separated using the following gradient conditions for the two groups of toxins. These gradient conditions are subject to modification to facilitate proper separation parameters.

Gradient

Time (min.)	% Solvent B	Flow Rate (mL/min.)		
		LC	Oxidant	Acid
Gonyautoxin and Saxitoxins				
0	0	0.8	0.4	0.4
7.9	0	0.8	0.4	0.4
8	100	0.8	0.4	0.4
18.5	100	0.8	0.4	0.4
18.6	0	0.8	0.4	0.4
24	0	0.8	0.4	0.4
C-toxins				
0	0	0.8	0.4	0.4
8	0	0.8	0.4	0.4
15	100	0.8	0.4	0.4
16	100	0.8	0.4	0.4
19	0	0.8	0.4	0.4
24	0	0.8	0.4	0.4

8.7. For GTX, STX and C toxins calibrate the instrument with duplicate injections of the matrix fortified working standards.

8.8. Inject aliquots of mixed working solutions (10 µL for the GTX and STX toxins and 5 µL for the C toxins) into the system and separate chromatographically using the gradient conditions shown in 8.6 to ensure system suitability conditions (shown below) are met.

8.9. The step time in the gradient for GTX and STX (see 8.6) and/or column temperature may be altered to facilitate the resolution of GTX3, artefact, and GTX2 peaks. If the artefact peak and GTX2 co-elute, reduce the column temperature to achieve the desired resolution.

Toxins	Conditions
GTXs & STXs	• Artefact peak must be at least 70% baseline resolved between GTX3 and GTX2 • GTX5 must be at least 40% baseline resolved between dcGTX3 and dcGTX2 • dcSTX and STX must be at least 70% baseline resolved • GTX4 retention time must be between 5 and 7 minutes • STX retention time must be between 17 and 23 minutes
C toxins	• C2 must be at least 70% baseline resolved between C1 and C3 • C1 retention time must be between 4 and 7 minutes

8.10. For GTX and STX inject 10 µL of samples (including checks, spikes, blanks and repeats). Peaks are identified by comparison of retention times with recently run standards.

8.11. For C toxins inject 5 µL of samples (including checks, spikes, blanks and repeats). Peaks are identified by comparison of retention times with recently run standards.

8.12. Flush the system regularly to prevent build-up of salts. This should be done at least once a week, and always before long periods of instrument inactivity.

8.12.1. Remove the column from the LC system and flush the LC with DIW or 10% acetonitrile.

8.12.2. Both post-column pumps should be flushed with acid and then with DIW

9. CALCULATIONS

9.1. Using a single point calibration, measure peak areas of the standards

9.2. Measure the peak areas of the sample(s).

9.3. Calculate the contribution of each toxin to the overall toxicity using the following formula:

$$mgSTXeq/kg = \sum \left(uM \left(372.2 / 1000 \right) * \left(Fvol / Ext.vol \right) * \left(10 / Wt \right) * \mathrm{Re}Tx \right)$$

Or $$mgSTXeq/kg = \sum \left(uM * \mathrm{Re}Tx * 8.33 \right)$$

Where :
- µM = Concentration in the extract
- Fvol = Final volume of the deproteinzed extract (560µL)
- Ext.vol = Volume of crude extract used (500µL)
- Wt= Weight of sample used
- ReTx= Toxicity of the analyte in relation to Saxitoxin from Table 1

To provide a total toxicity directly comparable to what would be obtained from the MBA when the laboratory uses the FDA STX standard a correction factor of 1.16 must be used with the result from the CFIA-PCOX. This is a result of the FDA STX standard having a nominal value of 100 µg/mL, when in fact that it is determined to be 86 µg/mL when analysed against the certified standard from NRC.

Table 1. Relative Toxicity Values

Toxin	**ReTx**	**Toxin**	**ReTx**
GTX1	0.9940	**NEO**	0.9243
GTX2	0.3592	**STX**	1.0000

GTX3	0.6379	**dcSTX**	0.5131
GTX4	0.7261	**C1**	0.0060
GTX5	0.0644	**C2**	.00963
dcGTX2	0.1538	**C3**	0.0133
dcGTX3	0.3766	**C4**	0.0576

9.4. Add the contributions of all of the individual toxins to obtain the overall toxin concentration for the sample in µg STX equivalents/100g

10. QA/QC CONSIDERATIONS

10.1. Representative chromatograms of the GTX and STX mixed working standards, an unspiked mussel sample and a spiked mussel sample run on the Agilent Zorbax Bonus RP, 4.6 x150 mm, 3.5 µ column are shown in Figure 1. Representative chromatograms of C toxin working standards, an unspiked mussel sample and a spiked mussel sample run on a Thermo BetaBasic 8, 4.6 x 250 mm, 5 µ column are shown in Figure 2.

10.2. Total toxicity spike recoveries (based on 5 determinations at 3 levels, two analysts) range from 94 to 106 %.

10.3. The reproducibility of the method (r) as determined from incurred material should be between 2 and 6 % at the regulatory limit of 80 ug STX·diHCl/100g.

10.4. LODs and LOQs for GTX, STX and C toxins are shown in Table 2.

Table 2. Estimated Limits of Detection and Quantitation for the individual PST in the validated matrices.

Toxin	Clams		Mussels		Scallops		Oysters	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
GTX4	0.0120	0.036	0.0160	0.048	0.016	0.048	0.026	0.078
GTX1	0.0210	0.063	0.0240	0.072	0.024	0.072	0.037	0.111
dcGTX3	0.0025	0.008	0.0008	0.002	0.018	0.054	0.003	0.008
GTX5	0.0060	0.018	0.0032	0.010	0.007	0.021	0.008	0.024
dcGTX2	0.0070	0.021	0.0021	0.006	0.005	0.014	0.007	0.021
GTX3	0.0025	0.008	0.0012	0.004	0.003	0.008	0.003	0.009
GTX2	0.0310	0.093	0.0220	0.066	0.024	0.072	0.029	0.087
NEO	0.0250	0.075	0.0240	0.007	0.024	0.072	0.026	0.078
dcSTX	0.0096	0.029	0.0077	0.023	0.008	0.023	0.010	0.029
STX	0.0170	0.051	0.0130	0.039	0.013	0.039	0.014	0.042
C-1	0.0004	0.001	0.0002	0.001	0.001	0.003	0.000	0.001
C-2	0.0008	0.002	0.0008	0.002	0.001	0.004	0.009	0.028
Total	0.135	0.404	0.115	0.345	0.143	0.430	0.172	0.515

LOD = 3 xS/N
LOQ= 3 x LOD

10.5. Store GTX & STX CRMs and standards in a refrigerator at 4 ^{o}C when not in use. Stock solutions are stable for two months

10.6. Store C toxin CRMs and standards in a freezer at <-18 ^{o}C when not in use. Stock solutions are stable for two months

10.7. Working standards and matrix fortified standards should be prepared fresh monthly.

10.8. The final extracts may be stored for at least 2 weeks when stored in the refrigerator

10.9. Single point calibration is recommended however depending on the equipment or columns used; a multi-point calibration may be required. In such cases the linearity of the standard curve (r^2) must be greater than 0.95.

