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

Justification for New Method

- Name of the New Method.
  Saxitoxin (PSP) Microtiter Plate Test Kit.

- Specify the Type of Method.
  Enzyme linked immunosorbent assay (ELISA) using anti-saxitoxin polyclonal antibody.

- Name of Method Developer.
  Drs. Titan Fan and Byungchul Kim

- Developer Contact Information.
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- Date of Submission.
  June 26, 2013

- Purpose and Intended Use of the Method.
  Rapid analysis of saxitoxin (PSP) from shellfish such as blue mussels, steamers and mahogany clams. This method can be used for screening purpose that screens out negative samples (below 30 µg/100g). Suspicious samples with PSP levels between 30 and 100 µg/100g will need confirmation with NSSP Approved Method, mouse bioassay (MBA). PSP levels higher than 100 µg/100g would be considered as positive, and may not need further confirmation.

- Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.
  The regulatory methods for PSP toxins are mouse bioassay (MBA) as NSSP Approved Method, and high performance liquid chromatography (HPLC) and liquid chromatography with post-column oxidation (PCOX) as NSSP Approved Limited Use Method. These methods are laborious, time consuming and expensive. Using these regulatory methods, it is difficult to process large amount of shellfish samples with limited resources. Therefore, there is a need of screening technique prior to the regulatory method that can screen out negative shellfish samples containing low levels of PSP (below 30 µg/100g). Only suspicious samples with PSP levels between 30 µg/100g and 100 µg/100g need further confirmation test with mouse bioassay. Therefore, this screening procedure will dramatically reduce the volume of samples to be confirmed with MBA, and save time and resources for the private, certified or state laboratories.

- Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.

Due to extremely high sensitivity of the method, sample can be easily diluted with buffer solution (total 15000 folds). This high degree of sample dilution results in reduction in sample preparation time and elimination of any potential matrix effects either positive or negative from shellfish samples. Therefore, it is possible that this method could be used for any shellfish species for the determination of PSP level.

- Other Comments.

Method Documentation

- Method Title.
  Saxitoxin (PSP) Microtiter Plate Test Kit.

- Method Scope.
  The method is a competitive enzyme linked immunosorbent assay (ELISA) for the detection of saxitoxins in blue mussels, steamers and mahogany clams from North Atlantic Ocean as an Approved Limited Use Method.

- References.
  1. B. J. Yakes, S. M. Prezioso, S. L. DeGrasse. Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta 2012, 99, 668-676.
  2. G. B. Inami, C. Crandall, D. Csuti, M. Oshiro, R. Brenden. Presence/Absence Screening for Saxitoxin in Frozen Acidified Mussel and Oyster Extracts from the Coast of California with In Vitro Methods, J AOAC. Int. 2004, 87 (5), 1133-1142.
  3. E. Usleber, R. Dietrich, C. Burk, E. Schneider, E. Martlbauer. Immunoassay Methods for Paralytic Shellfish Poisoning Toxins. J. AOAC. Int. 2001, 84 (5), 1649-1656.
  4. Anderson, D.M., P. Andersen, V. M. Bricelj, J. J. Cullen, and J. EE. Rensel, 2001. Monitoring and management strategies for harmful algal blooms in coastal waters, APEC #201-MR-01.1, Asia Pacific Economic Program and Intergovernmental Oceanographic Commission Technical Series No. 59, Paris.
  5. Fun S. Chu and Titan S. L. Fan. Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish. Journal-Association of Official Analytical Chemists 1985, 68 (1):13-16.

- Principle.
  The Beacon Saxitoxin (PSP) Microtiter Plate Kit is a competitive enzyme-labeled immunoassay. The Saxitoxin HRP conjugate, sample extract and calibrators are pipetted into the test wells followed by Saxitoxin antibody into the test wells to initiate the reaction. During the 30 minute incubation period, PSP toxins from the sample and Saxitoxin HRP conjugate compete for binding to Saxitoxin antibody. The Saxitoxin antibody is captured on the walls of the test well. Following this 30 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound PSP toxins, Saxitoxin HRP conjugate and free Saxitoxin antibody. After wash, a clear substrate is then added to the wells and any bound enzyme conjugate causes the conversion to a blue color. Following a 30 minute incubation, the reaction is stopped and the amount of color in each well is read. The color of the unknown samples is compared to the color of the calibrators and the Saxitoxin concentration of the samples is derived.

- Analytes/Measurands.
  Paralytic shellfish poisoning toxins (Saxitoxin, Neo-saxitoxin, Decarbamoylsaxitoxin, Gonyautoxin-2 and -3)

- Proprietary Aspects.
  Beacon Analytical Systems developed the kit including antibody and enzyme conjugate.

- Reagents provided.
  Antibody coated microplate
  Calibrators
  Enzyme conjugate
  Anti-saxitoxin rabbit polyclonal antibody
  Wash solution concentrate
  Substrate
  Stop solution

- Materials required but not provided.
  Laboratory quality distilled or deionized water
  20 mM Phosphate buffered saline
  Pipet with disposable tips capable of dispensing 50 µL
  Multi-channel pipet; 8-channel capable of dispensing 50 and 100 µL
  Paper towels or equivalent absorbent material
  Microwell plate or strip reader with 450nm filter
  Timer
  Wash bottle

- Media.
  A mixture of 70% Isopropanol and 5% Acetic acid in a ratio of 2 to 1 is used to extract PSP toxins from shellfish homogenate. To dilute the extract, 10% Methanol in 20 mM PBS buffer (10 mL of Methanol + 90 mL of 20 mM PBS) is used.
  Phosphate buffered saline with tween 20 (PBST) is used for washing.

- Matrices of Interest.
  Blue mussel (*Mytilus edulis*), steamer (*Mya arenaria*) and mahogany clam (*Arctica islandica*).

- Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.
  Preparation: Shellfish (12 animals) are shucked, rinsed and homogenized with a kitchen blender. Five grams (5 g) of homogenate is weighed in a 50 mL centrifuge tube, and 10 mL of extraction solvent (70% Isopropanol + 5% acetic acid solution, 2 + 1) is added. The tube is vortexed for 3 min. Approximately 1 mL of the extract is transferred into a microcentrifuge tube, and centrifuged for 5 min at 12,000 rpm. The supernatant is diluted with 10% Methanol in 20 mM PBS buffer to 5000 folds (e.g. 0.1 mL of supernatant + 9.9 mL of buffer and 0.1 mL of the diluted solution + 4.9 mL of buffer), and ready for the analysis.

- Safety Requirements.
  Personal safety items such as safety glasses, gloves and lab coat must be required. Calibrators and enzyme conjugate are toxic materials, and must be kept in the original vials when they are

not used. Since stop solution is a strong acidic solution (1 N hydrochloric acid), skin or eye contact must be avoided. Lab items with contact of toxins (sample extract and calibrator) such as pipette tips and lab wares must be soaked in 50% dilution of house bleach at least 1 hour before washing or disposal.

- Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]).
    - Cost of the method: Saxitoxin (PSP) Microtiter Plate Test Kit costs $325 and can test up to 44 samples in duplicate (approximately $7 per sample)
    - Special technical skills: Experience in ELISA assay would be preferable but not necessary. Basic lab experience is recommended such as pipetting and safety training.
    - Special equipment and cost:

Single channel micropipette (200 and 1000 µL)	$560
Multi channel micropipette (either 200 or 300 µL)	$680
Microplate reader (capable of reading at 450 nm, StatFax 303+)	$2,100

    - Abbreviations and acronyms:
      PSP – Paralytic Shellfish Poisoning
      ELISA – Enzyme Linked ImmunoSorbent Assay
      HRP – Horse Radish Peroxidase
      PBS – Phosphate Buffered Saline
      MBA – Mouse BioAssay
      HPLC-PCOX – High Performance Liquid Chromatography with Post-Column OXidation
    - Turnaround time: More than 200 samples can be tested in a day (8 hours). MBA and HPLC-PCOX may test up to 60 and 15 samples, respectively. (Reference 4, Page 33)

- Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures and indicate critical steps.). Please refer to the instructional booklet (Appendix).
    1. Prepare the 1X wash solution by adding the contents of the 10X wash concentrate bottle to 450 mL Lab grade water in a wash bottle.
    2. Allow reagents and sample extracts to reach room temperature prior to running the test. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
    3. Using a pipet with disposable tips, add 50 µL enzyme conjugate to the appropriate test wells. Be sure to use a clean pipet tip for each.
    4. Add 50 µL of Calibrators or Sample extract to each well. Dispense 50 µL of Antibody Solution into each test well. Shake the plate gently for 30 seconds and incubate the test wells for 30 minutes.
    5. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and dump. Repeat 3X for a total of four washes.
    6. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the wash solution. Dispense 100 µL of Substrate, and incubate for 30 minutes.
    7. Dispense 100 µL of Stop Solution into each test well. Read and record the absorbance of the wells at 450nm using a strip or plate reader.
    8. The concentration of PSP toxins in the sample is calculated based on the calibration curve (4-parameter fit). The dilution factor of 15000 must be applied to the calculated concentration (e.g., 1 ppb as calculated concentration X 15000 = 15000 ppb as real

concentration of PSP in sample). Then, the value in ppb (µg/1000 g) can be converted to µg/100 g by dividing by 10. For people who don't have 4-parameter fit in their readers, the Microsoft spreadsheet for the calculation would be provided upon request (Attached separately).

- Quality Control (Provide Specific Steps).
  Coefficient of variation (CV, %) of the results from duplicate wells for each test should be below 15%. If this CV (%) is not calculated by the reader, it can be manually calculated as standard deviation divided by average, and then multiply by 100. Coefficient of determination (R^2) from the calibration curve must be higher than 0.990, which indicates the assay performs accurately. Absorbance of zero calibrator should not be higher than 2.5. Customized QC protocol can be developed with assistance from Beacon Analytical Systems.

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

  Accuracy/Trueness
  The range chosen is 5 to 160 µg/100 g because any results higher than 100 µg/100 g are considered as positive. There is no reason to test the accuracy and trueness at such high levels.
  Sample type is shellfish tissue. Shellfish samples were purchased from a local fish market (Portland, Maine) and tested prior to the study to see if any considerable levels of PSP toxins are found. Only negligible levels of PSP were found (less than 3 µg/100 g). Samples used for spiking are blue mussel (*Mytilus edulis*), steamer (*Mya arenaria*) and mahogany clam (*Arctica islandica*). Since the regulatory limit of PSP toxin is 80 µg/100 g, a broad range of saxitoxin levels were spiked between 5 and 160 µg/100 g. The standard saxitoxin used for the spike was purchased from NIST (RM 8642, FDA Saxitoxin Dihydrocholoride Solution, National Institute of Standards and Technology). Detailed procedures are as below.

  1. Twelve animals (mussel, steamer or mahogany clam) were shucked and homogenized in a kitchen blender and stored in -20 °C freezer.
  2. Five grams of the homogenate was weighed in 50 mL centrifuge tube and saxitoxin was added into the tube so that the final concentration in 5 g is 5, 10, 20, 30, 40, 50, 55, 60, 65, 70, 75, 80, 90, 100, 110, 120, 130, 140, 150 or 160 µg/100 g.
  3. Extraction solvent of 10 mL was added into the tube, and vortexed for 3 minutes.
     (Extraction solvent is a mixture of 70% Isopropanol and 5% Acetic acid in a ratio of 2 to 1)
  4. Transfer 1 mL of extract into a microcentrifuge tube to spin at 12,000 rpm for 5 minutes.
  5. Mix 0.1 mL of the supernatant with 9.9 mL of 10% Methanol/20 mM PBS buffer for 100 times dilution, and transfer 0.1 mL of the diluted extract into a 4.9 mL of buffer solution. to make the final dilution of 5000 times. This diluted extract was used for the assay. As directed in the instructional booklet, multiply the assay result by 15000 in order to compensate the dilution. This results in saxitoxin concentration in the shellfish tissue (ppb). Then, the value in ppb (µg/1000 g) can be converted to µg/100 g by dividing by 10.

  Ruggedness

Sample type is shellfish tissue. Organisms used for spiking are blue mussel (*Mytilus edulis*), steamer (*Mya arenaria*) and mahogany clam (*Arctica islandica*). Spike and extraction were conducted same as Accuracy/Trueness. Spike levels chosen were 10, 20, 40, 50, 60, 70, 80, 90, 100 and 120 µg/100 g. However, two aliquots from each sample were tested with two different lots of the kit.

Precision

Working range, sample type and organism used for spiking are same as Accuracy/Trueness. Spike and extraction were conducted same as Accuracy/Trueness. However, each sample was spiked with three different levels. Spike levels chosen were 20, 80 and 160 µg/100 g. Each level of spiked sample was analyzed twice.

Recovery

Same sample procedure as Precision test.

Specificity

The cross reactivity was evaluated by US FDA. Betsy Jean Yakes, Samantha M. Prezioso, Stacey L. DeGrasse. Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta (2012), 99, 668-676.

Linearity/Limit of Detection/Limit of Quantitation/Sensitivity

Sample type is shellfish tissue. Working range is 10 to 240 µg/100 g. Range of interest is 20 to 80 µg/100 g. Range in spiking levels used is 5 to 240 µg/100 g. For the linearity, STX standards were used (0, 0.01, 0.08 and 0.32ppb), and this range of calibrators covers the saxitoxin levels of 15 through 480 µg/100 g in sample when the dilution was performed as directed in the instructional booklet. For the LOD, LOQ and sensitivity, blue mussel, steamer and mahogany clam were used for spiking. Spike and extraction were conducted same as Accuracy/Trueness. However, each sample was spiked with six different levels including zero. Ten samples were prepared. Spike levels chosen for each sample were 0, 5, 20, 80, 160 and 240 µg/100 g. Each spike level of the samples except zero was analyzed in triplicate.

Table 1. Accuracy/Trueness for blue mussel.

Mussel	Saxitoxin (µg/100 g)				
Sample	Spike level	Determined	Blank	Blank subtracted	Difference
A	5	5.86	1.72	4.14	0.86
B	10	11.19	1.64	9.55	0.45
C	20	20.73	1.56	19.17	0.83
D	30	31.85	1.89	29.96	0.04
E	40	41.88	1.71	40.17	-0.16
F	50	56.23	2.16	54.07	-4.07
G	55	63.23	2.16	61.07	-6.07
H	60	63.65	2.08	61.57	-1.57
I	65	70.6	2.18	68.42	-3.42
J	70	75.28	2.3	72.98	-2.98
K	75	83.61	2.12	81.49	-6.49
L	80	85.27	2.02	83.25	-3.25
M	90	90.82	2.05	88.77	1.23
N	100	122.34	2.27	120.07	-20.07
O	110	124.91	2.18	122.73	-12.73
P	120	138.26	2.35	135.91	-15.91
Q	130	151.76	2.31	149.45	-19.45
R	140	157.85	2.38	155.47	-15.47
S	150	180.79	2.64	178.15	-28.15
T	160	191.12	2.52	188.60	-28.60
Average	78	88.36	2.11	86.25	-8.25
Standard dev.					9.78
Accuracy/Trueness (%)	111				
Measurement uncertainty (µg/100 g)	4.29				

Table 2. Accuracy/Trueness for steamer.

Steamer	Saxitoxin (µg/100 g)				
Sample	Spike level	Determined	Blank	Blank subtracted	Difference
A	5	5.56	1.01	4.55	0.45
B	10	10.61	0.89	9.72	0.28
C	20	19.74	0.67	19.07	0.93
D	30	29.68	0.81	28.87	1.13
E	40	41.02	0.91	40.11	-0.11
F	50	53.74	1.21	52.53	-2.53
G	55	59.27	0.86	58.41	-3.41
H	60	58.31	1.16	57.15	2.85
I	65	67.78	1.17	66.61	-1.61
J	70	73.17	1.17	72.00	-2.00
K	75	76.69	1.07	75.62	-0.62
L	80	70.02	1.29	68.73	11.27
M	90	93.12	1.11	92.01	-2.01
N	100	102.03	1.24	100.79	-0.79
O	110	115.1	1.69	113.41	-3.41
P	120	127.62	1.61	126.01	-6.01
Q	130	131.82	1.78	130.04	-0.04
R	140	146.65	1.73	144.92	-4.92
S	150	161.68	1.79	159.89	-9.89
T	160	179.26	1.79	177.47	-17.47
Average	78	81.14	1.25	79.90	-1.90
Standard dev.					5.48
Accuracy/Trueness (%)	102				
Measurement uncertainty (µg/100 g)	2.40				

Table 3. Accuracy/Trueness for mahogany clam.

Mahogany clam	Saxitoxin (µg/100 g)				
Sample	Spike level	Determined	Blank	Blank subtracted	Difference
1	5	7.00	1.62	5.38	-0.38
2	10	11.72	1.62	10.10	-0.1
3	20	19.59	1.62	17.97	2.03
4	30	30.33	1.95	28.38	1.62
5	40	41.32	2.01	39.31	0.69
6	50	54.72	1.86	52.86	-2.86
7	55	60.24	1.84	58.40	-3.40
8	60	62.42	2.26	60.16	-0.16
9	65	64.70	2.27	62.43	2.57
10	70	70.83	2.38	68.45	1.55
11	75	76.86	2.55	74.31	0.69
12	80	81.45	2.29	79.16	0.84
13	90	92.70	2.64	90.06	-0.06
14	100	103.11	2.61	100.50	-0.50
15	110	113.75	2.29	111.46	-1.46
16	120	132.55	2.58	129.97	-9.97
17	130	137.53	2.56	134.97	-4.97
18	140	138.86	2.39	136.47	3.53
19	150	150.25	2.54	147.71	2.29
20	160	170.89	2.63	168.26	-8.26
Average	78	81.04	2.23	78.82	-0.82
Standard dev.					3.44
Accuracy/Trueness (%)	101				
Measurement uncertainty (µg/100 g)	1.55				

Table 4. Ruggedness analysis.

	Mussel (µg/100 g)		Steamer (µg/100 g)		Mahogany (µg/100 g)	
Sample	Lot #1	Lot #2	Lot #1	Lot #2	Lot #1	Lot #2
1	9.75	9.84	8.58	8.61	10.89	12.71
2	17.66	18.12	14.09	15.73	21.74	21.14
3	33.46	33.97	32.92	32.95	39.91	39.69
4	46.52	45.17	42.39	41.12	49.29	55.45
5	57.9	56.55	48.16	50.02	62.33	69.42
6	65.32	66.89	61.67	64.72	79.93	70.44
7	77.52	74.98	69.05	67.32	84.86	87.97
8	79.31	78.15	72.38	76.77	88.01	97.9
9	90.31	98.38	82.33	87.42	101.3	103.77
10	112.45	116.24	102.51	103.14	130.54	124.97
Skewness	-0.04	0.10	-0.04	-0.05	0.06	-0.12
Variance	1064.05	1157.37	893.83	942.89	1378.60	1337.38
Ratio of variances	1.09		1.05		1.03	
P-value (Paired t-test)	0.43		0.09		0.45	
Significant difference	NO		NO		NO	

Table 5. Precision for blue mussel.

Sample	Concentration	Determination	STX Score	Sample	Concentration	Determination	STX Score
1	L*	1	20.32	6	L	1	15.54
1	L	2	24.59	6	L	2	21.30
1	M*	1	81.33	6	M	1	83.87
1	M	2	79.36	6	M	2	86.25
1	H*	1	162.73	6	H	1	175.71
1	H	2	152.45	6	H	2	168.75
2	L	1	19.77	7	L	1	16.99
2	L	2	14.40	7	L	2	19.75
2	M	1	78.70	7	M	1	86.41
2	M	2	79.60	7	M	2	85.06
2	H	1	149.92	7	H	1	172.50
2	H	2	158.63	7	H	2	167.14
3	L	1	25.15	8	L	1	21.48
3	L	2	18.34	8	L	2	21.10
3	M	1	72.79	8	M	1	85.97
3	M	2	76.45	8	M	2	86.22
3	H	1	152.89	8	H	1	166.83
3	H	2	152.19	8	H	2	170.77
4	L	1	23.05	9	L	1	23.24
4	L	2	18.48	9	L	2	24.06
4	M	1	73.24	9	M	1	84.01
4	M	2	74.61	9	M	2	92.05
4	H	1	149.37	9	H	1	170.87
4	H	2	141.79	9	H	2	171.03
5	L	1	20.60	10	L	1	20.16
5	L	2	16.54	10	L	2	17.87
5	M	1	75.65	10	M	1	84.29
5	M	2	78.27	10	M	2	86.37
5	H	1	152.04	10	H	1	175.94
5	H	2	158.34	10	H	2	170.48

*L; Low level spike (20 µg/100 g), M; Medium level spike (80 µg/100 g), H; High level spike (160 µg/100 g)

Table 6. Nested ANOVA for blue mussel sample.