10.10. Monitor dcGTX3 for signs of a shoulder on the front of the peak, as this peak will sometimes split. Both the main peak and the front shoulder are dcGTX3.

10.11. The retention times of GTX1 and GTX4 are affected by the matrix, so matrix fortified standards must be used.

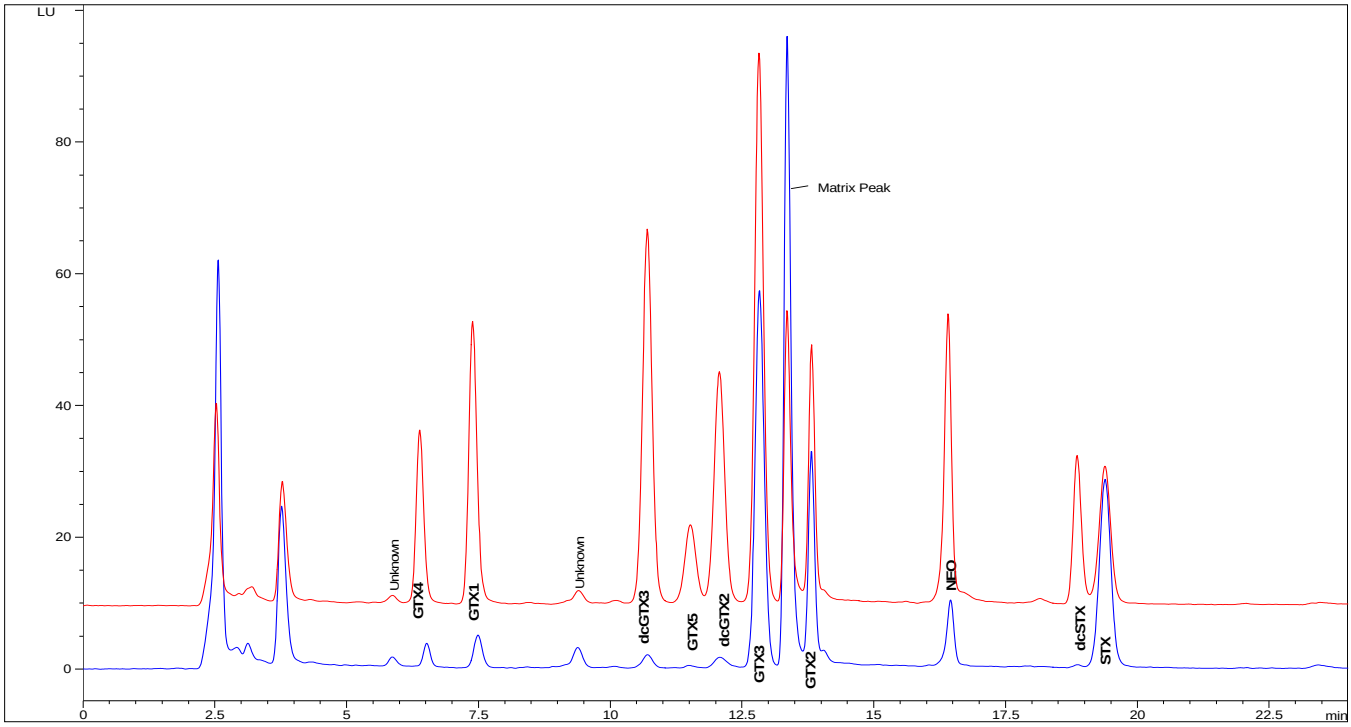

Figure 1. Overlaid chromatographic separation of the Gonyautoxins and Saxitoxins working standard (top) and an incurred mussel tissue (bottom) obtained by the CFIA-PCOX method of analysis.

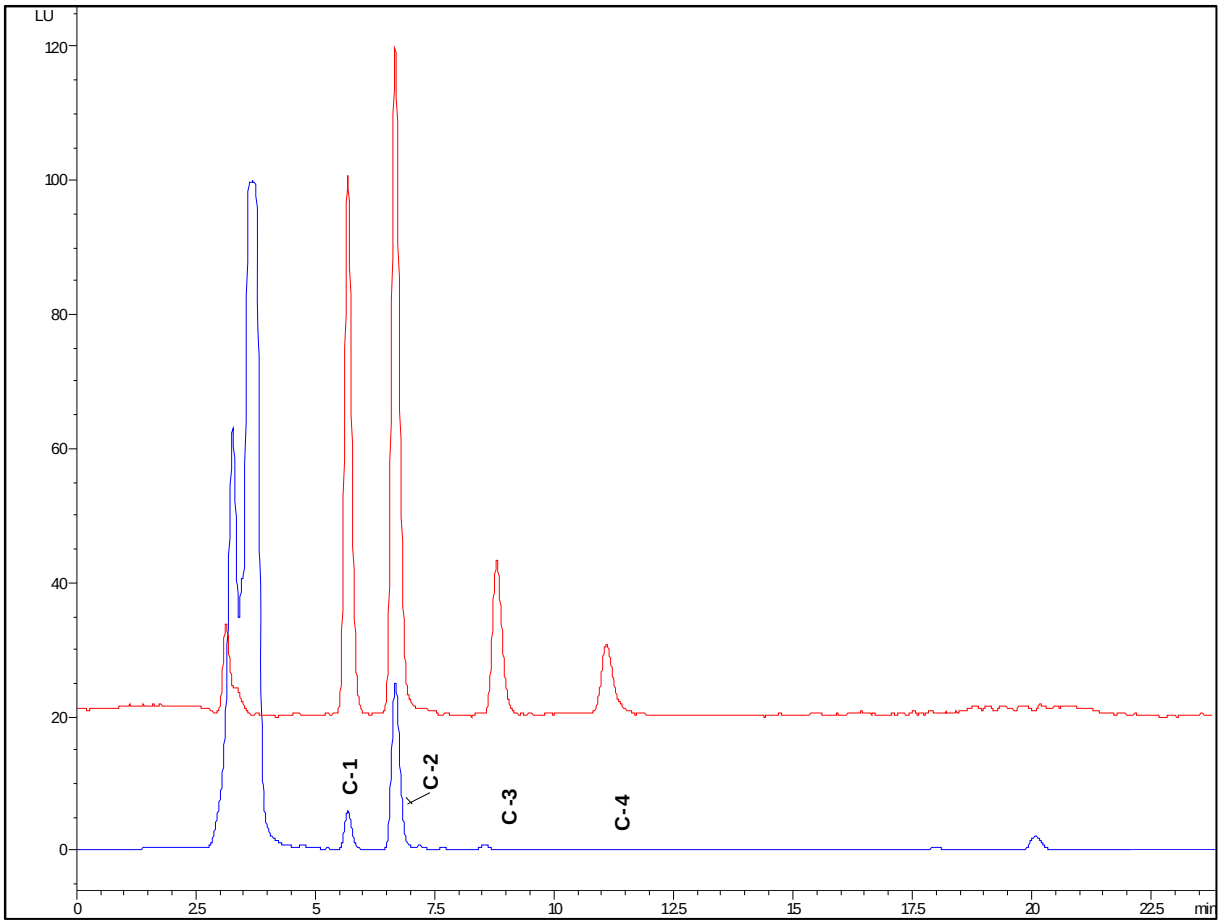

Figure 2. Overlaid chromatographic separation of the N-sulfocarbamoyl-gonyautoxins (C-toxins) working standard (top) and an incurred mussel tissue (bottom) obtained by the CFIA-PCOX method of analysis.