Source of variation	DF	SS	MS	F value
Samples	9	1384.16	153.80	0.02
Concentrations in samples	20	203507	10175	894.24
Determinations within concentrations	30	341.36	11.38	
Total	59	205233	3478.52	

Table 7. Precision for steamer.

Sample	Concentration	Determination	STX Score	Sample	Concentration	Determination	STX Score
1	L	1	18.68	6	L	1	16.87
1	L	2	23.16	6	L	2	21.63
1	M	1	77.46	6	M	1	81.76
1	M	2	76.23	6	M	2	82.96
1	H	1	154.04	6	H	1	167.80
1	H	2	154.51	6	H	2	163.75
2	L	1	20.14	7	L	1	18.73
2	L	2	21.66	7	L	2	24.59
2	M	1	85.17	7	M	1	83.62
2	M	2	79.32	7	M	2	80.45
2	H	1	160.30	7	H	1	155.48
2	H	2	165.08	7	H	2	165.04
3	L	1	23.13	8	L	1	23.69
3	L	2	19.95	8	L	2	20.61
3	M	1	79.99	8	M	1	87.19
3	M	2	80.85	8	M	2	87.25
3	H	1	153.02	8	H	1	169.35
3	H	2	151.95	8	H	2	166.76
4	L	1	26.14	9	L	1	23.20
4	L	2	23.63	9	L	2	26.76
4	M	1	82.59	9	M	1	85.59
4	M	2	84.22	9	M	2	92.55
4	H	1	163.35	9	H	1	174.61
4	H	2	155.64	9	H	2	167.74
5	L	1	21.51	10	L	1	20.71
5	L	2	23.76	10	L	2	22.71
5	M	1	78.95	10	M	1	88.30
5	M	2	81.70	10	M	2	88.46
5	H	1	168.83	10	H	1	171.01
5	H	2	160.60	10	H	2	169.67

Table 8. Nested ANOVA for steamer sample.

Source of variation	DF	SS	MS	F value
Samples	9	666.39	74.04	0.01
Concentrations in samples	20	199960	9997.98	1093.56
Determinations within concentrations	30	274.28	9.14	
Total	59	200900	3405.09	

Table 9. Precision for mahogany clam.

Sample	Concentration	Determination	STX Score	Sample	Concentration	Determination	STX Score
1	L	1	16.52	6	L	1	22.28
1	L	2	23.15	6	L	2	22.54
1	M	1	81.58	6	M	1	78.12
1	M	2	78.26	6	M	2	79.69
1	H	1	155.89	6	H	1	171.77
1	H	2	158.16	6	H	2	162.53
2	L	1	20.78	7	L	1	21.89
2	L	2	19.63	7	L	2	27.09
2	M	1	80.07	7	M	1	89.17
2	M	2	74.56	7	M	2	89.24
2	H	1	161.16	7	H	1	168.43
2	H	2	152.61	7	H	2	161.55
3	L	1	26.17	8	L	1	29.98
3	L	2	21.59	8	L	2	26.59
3	M	1	76.65	8	M	1	96.99
3	M	2	77.17	8	M	2	83.19
3	H	1	152.00	8	H	1	164.97
3	H	2	159.93	8	H	2	165.68
4	L	1	22.83	9	L	1	24.67
4	L	2	23.56	9	L	2	25.65
4	M	1	80.57	9	M	1	83.07
4	M	2	80.61	9	M	2	86.46
4	H	1	162.48	9	H	1	169.98
4	H	2	155.49	9	H	2	156.82
5	L	1	21.18	10	L	1	22.74
5	L	2	26.06	10	L	2	28.91
5	M	1	82.48	10	M	1	88.31
5	M	2	85.91	10	M	2	89.45
5	H	1	165.33	10	H	1	165.92
5	H	2	156.10	10	H	2	162.24

Table 10. Nested ANOVA for mahogany clam sample.

Source of variation	DF	SS	MS	F value
Samples	9	646.57	71.84	0.01
Concentrations in samples	20	191207	9560.33	561.00
Determinations within concentrations	30	511.25	17.04	
Total	59	192364	3260.41	

Table 11. Recovery for blue mussel.

Spike (µg/100 g)	Replicate 1	Replicate 2	Average	Spike - Average
L (20)	20.32	24.59	22.46	-2.46
L (20)	19.77	14.40	17.09	2.92
L (20)	25.15	18.34	21.75	-1.75
L (20)	23.05	18.48	20.77	-0.77
L (20)	20.60	16.54	18.57	1.43
L (20)	15.54	21.30	18.42	1.58
L (20)	16.99	19.75	18.37	1.63
L (20)	21.48	21.10	21.29	-1.29
L (20)	23.24	24.06	23.65	-3.65
L (20)	20.16	17.87	19.02	0.99
M (80)	81.33	79.36	80.35	-0.35
M (80)	78.70	79.60	79.15	0.85
M (80)	72.79	76.45	74.62	5.38
M (80)	73.24	74.61	73.93	6.08
M (80)	75.65	78.27	76.96	3.04
M (80)	83.87	86.25	85.06	-5.06
M (80)	86.41	85.06	85.74	-5.74
M (80)	85.97	86.22	86.10	-6.10
M (80)	84.01	92.05	88.03	-8.03
M (80)	84.29	86.37	85.33	-5.33
H (160)	162.73	152.45	157.59	2.41
H (160)	149.92	158.63	154.28	5.73
H (160)	152.89	152.19	152.54	7.46
H (160)	149.37	141.79	145.58	14.42
H (160)	152.04	158.34	155.19	4.81
H (160)	175.71	168.75	172.23	-12.23
H (160)	172.50	167.14	169.82	-9.82
H (160)	166.83	170.77	168.80	-8.80
H (160)	170.87	171.03	170.95	-10.95
H (160)	175.94	170.48	173.21	-13.21

Table 12. One-way ANOVA for blue mussel sample.

Source of variation	DF	SS	MS	F	P-value	F critcal
Concentration	2	19.045	9.52	0.22	0.81	3.35
Error	27	1184.75	43.88			
Total	29	1203.79				

Table 13. Recovery for steamer.

Spike (µg/100 g)	Replicate 1	Replicate 2	Average	Spike - Average
L (20)	18.68	23.16	20.92	-0.92
L (20)	20.14	21.66	20.90	-0.90
L (20)	23.13	19.95	21.54	-1.54
L (20)	24.14	23.63	24.89	-4.89
L (20)	21.51	23.76	22.64	-2.64
L (20)	16.87	21.63	19.25	0.76
L (20)	18.73	24.59	21.66	-1.66
L (20)	23.69	20.61	22.15	-2.15
L (20)	23.20	26.76	24.98	-4.98
L (20)	20.71	22.71	21.71	-1.71
M (80)	77.46	76.23	76.85	3.16
M (80)	85.17	79.32	82.24	-2.24
M (80)	79.99	80.85	80.42	-0.42
M (80)	82.59	84.22	83.41	-3.41
M (80)	78.95	81.70	80.33	-0.33
M (80)	81.76	82.96	82.36	-2.36
M (80)	83.62	80.45	82.04	-2.04
M (80)	87.19	87.25	87.22	-7.22
M (80)	85.59	92.55	89.07	-9.07
M (80)	88.30	88.46	88.38	-8.38
H (160)	154.04	154.51	154.28	5.73
H (160)	160.30	165.08	162.69	-2.69
H (160)	153.02	151.95	152.49	7.52
H (160)	163.35	155.64	159.50	0.51
H (160)	168.83	160.60	164.72	-4.72
H (160)	167.80	163.75	165.77	-5.77
H (160)	155.48	165.04	160.26	-0.26
H (160)	169.35	166.76	168.05	-8.05
H (160)	174.61	167.74	171.18	-11.18
H (160)	171.01	169.67	170.34	-10.34

Table 14. One-way ANOVA for steamer sample.

Source of variation	DF	SS	MS	F	P-value	F critcal
Concentration	2	7.34	3.67	0.19	0.83	3.35
Error	27	528.18	19.56			
Total	29	535.51				

Table 15. Recovery for mahogany clam.

Spike (µg/100 g)	Replicate 1	Replicate 2	Average	Spike - Average
L (20)	16.52	23.15	19.84	0.17
L (20)	20.78	19.63	20.21	-0.21
L (20)	26.17	21.59	23.88	-3.88
L (20)	22.83	23.56	23.20	-3.20
L (20)	21.18	26.06	23.62	-3.62
L (20)	22.28	22.54	22.41	-2.41
L (20)	21.89	27.09	24.49	-4.49
L (20)	29.98	26.59	28.29	-8.29
L (20)	24.67	25.65	25.16	-5.16
L (20)	22.74	28.91	25.83	-5.83
M (80)	81.58	78.26	78.26	1.74
M (80)	80.07	74.56	74.56	5.44
M (80)	76.65	77.17	77.17	2.83
M (80)	80.57	80.61	80.61	-0.61
M (80)	82.48	85.91	85.91	-5.91
M (80)	78.12	79.69	78.91	1.10
M (80)	89.17	89.24	89.21	-9.21
M (80)	96.99	83.19	90.09	-10.09
M (80)	83.07	86.46	84.77	-4.77
M (80)	88.31	89.45	88.88	-8.88
H (160)	155.89	158.16	157.03	2.98
H (160)	161.16	152.61	156.89	3.12
H (160)	152.00	159.93	155.97	4.04
H (160)	162.48	155.49	158.99	1.02
H (160)	165.33	156.10	160.72	-0.72
H (160)	171.77	162.53	167.15	-7.15
H (160)	168.43	161.55	164.99	-4.99
H (160)	164.97	165.68	165.33	-5.33
H (160)	169.98	156.82	163.40	-3.40
H (160)	165.92	162.24	164.08	-4.08

Table 16. One-way ANOVA for steamer sample.

Source of variation	DF	SS	MS	F	P-value	F critcal
Concentration	2	25.52	12.76	0.70	0.50	3.35
Error	27	490.11	18.15			
Total	29	515.63				

Table 17. Percent recovery of the method for each tissue type.

		Blue Mussel		Steamer		Mahogany Clam	
	Spike (µg/100 g)	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
	20	20.32	24.59	18.68	23.16	16.52	23.15
	20	19.77	14.40	20.14	21.66	20.78	19.63
	20	25.15	18.34	23.13	19.95	26.17	21.59
	20	23.05	18.48	24.14	23.63	22.83	23.56
	20	20.60	16.54	21.51	23.76	21.18	26.06
	20	15.54	21.30	16.87	21.63	22.28	22.54
	20	16.99	19.75	18.73	24.59	21.89	27.09
	20	21.48	21.10	23.69	20.61	29.98	26.59
	20	23.24	24.06	23.20	26.76	24.67	25.65
	20	20.16	17.87	20.71	22.71	22.74	28.91
	80	81.33	79.36	77.46	76.23	81.58	78.26
	80	78.70	79.60	85.17	79.32	80.07	74.56
	80	72.79	76.45	79.99	80.85	76.65	77.17
	80	73.24	74.61	82.59	84.22	80.57	80.61
	80	75.65	78.27	78.95	81.70	82.48	85.91
	80	83.87	86.25	81.76	82.96	78.12	79.69
	80	86.41	85.06	83.62	80.45	89.17	89.24
	80	85.97	86.22	87.19	87.25	96.99	83.19
	80	84.01	92.05	85.59	92.55	83.07	86.46
	80	84.29	86.37	88.30	88.46	88.31	89.45
	160	162.73	152.45	154.04	154.51	155.89	158.16
	160	149.92	158.63	160.30	165.08	161.16	152.61
	160	152.89	152.19	153.02	151.95	152.00	159.93
	160	149.37	141.79	163.35	155.64	162.48	155.49
	160	152.04	158.34	168.83	160.60	165.33	156.10
	160	175.71	168.75	167.80	163.75	171.77	162.53
	160	172.50	167.14	155.48	165.04	168.43	161.55
	160	166.83	170.77	169.35	166.76	164.97	165.68
	160	170.87	171.03	174.61	167.74	169.98	156.82
	160	175.94	170.48	171.01	169.67	165.92	162.24
Average	86.67		87.89		89.37		89.41
Recovery (%)			101.42		103.12		103.16

Table 18. Cross reactivity of antibody to saxitoxin-related congeners.

Compound	Cross reactivity (%)
Saxitoxin dihydrochloride	100.00
Neosaxitoxin	2.28
Decarbamoyl STX	42.30
GTX2 & 3	50.00
GTX1 & 4	0.48
Decarbamoyl GTX2 & 3	2.36
Decarbamoyl NeoSTX	1.50
B1	16.95
C1& 2	12.10

*The cross reactivity was evaluated by US FDA. Betsy Jean Yakes, Samantha M. Prezioso, Stacey L. DeGrasse. Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta (2012), 99, 668-676.