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FOOD CHEMICAL CONTAMINANTS

Rapid Postcolumn Methodology for Determination of Paralytic Shellfish Toxins in Shellfish Tissue

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A rapid liquid chromatographic (LC) method with postcolumn oxidation and fluorescence detection (excitation 330 nm, emission 390 nm) for the determination of paralytic shellfish toxins (PSTs) in shellfish tissue has been developed. Extracts prepared for mouse bioassay (MBA) were treated with trichloroacetic acid to precipitate protein, centrifuged, and pH-adjusted for LC analysis. Saxitoxin (STX), neoSTX (NEO), decarbamoylSTX (dcSTX), and the gonyautoxins, GTX1, GTX2, GTX3, GTX4, GTX5, dcGTX2, and dcGTX3, were separated on a polar-linked alkyl reversed-phase column using a step gradient elution; the *N*-sulfocarbamoyl GTXs, C1, C2, C3, and C4, were determined on a C-8 reversed-phase column in the isocratic mode. Relative toxicities were used to determine STX-dihydrochloride salt (diHCl) equivalents (STXeq). Calibration graphs were linear for all toxins studied with STX showing a correlation coefficient of 0.999 and linearity between 0.18 and 5.9 ng STX-diHCl injected (equivalent to 3.9–128 μg STXeq/100 g in tissue). Detection limits for individual toxins ranged from 0.07 μg STXeq/100 g for C1 and C3 to 4.1 μg STXeq/100 g for GTX1. Spike recoveries ranged from 76 to 112% in mussel tissue. The relative standard deviation (RSD) of repeated injections of GTX and STX working standard solutions was <4%. Uncertainty of measurement at a level of 195 μg STXeq/100 g was 9%, and within-laboratory reproducibility expressed as RSD was 4.6% using the same material. Repeatability of a 65 μg STXeq/100 g sample was 3.0% RSD. Seventy-three samples were analyzed by the new postcolumn method and both AOAC Official Methods for PST determination: the MBA ($y = 1.22x + 13.99$, $r^2 = 0.86$) and the precolumn LC oxidation method of Lawrence ($y = 2.06x + 12.21$, $r^2 = 0.82$).

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Testing shellfish for the group of potent neurotoxins responsible for paralytic shellfish poisoning is critical for consumers and for the shellfish industry in general. Paralytic shellfish toxins (PSTs) accumulate in shellfish, and consumption of these shellfish can lead to serious illness and death. Monitoring programs are needed to determine when it is safe to harvest and consume shellfish. The PST group comprises more than 20 different naturally occurring analogs of saxitoxin (STX). The toxins can be subgrouped into 4 categories: the most toxic, carbamate group, which includes STX and neosaxitoxin (NEO); the decarbamoyl group; the deoxy-decarbamoyl group; and the least toxic, *N*-sulfocarbamoyl group. The individual toxin levels of these analogs are usually expressed as STX equivalents (STXeq) so that an overall toxicity of a sample may be calculated (1, 2) when chemical or biological tests other than the mouse bioassay (MBA) are used. The dihydrochloride salt of STX (STX-diHCl) is used as the standard for the MBA; therefore, the regulatory limit is actually 80 μg STX-diHCl equivalents per 100 g of whole tissue. Ensure that the proper units are used when comparing chemical test results to MBA results. All references to STXeq in this paper refer to the diHCl salt.

The MBA has been the regulatory method for over 50 years and is an Official Method of AOAC INTERNATIONAL (3). The MBA method currently serves as the reference method in the European Union (EU) with the EU council directive 91/492/EEC (4) stating that the total PST content must not exceed 80 μg STXeq/100 g tissue. The time from exposure to death is used in the MBA to estimate the amount of toxin present in shellfish, with a detection limit for the method at 40 μg STXeq/100 g. Although the MBA method has proved to be very reliable, there is international pressure to reduce or eliminate testing involving animals (5, 6). The MBA provides little toxin profile information, but has the advantage of reporting the total toxicity of the sample. This method also is subject to considerable variability (7). Alternative methods that could reduce or completely eliminate MBA testing for PSTs in a regulatory environment are becoming very desirable.

A number of different approaches have been investigated to replace the MBA as a regulatory tool, including biological assays (8–11), electrophoresis (12), chemosensors (13), and immunoassays (14, 15). The most common chemical method

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Table 1. Relative PST toxicities and concentrations of reference, stock, and working standard solutions

Toxin	Mouse units (MU)/μmole	CRM, μM[a]	Stock standard solution, μM	Working standard solution, μM
GTX4	1803	35	8.3	0.66
GTX1	2468	106	25.2	2.0
dcGTX3	935	32	7.9	0.32
dcGTX2	382	114	28.1	1.1
GTX5	160	65	17.5	1.4
GTX3	1584	39	10.3	0.41
GTX2	892	118	31.0	1.2
NEO	2295	65	16.6	1.3
dcSTX	1274	62	16.1	0.64
STX	2483	65	15.9	0.64
C1	15	114	31.2	2.5
C2	239	35	9.5	0.76
C3	33	34	2.5	0.34
C4	143	27	0.76	0.27

[a] CRM = Certified Reference Material.

uses a combination of liquid chromatography (LC) with either pre- or postcolumn oxidation followed by fluorescence detection (FLD; 16–19). This instrumental technology can screen samples while providing detailed toxin profile information, now that a variety of calibration solutions are available (20). The LC-FLD method of Lawrence et al. has been the subject of a successful interlaboratory study (2) and collaborative study (21) and has been accepted by AOAC as the first analytical alternative to the MBA (22). Although it meets the major safety criteria of equivalency to the MBA, the Lawrence method suffers from several drawbacks when applied in a regulatory environment. The major impediment to widespread use of the Lawrence method is the amount of time required to process samples containing significant amounts of PSTs (23). The Lawrence method also cannot distinguish isomeric toxins that may exhibit significantly different toxicities. This study describes the modification of a postcolumn approach previously reported by Oshima (18) and Thomas et al. (19) to address these shortcomings.