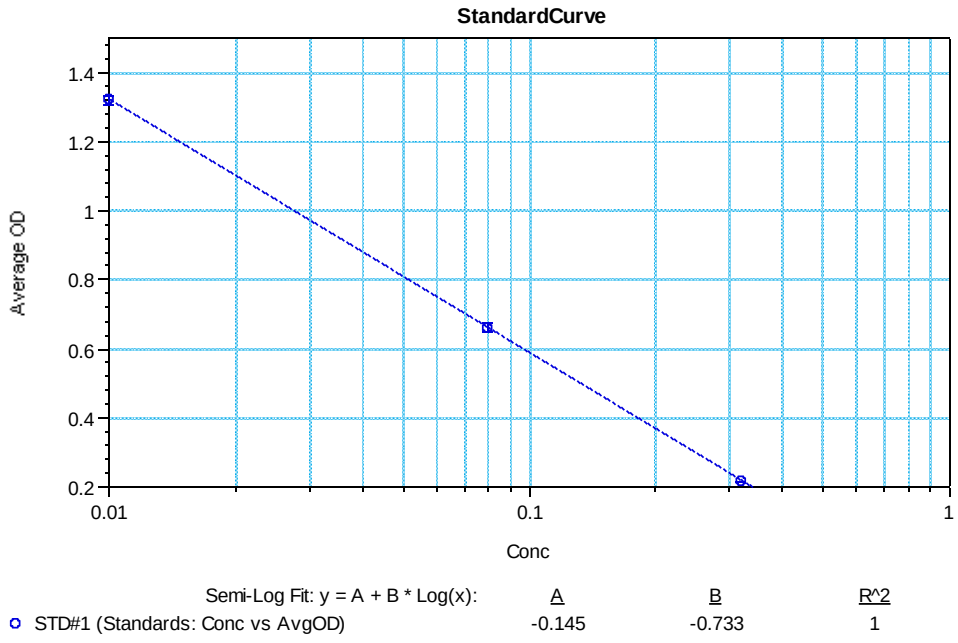

Figure 1. Calibration curve.

Table 19. LOD and LOQ for blue mussel.

Sample	Replicate	Spike (µg/100 g)				
		5	20	80	160	240
1	1	8.00	19.44	81.56	154.58	218.59
	2	5.23	22.13	79.71	151.94	222.88
	3	6.70	20.32	76.53	150.97	227.44
2	1	8.98	20.01	77.37	158.08	222.52
	2	6.22	21.09	79.20	153.75	225.14
	3	8.17	19.67	76.63	153.63	229.74
3	1	5.51	19.89	79.46	165.71	220.13
	2	7.55	21.71	80.02	167.96	231.76
	3	6.21	22.31	77.98	174.00	232.58
4	1	6.21	19.33	78.04	155.51	232.76
	2	6.91	22.19	83.81	156.42	237.66
	3	7.01	22.34	85.43	162.98	229.39
5	1	7.87	22.06	86.31	163.41	234.75
	2	10.65	24.62	80.05	160.72	231.98
	3	9.46	22.04	79.33	161.27	228.36
6	1	3.17	21.17	81.48	158.63	234.11
	2	4.90	23.31	81.72	161.60	237.80
	3	10.09	22.82	84.49	166.57	241.66
7	1	7.54	22.04	83.74	167.89	242.64
	2	7.25	21.40	80.08	160.91	238.06
	3	6.04	20.19	80.64	158.23	235.43
8	1	4.43	20.31	69.33	155.01	228.79
	2	6.31	22.70	75.59	161.16	243.85
	3	6.86	23.74	77.60	162.91	244.77
9	1	4.88	20.13	68.81	154.66	228.89
	2	5.83	22.57	74.57	160.32	243.81
	3	8.13	23.92	77.61	163.21	246.43
10	1	6.30	24.06	76.86	161.50	246.37
	2	8.02	22.43	81.28	168.06	244.21
	3	8.23	24.54	82.96	170.67	243.64
Average		6.89	22.16	79.47	162.47	236.66
Standard Deviation		1.76	1.47	4.40	4.98	7.03
CV (%)		25.48	6.63	5.54	3.06	2.97

Limit of quantitation (LOQ) is 13 µg/100 g and limit of detection (LOD) is 3.9 µg/100 g.

Table 20. LOD and LOQ for steamer.

Sample	Replicate	Spike (µg/100 g)				
		5	20	80	160	240
1	1	5.89	19.23	74.16	154.92	228.85
	2	4.93	20.26	75.06	155.50	235.79
	3	5.84	21.23	76.36	154.92	233.99
2	1	4.92	18.49	72.48	153.03	228.16
	2	4.24	18.74	73.04	153.62	233.76
	3	5.25	20.56	75.21	154.07	235.08
3	1	5.26	15.70	70.34	142.34	225.09
	2	5.68	19.10	75.49	147.96	221.74
	3	5.94	18.87	76.12	150.07	228.19
4	1	4.46	19.77	75.80	147.91	234.36
	2	2.53	21.12	77.35	152.37	229.56
	3	3.16	22.37	79.89	156.60	238.98
5	1	4.91	20.53	82.73	154.60	237.12
	2	4.33	21.12	84.16	165.37	244.30
	3	6.82	21.95	85.43	161.95	244.16
6	1	5.93	22.43	84.55	160.50	240.98
	2	2.43	20.55	82.53	162.05	239.87
	3	3.93	19.76	81.05	153.58	233.75
7	1	6.80	22.04	78.88	161.82	249.64
	2	4.74	19.42	87.73	169.54	258.67
	3	7.70	21.53	84.61	170.70	250.27
8	1	5.37	21.95	82.42	167.55	246.71
	2	3.99	17.22	82.09	164.19	247.66
	3	5.13	18.27	78.32	161.26	243.13
9	1	8.23	20.57	76.89	150.28	240.76
	2	7.65	19.04	74.81	148.37	235.24
	3	9.36	19.46	76.15	156.55	243.71
10	1	6.86	22.15	77.17	152.94	242.10
	2	7.05	23.59	76.91	155.44	244.50
	3	8.62	22.69	83.93	157.17	249.33
Average		5.60	20.32	78.72	156.57	238.85
Standard Deviation		1.70	1.76	4.42	6.77	8.45
CV (%)		30.39	8.67	5.61	4.32	3.54

Limit of quantitation (LOQ) is 17 µg/100 g and limit of detection (LOD) is 5.2 µg/100 g.

Table 21. LOD and LOQ for Mahogany clam.