The new postcolumn method performance was compared with the "gold standard" MBA as well as the Lawrence precolumn oxidation method. Fourteen of the most toxic and most commonly occurring PSTs were chosen for the study, including STX; NEO; decarbamoylsaxitoxin (dcSTX); gonyautoxin (GTX)-1,2,3,4,5; decarbamoylgonyautoxin (dcGTX)-2,3; and *N*-sulfocarbamoyl gonyautoxin (C)-1,2,3,4 to ensure that the majority of the toxin profiles could be addressed. This method was evaluated against a number of criteria essential to meeting the needs of a regulatory environment, including the practicality for regulatory work, equivalency of results to the MBA and/or the Lawrence method results, applicability to a variety of toxin profiles, reliability on a daily basis, cost, and ease of use. Instrument and analyst time were also considered as factors. The most important consideration in method acceptance for regulatory use was and continues to be the safety of the consumer. The method was applied to a variety of shellfish matrixes, containing numerous toxin profiles, collected throughout eastern Canada.

METHOD

Apparatus

(**a**) *LC system.*—Agilent 1200 quaternary solvent delivery system, autosampler equipped with 0.1–100 μL variable volume injector, column oven, column-switching valve, and data-handling module (Agilent Technologies, Kirkland, QU, Canada).

(**b**) *Postcolumn reaction system.*—Waters postcolumn reaction module capable of maintaining temperature at 85°C with reagents delivered by Waters Reagent Manager pumps (Waters, Milford, MA).

(**c**) *Reaction coil.*—Supelco knitted teflon tube with total volume of 1.0 mL (Sigma-Aldrich Canada, Oakville, ON, Canada).

(**d**) *Fluorescence detector.*—Agilent 1200 FLD operated at an excitation wavelength of 330 nm and an emission wavelength of 390 nm.

(**e**) *LC columns.*—(*1*) Agilent Zorbax Bonus RP, 4.6 × 150 mm, 3.5 μm; (*2*) Thermo BetaBasic 8, 4.6 × 250 mm, 5 μm (Fisher Scientific, Nepean, ON, Canada).

(**f**) *Centrifuge.*—Eppendorf 5415C equipped with F-45-18-11 rotor; maximum 16 000 × *g*.

Reagents

All solvents and reagents were analytical or LC grade materials. All mobile phase and postcolumn reagents were filtered through a 0.2 μm membrane before use.

(**a**) *Water.*—Glass-distilled or deionized (DIW).

(**b**) *DIW (pH 5.0).*—Acidify DIW to pH 5.0 by dropwise addition of 10% acetic acid (HOAc).

Table 2. Postcolumn LC system suitability conditions

Toxins	Conditions
GTXs and STXs	Artifact peak must be at least 70% baseline-resolved between GTX3 and GTX2
	GTX5 must be at least 40% baseline-resolved between dcGTX3 and dcGTX2
	dcSTX and STX must be at least 70% baseline-resolved
	GTX4 retention time must be between 5 and 7 min
	STX retention time must be between 17 and 23 min
C toxins	C2 must be at least 70% baseline-resolved between C1 and C3
	C1 retention time must be between 4 and 7 min

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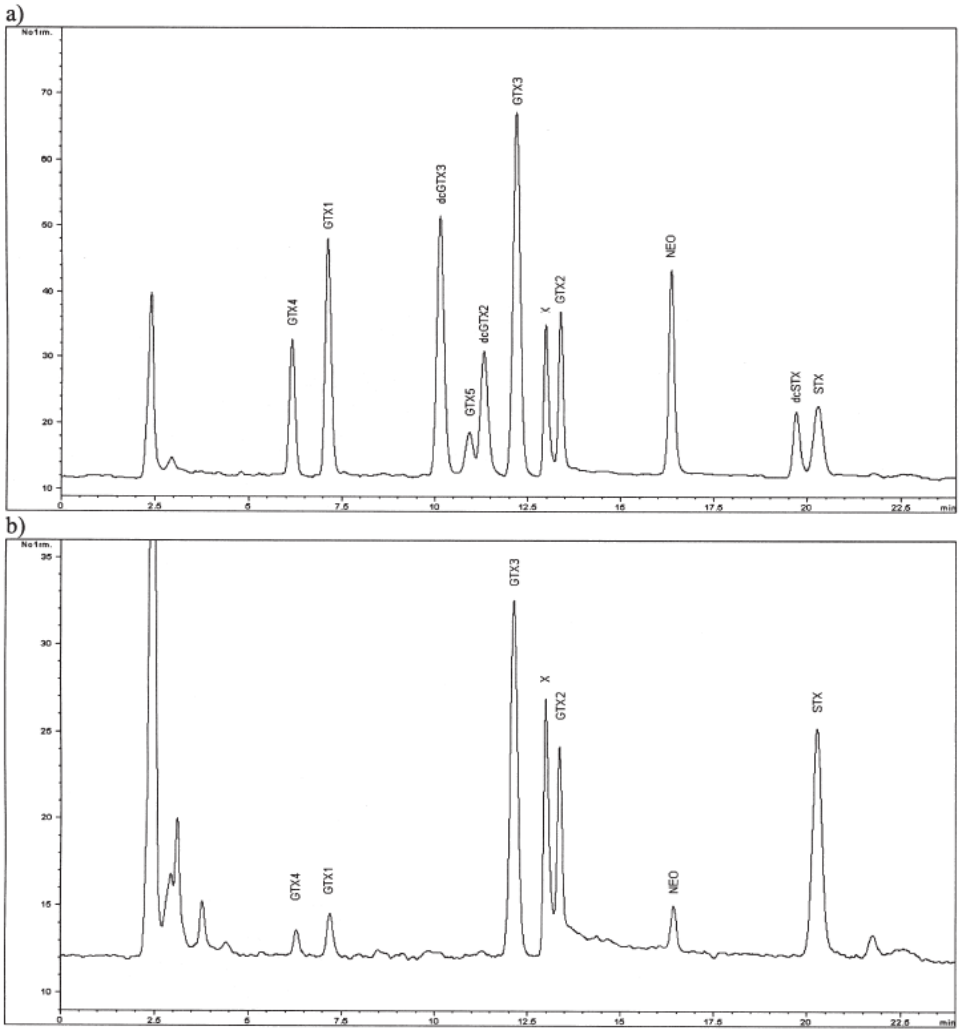

Figure 1. Chromatograms of (a) GTX and STX matrix-matched mixed working solution (10 μL) on an Agilent Zorbax Bonus RP (4.6 × 150 mm, 3.5 μm). Mobile phase: (A) 5.5 mM H_3PO_4, 11 mM heptane sulfonate, pH 7.1. (B) 16.5 mM H_3PO_4, 11 mM heptane sulfonate, pH 7.1 containing 11.5% MeCN. Gradient: 100% mobile phase A for 7.9 min; step to 100% mobile phase B at 8 min; hold for 10.5 min, step to 100% mobile phase A at 18.6 min, 0.8 mL/min. Ox = 5 mM H_5IO_6, 100 mM H_3PO_4, pH 7.8, 0.4 mL/min; H+ = 0.75 M HNO_3, 0.4 mL/min. (b) Mussel sample containing 119 μg STXeq/100 g GTX and STX toxins, conditions as above. In both chromatograms, the artifact peak is labeled "X."

(c) *LC mobile phases (GTXs and STXs).—Solvent A.*—11 mM heptane sulfonate, 5.5 mM phosphoric acid (H_3PO_4) aqueous solution adjusted to pH 7.1 with ammonium hydroxide (NH_4OH). *Solvent B.*—11 mM heptane sulfonate, 16.5 mM H_3PO_4, 11.5% acetonitrile (MeCN) aqueous solution adjusted to pH 7.1 with NH_4OH.

(d) *LC mobile phase (C toxins).*—2 mM tetrabutyl ammonium phosphate aqueous solution adjusted to pH 5.8 using 10% HOAc if too basic or 1% NH_4OH if too acidic. The pH must only be adjusted in one direction, and if the pH is overshot the solution must be remade.

(e) *Postcolumn oxidant.*—100 mM H_3PO_4, 5 mM periodic acid (H_5IO_6) aqueous solution adjusted to pH 7.8 with 5 M sodium hydroxide (NaOH).

(f) *Postcolumn acid.*—0.75 M nitric acid (HNO_3).

(g) *Primary standards.*—National Research Council Canada (NRC) Certified Reference Materials (CRMs) for C1, C2, dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX; NRC in-house reference materials for C3 and C4 (NRC Institute for Marine Biosciences, Halifax, NS, Canada). These CRMs were used as supplied by the NRC. The lack of a specific salt does not imply the free-base form of

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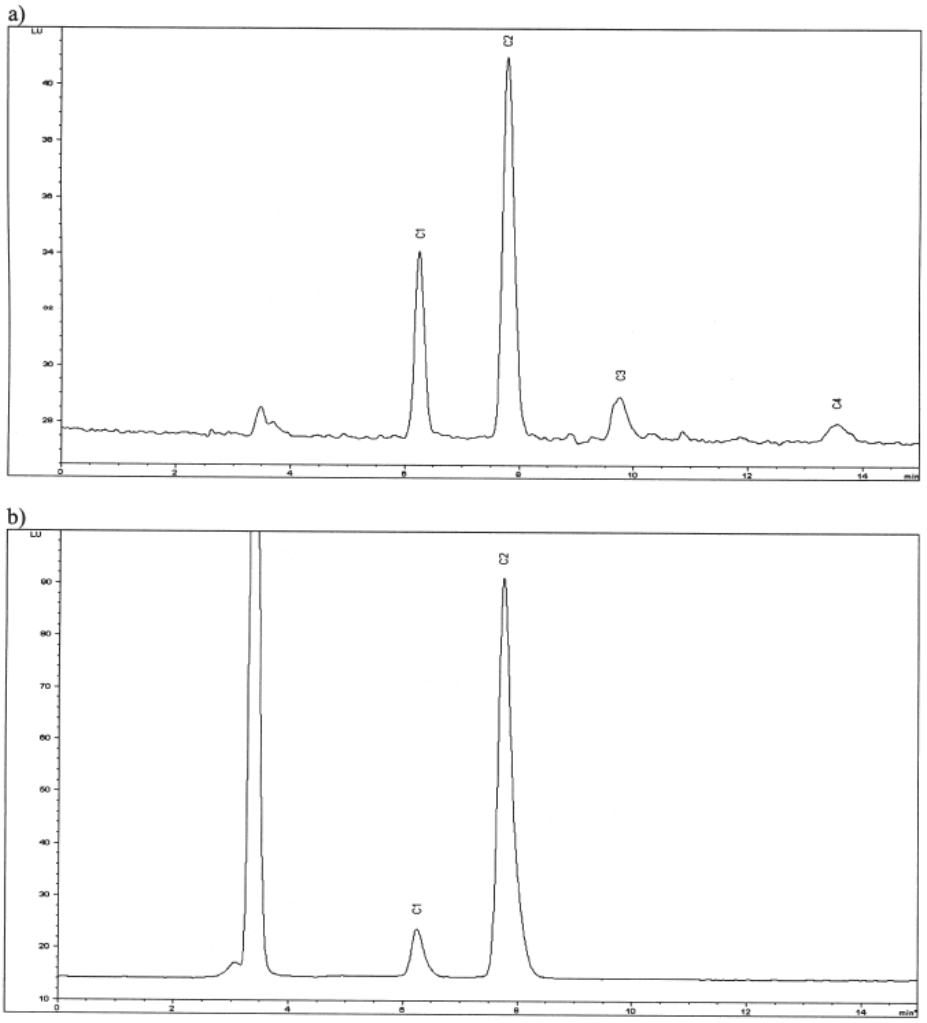

Figure 2. Chromatograms of (a) C toxin mixed working solution (5 μL) on a Thermo BetaBasic 8 (4.6 × 250 mm, 5 μm). Mobile phase (isocratic): 2 mM tetrabutyl ammonium phosphate, pH 5.8, at 0.8 mL/min. Ox = 5 mM H_5IO_6, 100 mM H_3PO_4, pH 7.8, 0.4 mL/min; H+ = 0.75 M HNO_3, 0.4 mL/min. (b) Mussel sample containing 51.4 μg STXeq/100 g C toxins, conditions as above.

toxins in the preceding list, but is simply a list of the toxins used. The NRC has only one form of each toxin available. STX for standardization of MBA was obtained from the U.S. Food and Drug Administration (FDA).

(**h**) *Stock solutions (0.76–31 μM; see Table 1).*—Prepare individual stock standards gravimetrically as per NRC instructions (24). Perform dilutions with 0.003 M HCl for the GTXs and STXs and DIW (pH 5.0) for C toxins.

(**i**) *Neat mixed working solutions (0.269–2.496 μM; see Table 1).*—Prepare 2 solutions, the first containing dcGTX2, dcGTX3, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5, NEO, and STX, and the second containing C1, C2, C3, and C4 (Table 1). Perform dilutions with 0.003 M HCl for the GTXs and STXs and DIW (pH 5.0) for C toxins.

(**j**) *Matrix-matched mixed working solutions.*—Follow the instructions for the neat mixed working solutions but dilute matrix-matched working solutions using a toxin-free mussel extract as the diluent.