Sample	Replicate	Spike (µg/100 g)				
		5	20	80	160	240
1	1	4.79	20.72	74.76	156.09	235.92
	2	3.66	20.58	73.55	152.94	238.18
	3	5.52	22.54	78.00	146.95	241.35
2	1	5.30	21.52	76.18	151.08	240.92
	2	2.32	20.46	71.91	150.13	239.19
	3	4.50	19.50	75.17	154.73	233.91
3	1	5.14	20.74	75.36	150.57	239.47
	2	7.62	19.13	70.52	148.60	237.14
	3	3.80	18.70	74.66	154.31	233.02
4	1	4.75	20.38	75.15	150.26	240.21
	2	3.13	18.20	74.55	153.86	233.42
	3	6.36	18.37	69.73	147.80	237.09
5	1	5.67	18.83	75.84	149.32	229.98
	2	6.38	21.11	77.56	155.13	242.33
	3	6.64	20.18	81.71	160.98	246.92
6	1	6.09	18.92	82.98	161.94	248.69
	2	5.09	19.40	76.25	155.23	243.44
	3	4.92	17.48	75.18	148.89	231.89
7	1	3.37	18.46	76.29	154.20	240.17
	2	6.03	18.95	83.97	161.17	246.89
	3	4.46	17.25	75.97	149.58	231.00
8	1	9.50	22.67	83.28	153.81	231.91
	2	8.39	23.87	82.56	158.22	231.64
	3	6.29	22.53	86.47	149.23	232.41
9	1	7.62	19.18	73.82	158.28	237.57
	2	7.13	22.08	75.80	156.14	223.80
	3	7.71	21.37	77.12	153.62	223.58
10	1	5.36	19.00	73.77	157.04	235.65
	2	2.84	21.28	74.66	155.53	221.84
	3	5.85	21.49	78.05	156.17	224.65
Average		5.54	20.16	76.69	153.73	235.81
Standard Deviation		1.69	1.66	3.98	4.08	6.93
CV (%)		30.45	8.24	5.19	2.66	2.94

Limit of quantitation (LOQ) is 16 µg/100 g and limit of detection (LOD) is 4.9 µg/100 g.

Table 22. Comparison between ELISA and MBA for samples collected from same location over the seasons.

Date of collection	ELISA (µg/100g)	MBA (µg/100g)	Result from ELISA	Result from MBA
6/7/2010	35	45	Suspicious	Below limit
6/9/2010	5	0	Negative	Negative
6/14/2010	31	45	Suspicious	Below limit
6/16/2010	18	0	Negative	Negative
6/21/2010	66	59	Suspicious	Below limit
6/23/2010	31	48	Suspicious	Below limit
6/28/2010	133	274	Positive	Positive
6/30/2010	433	567	Positive	Positive
7/5/2010	1006	1204	Positive	Positive
7/7/2010	115	163	Positive	Positive
7/12/2010	276	264	Positive	Positive
7/14/2010	11	0	Negative	Negative
7/19/2010	10	0	Negative	Negative
7/21/2010	6	0	Negative	Negative
7/26/2010	0	0	Negative	Negative
7/28/2010	1	0	Negative	Negative
8/2/2010	0	0	Negative	Negative
8/9/2010	0	0	Negative	Negative
8/11/2010	12	0	Negative	Negative
8/16/2010	8	0	Negative	Negative
8/18/2010	8	0	Negative	Negative
5/9/2011	42	54	Suspicious	Below limit
5/16/2011	34	42	Suspicious	Below limit
5/30/2011	25	0	Negative	Negative
6/6/2011	184	124	Positive	Positive
6/13/2011	288	382	Positive	Positive

Table 23. Comparison between ELISA and MBA for samples collected from different locations.

Location of collection	ELISA (µg/100g)	MBA (µg/100g)	Result from ELISA	Result from MBA
Black Rock	101	121	Positive	Positive
Lumbos Hole	124	156	Positive	Positive
Ogunquit R.	24	48	Negative	Below limit
Hermit Island East	120	136	Positive	Positive
Long Point	72	67	Suspicious	Below limit
Gurnet	264	156	Positive	Positive
Head Beach	32	39	Suspicious	Below limit
Little johns Bridge	52	52	Suspicious	Below limit
Black Rock	32	50	Suspicious	Below limit
Bangs Island	40	54	Suspicious	Below limit
Ash Point	99	74	Suspicious	Below limit
Basin Point	44	44	Suspicious	Below limit
Ash Point	44	48	Suspicious	Below limit
Lumbos Hole	36	49	Suspicious	Below limit
CB 004 Youngs Point	64	55	Suspicious	Below limit
Matthews Island	56	47	Suspicious	Below limit

Discussion

The accuracy/trueness of the method for mussel, steamer and mahogany clam were 111%, 102% and 101%, respectively. The measurement uncertainty for mussel, steamer and mahogany clam were 4.29, 2.40 and 1.55 µg/100 g, respectively. Based on these results, mahogany clam sample gives better accuracy/trueness and measurement uncertainty for the method (Table 1 – 3).

The skewness (within -2 to +2) and the ratio of variances (close to 1) indicate that this is a symmetric distribution and the variance is homogeneous, respectively. Therefore, the paired t-test was chosen for the analysis instead of Welch's t-test in order to see the significant difference between two lots of each tissue type test. P-value below 0.05 is considered statistically significant at a significance level of 0.05, while one of 0.05 or greater indicates no significant difference between the groups. Therefore, all three tissue types had no significant differences between lots when tested with the method over the tested range of 10 – 120 µg/100 g. This indicates that the method is sufficiently rugged to be used routinely (Table 4).

For mussel sample, we obtained an F value of 0.02 for sample which is less than the critical value of 2.39 for 9 and 20 degrees of freedom at the 0.05 significance level (Table 6). This indicates that the mean values from the samples are not significantly different. The F value for concentration in sample is greater than the critical value of 1.93 for 20 and 30 degrees of freedom a the 0.05 significance level. This indicates that the mean values of each concentration within samples are significantly different. From this nested ANOVA table, we can conclude that the precision of the method is consistent over the samples. Each sample has three different levels of saxitoxin contamination. However, the precision of the method is not consistent for each spiked concentration when repeated with different samples. The other two samples, steamer and mahogany had the same results as mussel sample (Table 5 – 10).

From one-way ANOVA for recovery, since F value for each shellfish type is smaller than F critical (tabulated F value at 95% confidence level), there is no significance between groups (20, 80 and 160). Therefore, the recoveries for mussel, steamer and mahogany samples are consistent over the range tested by the method (20 through 160 µg/100 g) (Table 11 – 17).

Cross reactivity of the antibody used for the kit was evaluated by the third party (Table 18). The detailed information regarding the cross reactivity can be achieved from the reference 1 (Yakes et al., 2012). However, it is common that the results may vary depending on the assay formats used to evaluate the cross reactivity.

For the linearity of the method, calibrators were run by the method. OD (optical density) values at 450 nm were graphed on the y-axis, and log concentrations of calibrators (0.01 – 0.32 µg/L) were graphed on the x-axis. A linear curve fit was applied to the data. The curve in Figure 1 is very good with coefficient of determination of 1.0. This indicates the method can detect the levels of saxitoxin between 15 and 480 µg/100 g in sample with good linearity.