Sampling

Samples of shellstock collected during the summer of 2005 as part of the toxin monitoring program of the Canadian Food Inspection Agency, Dartmouth, NS, Canada, were used in this study. The majority of the samples were collected from coastal regions of New Brunswick, Canada; Nova Scotia, Canada; and Prince Edward Island, Canada but also included offshore and imported products. Samples consisted mainly of mussels (*Mytilus edilus*) and clams (*Mya arenaria*) but included a small number of other species such as scallops and oysters. Samples were shucked and analyzed by MBA on receipt. AOAC MBA extracts were stored at 4°C prior to postcolumn LC analysis, and

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Table 3. Method performance statistics for the new postcolumn method and the Lawrence method as applied in the authors' laboratory

Toxins	Lawrence method LOD, μg STXeq/100 g	New postcolumn method LOD, μg STXeq/100 g	New postcolumn method spike recovery, % ± SD[a,b]
GTX4	2.8	1.6	99 ± 13
GTX1	2.8	4.1	112 ± 7
dcGTX3	0.98	0.25	101 ± 8
dcGTX2	0.98	0.67	100 ± 4
GTX5	1.5	0.90	98 ± 5
GTX3	0.80	0.38	102 ± 2
GTX2	0.80	1.5	76 ± 5
NEO	2.8	2.3	106 ± 6
dcSTX	2.0	2.1	102 ± 2
STX	3.0	3.9	100 ± 3
C1	0.002	0.07	100 ± 2
C2	0.002	0.15	95 ± 3
C3	0.05	0.07	NA
C4	0.05	0.41	NA

[a] Average of 5 replicate analyses.
[b] Spiked at approximately 3 × LOD for each toxin.

tissue homogenate was stored at –20°C prior to precolumn LC analysis.

Sample Extraction and Cleanup

Thoroughly clean the outside of the shellfish with fresh water. Shuck the samples onto a No. 10 sieve and drain for 5 min. Homogenize the soft tissue in a standard household blender in preparation for extraction. Prepare a sufficient amount of tissue for MBA, LC-FLD precolumn and postcolumn analyses.

Postcolumn LC-FLD and MBA.—Extract 100 g samples of homogenized shellfish tissue according to the AOAC MBA method (3) using 0.1 M HCl. Store aliquots of the extract in scintillation vials for later injections into mice or for further cleanup and LC postcolumn analysis. Deproteinate samples destined for postcolumn FLD analysis by adding 25 μL 30% (w/v) trichloroacetic acid (TCA) to 500 μL shellfish extract in a microcentrifuge tube. Mix in a Vortex mixer and centrifuge at 16 000 × *g* for 5 min. Add 20 μL 1.0 M NaOH, mix, and centrifuge at 16 000 × *g* for 5 min. Filter through 0.2 μm syringe filter into an autosampler vial in preparation for LC analysis.

Precolumn oxidation LC-FLD.—Extract 5 g homogenized shellfish tissue with 1% HOAc, boil for 5 min, and clean up using C18 solid-phase extraction (SPE) and COOH SPE cartridges according to the method of Lawrence (22) in preparation for LC analysis. Apply the method for "Application of the Method for Routine Analysis" as described by Lawrence (Lawrence screen; 22). If toxins are detected, continue with the full Lawrence method.

LC Postcolumn Determinations

GTX and STX toxins.—Equilibrate the LC system for ≥20 min at a column oven temperature of 40°C with 100% solvent A flowing at 0.8 mL/min. Construct a step gradient as follows: 100% solvent A for 7.9 min; step to 100% solvent B at 8 min; hold for 10.5 min; step to 100% A at 18.6 min; equilibrate for 5.4 min.

C toxins.—Equilibrate the LC system for ≥20 min at a column oven temperature of 20°C with mobile phase flowing at 0.8 mL/min. Operate the system in the isocratic mode.

Postcolumn reaction module.—Oxidant flow rate, 0.4 mL/min; acid flow rate, 0.4 mL/min; reaction oven temperature, 85°C; reaction coil, 5 m × 0.50 mm id.

Inject mixed working solutions (10 μL for GTX and STX toxins and 5 μL for C toxins) to ensure that system suitability conditions (Table 2) are met, and construct a linear regression curve of peak area vs concentration in μM. Inject 10 μL sample extracts, blanks, and spikes for GTX and STX toxins, and 5 μL sample extracts, blanks, and spikes for the C toxins. Calculate the μmoles of STXeq for each toxin in the sample extracts using the linear regression of the calibration graph and the specific relative toxicities of each individual PST (Table 1). For comparison to MBA results, use the following equation to calculate the toxicity in the traditional units of "μg STXeq per 100 g tissue" in the specific case of 0.1 kg tissue being extracted with 0.1 L solvent in a single-step dispersive extraction (final volume = 0.2 L):

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Table 4. Percent relative standard deviation (% RSD) of retention time (RT) and instrument response of repeated injections of PST standard solutions determined by this method

Toxin (μg STXeq/100 g)	RT (% RSD)	Peak area (% RSD)
GTX4 (20.69)	0.11[a]	2.2[a]
GTX1 (85.79)	0.16[a]	1.2[a]
dcGTX3 (4.98)	0.23[a]	3.1[a]
dcGTX2 (7.25)	0.18[a]	2.8[a]
GTX5 (3.45)	0.19[a]	2.4[a]
GTX3 (9.37)	0.07[a]	1.5[a]
GTX2 (15.97)	0.03[a]	1.5[a]
NEO (47.79)	0.24[a]	2.8[a]
dcSTX (13.07)	0.45[a]	1.8[a]
STX (25.19)	0.49[a]	1.8[a]
C1 (0.56)	0.46[b]	7.3[b]
C2 (2.72)	0.85[b]	4.5[b]
C3 (0.17)	1.8[b]	15[b]
C4 (0.58)	2.4[b]	11[b]

[a] Average of five 10 μL injections.
[b] Average of five 5 μL injections.

Sample toxicity (μg STXeq/100 g) =

$$\sum_{i=1}^{n} C_i \times T_i \times \frac{0.20}{0.10} \times \frac{372.2}{2483} \times F \times 0.1$$

This is simplified to:

Sample toxicity (μg STXeq/100 g) =

$$\sum_{i=1}^{n} C_i \times T_i \times F \times 0.03$$

where C_i = concentration of each toxin "i" in micromoles per liter (μM); T_i = specific toxicity of each toxin "i" in mouse units per micromole (MU/μmole); F = 1.16 for MBA data calibrated against the FDA STX solution (if the MBA was calibrated against the NRC standard, a value of F = 1.00 would be used); 372.2 = molecular weight of STXdiHCl (g/mole).

This F factor of 1.16 must be applied when comparing data calibrated against the NRC STX CRM with the MBA data, which has been calibrated against the FDA STX standard (100 μg/mL stated concentration). A concentration of 86 μg STX-diHCl/mL is observed for the FDA STX standard when calibrated using the NRC STX CRM (1).

LC Precolumn Determinations

Inject 50 μL cleaned-up extract and the periodate oxidation of the cleaned-up extract onto a Supelcosil LC-18-DB, 4.6 × 15 cm, 5 μm column as described by Lawrence (22). If toxins are detected, inject periodate and/or peroxide oxidations of required fractions according to Lawrence (22). Quantify each toxin by direct comparison to analytical standards. Calculate the amount of PSTs present as μg STXeq/100 g sample using the PST relative toxicity values as described by Lawrence (22) in order to compare to the MBA. Calculate total toxicity by summing the individual toxin contributions. Apply factor of 1.16 as in postcolumn determinations for comparison with MBA data.

MBA Determinations

Inject 17–23 g mice intraperitoneally with 1 mL HCl extract according to the AOAC Official Method **959.08** (3) and record death times. Calculate the amount of PSTs present as μg STXeq/100 g sample using Sommer's Table (3).

Results and Discussion

A new postcolumn method for the determination of PSTs was developed and compared to AOAC Official Methods for PST determination. Oshima's postcolumn method (18) required 3 injections to quantify the 14 toxins included in this study. The number of injections was decreased to 2 by Thomas et al. (19), but the separation of GTXs and STXs took 60 min, and used a trinary mobile phase system. The GTX and STX toxin method was improved by consolidating the trinary mobile phase system into a binary step gradient, which allowed a decreased run time of 24 min. All GTX and STX toxins studied were baseline-resolved with the exception of GTX5, which was 50% baseline-resolved (Figure 1). The C toxins were baseline-resolved and quantified in <15 min (Figure 2) in an isocratic system very similar to that described by Oshima (18). Differences between the new postcolumn method for C toxin determination and Oshima's method (18) include a different cleanup procedure, a different concentration of tetrabutyl ammonium phosphate, a different LC column, and different oxidation conditions. This study included 14 currently available commercial standards. An additional standard, decarbamoylneosaxitoxin (dcNEO), was not included at this time due to co-elution with NEO under the rapid separation system. It is possible to resolve dcNEO and NEO with a 75 min trinary step gradient (19). The oxidation products of dcNEO co-elute with the oxidation products of both dcSTX and STX when the Lawrence method is used (B. Niedzwiadek, Health Canada, Ottawa, ON, Canada, personal communication, 2006). From a regulatory perspective, this was not a major issue in the postcolumn method, as the relative toxicity of dcNEO is less than that of NEO. The worst case scenario would be a slight overestimation of total toxicity, further protecting the consumer. Gonyautoxin-6 (GTX6) was not included in this study due to the lack of standard availability, but elutes

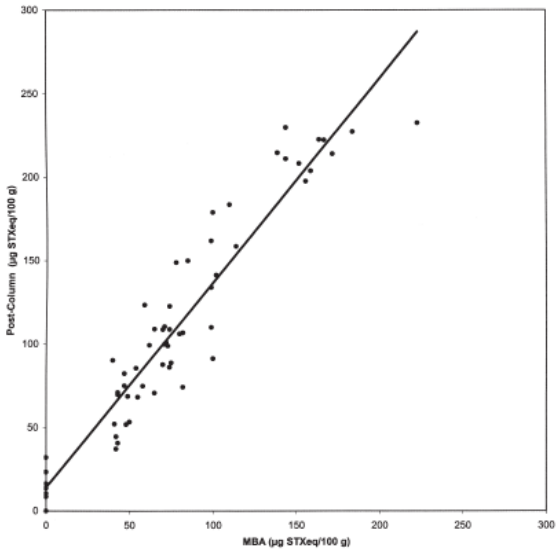

Figure 3. Correlation between results of the MBA method and the new postcolumn method for samples up to 250 µg STXeq/100 g; $y = 1.22x + 13.99$; $r^2 = 0.86$.

immediately before GTX4 under the described chromatographic conditions.

PSTs were extracted using the AOAC MBA method (3) for postcolumn LC analysis; therefore, the toxin profile quantified using the postcolumn method was very similar to that injected into the mouse. Protein remaining in the AOAC MBA extract can be trapped on column frits, leading to the rapid development of backpressure, an attendant decrease in column performance, and possible damage to LC pumping systems. TCA was used to remove protein from the AOAC MBA extract and, in so doing, extended column life. Some concern was expressed that the use of TCA might change the toxin profile even though the pH was returned to its original level quickly. No differences were observed in the toxin profiles following treatment with TCA. However, treatment with TCA increased column life so that approximately 600 samples could be analyzed before significant deterioration of the column was observed. Without TCA treatment, column deterioration is evident after approximately 100 samples have been analyzed.

The LC system performed reliably and was simply shut down at the end of each daily run. No problems were associated with start-up the next day. The postcolumn system (pumps and reaction coil) was flushed once a week with 0.75 M HNO_3 followed by DIW. As a precaution, the column was removed from the LC and the entire fluid path was flushed with 10% MeCN in DIW to prevent line blockage due to the precipitation of buffers. If the system is to be shut down for extended periods, it is recommended that the pumps are not left in the harsh acid or oxidant environment. Following these maintenance procedures, no problems were experienced other than the very minor difficulties that are typically encountered with modern LC pumping systems.

The maximum sample throughput of the new postcolumn method and the Lawrence method was compared because of its importance in a regulatory environment. A single LC system could analyze 31 samples per 24 h period with the postcolumn method, including attendant standards and quality assurance samples. In those situations where the Lawrence screen could be used, approximately 40 samples could be processed in a 24 h period. However, if positive samples are encountered, as is the case in our laboratory where approximately 30% of samples received are positive for PSTs, a combination of the Lawrence screen and full methodologies is required. Using a combination of full and screen methodologies allows only an average of 16 samples to be processed each day. In addition, results from those samples requiring the full method will be delayed up to a further 24 h while the COOH SPE fractions are prepared and oxidized prior to LC analysis. This is a major limitation of the Lawrence methodology in a regulatory environment (23).

Limits of detection (LODs) of the new postcolumn method and the Lawrence method are shown in Table 3. LODs ranged from 0.07 µg STXeq/100 g for C3 to 4.1 µg STXeq/100 g for GTX1 for the postcolumn method. This compared quite favorably with detection limits for the Lawrence method, which ranged from 0.002 µg STXeq/100 g for C1, C2 to 3.0 µg STXeq/100 g for STX as applied in our laboratory. Adequate detection capability for regulatory purposes was supplied by both LC methods. A spiking study near the limit of quantitation for individual toxins demonstrated that the new postcolumn method recovered between 76% (GTX2) and 112% (GTX1) of toxins (Table 3). No spiking data are currently available for C3 or C4 due to the limited supply of standards, but these recoveries are expected to fall within the range of recoveries for other toxins examined.

A calibration graph for STX was linear between 0.18 and 5.9 ng STX injected, which was equivalent to 3.9–128 µg STXeq/100 g in tissue. Calibration graphs for other toxins showed very similar results. The correlation coefficients of the calibration graphs for all toxins ranged from 0.999 to 1.00. Stock and working solutions of GTXs and STXs were stored at 4°C; stock and working solutions for C toxins were stored at ≤–20°C. Standard solutions have been stored for >12 months with no noticeable deterioration.

Working standards were prepared using a mussel tissue extract to assist in the identification of toxins present in the samples, as matrixes caused a slight positive retention time shift for GTX4 and GTX1 in the new postcolumn method. Exact matrix matching of standards was not required for any matrixes studied, including various species of mussels, clams, scallops, and oysters. Matrix-matched standards assisted in resolving interfering peaks, as most samples have an artifact peak (Figure 1, peak X) corresponding to the step gradient solvent front. This artifact peak did not contain any toxins included in this study and was generally well resolved, but over time may co-elute with GTX3 or GTX2. It was found

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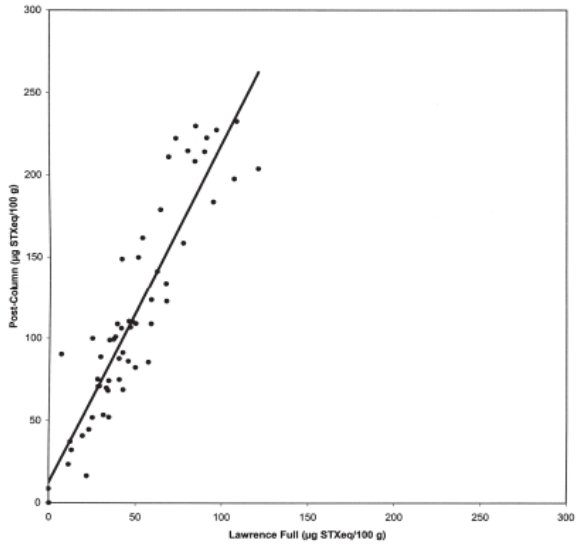

Figure 4. Correlation between results of the full Lawrence method and the new postcolumn method for samples up to 250 µg STXeq/100 g; $y = 2.06x + 12.21$; $r^2 = 0.82$.

that a temperature adjustment of <±5°C easily resolved all 3 peaks with no significant impact on overall run time or separation of other toxins. This adjustment may be effective for up to several weeks, depending on column usage. The first injection each day should contain GTX2, GTX3, and the artifact peak (matrix standard or check sample), and this injection will be used to adjust the column temperature to meet system suitability criteria. The elution conditions (gradient step time, column temperature) must be confirmed each time a new column is used, and after this only small changes to column temperature should be needed. No unresolvable interferences were observed in any of the mussel, clam, oyster, or scallop samples tested. Retention times were stable; the relative standard deviation (RSD) varied from 0.03 to 2.4% (Table 4). Replicate injections of standard and tissue extract solutions indicated good peak response repeatability over the range of concentrations studied with RSDs ranging from 1.2 to a maximum of 15% (Table 4). Quantification was based on peak areas. The method showed good within-laboratory reproducibility; a mussel tissue extract containing 195 µg STXeq/100 g analyzed over 21 days showed an RSD of only 4.6%. The uncertainty of measurement based on precision data for the same mussel tissue extract was 9%. Repeatability RSD of a 65 µg STXeq/100 g mussel tissue analyzed 5 times was 3.0%.

The MBA has a long successful history of preventing consumer illnesses and deaths. Therefore, equivalency to the MBA is essential. More than 50 positive shellfish samples with MBA results between 40 and 223 µg STXeq/100 g were analyzed by MBA, pre- and postcolumn methods. The MBA results were plotted against the postcolumn results in Figure 3; the slope was 1.22 and the correlation coefficient was 0.86. It was expected that the postcolumn results would be slightly higher than the MBA results. It has been reported widely that salt effects lead to an underestimation of the toxicity of shellfish especially with samples near the MBA detection limit (7, 18, 25). The vast majority of samples with MBA results near the regulatory limit show very similar postcolumn results.

The Lawrence method has been approved by AOAC as the first official LC method for PSTs (22). The comparison of MBA and Lawrence screen results exhibited a slope of 0.79 and a correlation coefficient of 0.36. Although the correlation was poor, samples with higher MBA values generally produced higher values in the Lawrence screen method. This points out the necessity of running the full Lawrence method when PSTs are detected if accurate results are to be obtained. The MBA is known to have a large variation (17, 26), due in large part to the fact that it uses a biological system. It was expected that the results from the pre- and postcolumn methods would be quite comparable since neither method uses a biological system. Figure 4 compares the full Lawrence method and the postcolumn method results. A slope of 2.06 indicates that the postcolumn results were approximately 50% higher than the results of the full Lawrence method but the correlation coefficient was good (0.82). Lawrence and Menard (27) initially noted this trend of postcolumn methods producing higher results than precolumn methods. Experiments carried out to determine where toxicity might be lost while using the Lawrence method highlighted 3 stages for potential toxin loss. Standard solutions and positive samples were extracted using the Lawrence method, and monitored at various stages using the new postcolumn LC system. In our laboratory, approximately 7% of the total toxicity was lost during C18 SPE cartridge cleanup, 11% was lost to the pH adjustment after the C18 SPE, and an additional 11% was lost during the COOH SPE cleanup. These losses totaled 29% of overall toxicity, resulting from the full Lawrence cleanup procedure. Correcting for these losses provided a simple solution and provided a corrected slope of 1.4 with the new postcolumn data. There is also an expected difference due to different extractant acids. The HOAc extraction used by the Lawrence method is milder than the HCl extraction used by the AOAC MBA method and is not subject to the Proctor enhancement, which converts *N*-sulfocarbamoyl toxins to the more toxic carbamate forms (28).

Both LC methods were compared in our laboratory to determine the pros and cons of each method in a regulatory environment. The positive aspects of the postcolumn method were easier interpretation of data, separation of all analytes tested, and faster turnaround times for positive samples (31 versus 16 samples/day/LC system assuming a 30% positive rate). The precolumn advantages were excellent chromatographic performance, faster turnaround time when most samples tested negative for PSTs, and no postcolumn system required. One concern with the Lawrence method is the possibility of a single sample accidentally not being

oxidized; a sample would be reported as a false negative if it was not oxidized. Caution must be exercised to ensure that the proper volumes and reagents have been added to each vial before LC injection. Although the postcolumn equipment has a few additional moving parts which may fail in day-to-day operation, postcolumn system failure is very obvious, as all standards, spikes, and control samples would also be affected. The total analysis cost (capital purchases and consumables) for the new postcolumn method was less than that of MBA analysis if capital costs are depreciated over 7 years. The Lawrence screen cost approximately the same as MBA analysis and the full Lawrence method was nearly triple the cost of MBA analysis, due to increased consumable costs (SPE cartridges, filters).

Both the pre- and postcolumn methods have demonstrated that they are viable alternatives to MBA analysis. These LC methods effectively measured the toxin content in shellfish tissue containing a variety of toxin profiles. The main advantages of the new postcolumn method in a regulatory setting were higher throughput and faster turnaround of positive samples. The speed of analysis provided by this method is essential in a regulatory environment where decisions are required on a timely basis.

Future work will concentrate on running the new postcolumn method in parallel with the MBA over one shellfish season to ensure that the method is robust, reliable, and accurate and can be counted upon to protect the health and safety of consumers. Approximately 1000 samples have been analyzed concurrently with no significant problems. Validation data for additional toxins will be generated when standards become available, and alternate extraction methods which may reduce turnaround time will be evaluated.

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