To determine the LOQ for each tissue type, coefficient of variation (%) was plotted on the y-axis and saxitoxin spike level was plotted on the x-axis. From this graph, LOQ was determined by finding the saxitoxin concentration where the CV (%) is 10%. LOQs for mussel, steamer and mahogany samples are 13, 17 and 16 µg/100 g, respectively. LOD was determined by dividing the LOQ by 3.3. LODs for mussel, steamer and mahogany samples are 3.9, 5.2 and 4.9 µg/100 g, respectively (Table 19 – 21).

ELISA and MBA methods were compared for the determination of PSP from naturally contaminated blue mussels (Table 22 – 23). All the suspicious results from ELISA (between 30 µg/100 g and 100 µg/100 g) agreed with the results from MBA (Detectable but below limit, < 80 µg/100 g). Therefore, these suspicious results from ELISA testing can be retested by MBA to confirm the PSP level. All the results either negative or positive from ELISA well matched with the results from MBA except one negative result from ELISA (Ogunquit R.). However, the result from MBA for the sample is quite below the legal limit (48 µg/100 g) as well. Therefore, all the naturally contaminated samples were correctly screened by the ELISA indicating this ELISA method can be used as a screening tool for PSP analysis in the laboratory.

Summary

Rapid screening method for saxitoxin (PSP) in blue mussels, steamers and mahogany clams was developed and evaluated. This method can be used in the private, certified or state laboratories for the determination of PSP toxins in order to screen out negative samples (below 30 µg/100g of saxitoxin equivalent) prior to mouse bioassay (MBA) as a confirmation method. Suspicious samples with PSP levels between 30 µg/100g and 100 µg/100 g will need confirmation with MBA. However, PSP levels higher than 100 µg/100g would be considered as positive, and may not need further confirmation. Therefore, this screening method will dramatically reduce the volume of samples to be confirmed with MBA, and save time and resources for the laboratories.

CALCULATE RESULTS

1. Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbance of the calibrator wells. Samples containing less color than a calibrator will have a concentration of Saxitoxin greater than the concentration of the calibrator. Samples containing more color than a calibrator well have a concentration less than the concentration of the calibrator.
2. Quantitative interpretation requires graphing the absorbances of the calibrators (Y axis) versus the log of the calibrator concentration (X axis) on semi-log graph paper. A straight line is drawn through the calibrator points and the sample absorbances are located on the line. The corresponding point on the X axis is the concentration of the sample Alternatively, Beacon can supply a spreadsheet template which can be used for data reduction. Please contact Beacon for further details.

SAMPLE CALCULATIONS

Well Contents	OD	Average OD ± SD*	%RSD	%Bo**	STX conc. (ppb)
Negative Control	1.438 1.461	1.449 ± 0.016	1.1	100	N/A
0.01 ppb Calibrator	1.312 1.330	1.321 ± 0.012	0.9	91.2	N/A
0.08 ppb Calibrator	0.669 0.653	0.661± 0.011	1.7	45.6	N/A
0.32 ppb Calibrator	0.217 0.216	0.217 ± 0.001	0.4	15.0	N/A
Sample	0.491 0.511	0.501 ± 0.014	2.8	34.6	0.119

Actual values may vary; this data is for example purposes only.

* standard deviation

** %Bo equals average sample absorbance divided by average negative control absorbance times 100%.

TECHNICAL ASSISTANCE

For questions regarding this kit or for additional information about Beacon products, call (207) 571-4302.

SAFETY

To receive complete safety information on this product, contact Beacon Analytical Systems, Inc. and request Material Safety Data Sheets. Stop Solution is 1N hydrochloric acid. Handle with care.

General Limited Warranty

Beacon Analytical Systems, Inc. ("Beacon") warrants the products manufactured by it against defects in materials and workmanship when used in accordance with the applicable instructions for a period not to extend beyond a product's printed expiration date. BEACON MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. The warranty provided herein and the data, specifications and descriptions of Beacon products appearing in published catalogues and product literature may not be altered except by express written agreement signed by an officer of Beacon. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and, if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Beacon's sole obligation shall be to repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies Beacon promptly of any such defect. The exclusive remedy provided herein shall not be deemed to have failed of its essential purpose so long as Beacon is willing and able to repair or replace any nonconforming Beacon product or part. Beacon shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by a customer from the use of its products. However, in some states the purchaser may have rights under state law in addition to those provided by this warranty.

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Beacon Analytical Systems Inc.

Saxitoxin (PSP) Microtiter Plate Test Kit

Instructional Booklet

READ COMPLETELY BEFORE USE.

INTENDED USE

The Beacon Saxitoxin Plate Kit is a competitive ELISA for the quantitative analysis of Saxitoxin in contaminated samples

USE PRINCIPLES

The Beacon Saxitoxin (PSP) Microtiter Plate Test Kit is a competitive enzyme-labeled immunoassay. The Saxitoxin HRP conjugate, sample extract and calibrators are pipetted into the test wells followed by Saxitoxin antibody into the test wells to initiate the reaction. During the 30 minute incubation period, PSP toxins from the sample and Saxitoxin HRP conjugate compete for binding to Saxitoxin antibody. The Saxitoxin antibody is captured on the walls of the test well. Following this 30 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound PSP toxins, Saxitoxin HRP conjugate and free Saxitoxin antibody. After wash, a clear substrate is then added to the wells and any bound enzyme conjugate causes the conversion to a blue color. Following a 30 minute incubation, the reaction is stopped and the amount of color in each well is read. The color of the unknown samples is compared to the color of the calibrators and the Saxitoxin concentration of the samples is derived.

MATERIALS PROVIDED

The kit in its original packaging can be used until the end of the month indicated on the box label when stored at 2 – 8ºC.

1 plate containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.

1 vial of Negative control (Zero ppb Saxitoxin)

3 vials each containing 2 mL of Saxitoxin calibrators corresponding to 0.01, 0.08 and 0.32 µg/L (ppb) of Saxitoxin.

1 vial containing 7 mL Saxitoxin HRP Enzyme Conjugate.

1 vial containing 7 mL of Polyclonal anti-Saxitoxin antibody.

1 bottle containing 50 mL 10X Wash solution concentrate.

1 vial containing 14 mL of Substrate.

1 vial containing 14 mL of Stop Solution. (Caution! 1N HCl. Handle with care.)

1 Instructional Booklet

MATERIALS REQUIRED BUT NOT PROVIDED

- Laboratory quality distilled or deionized water.
- 20 mM PBS (phosphate buffered saline).
- Pipet with disposable tips capable of dispensing 50 µL.
- Multi-channel pipet; 8-channel capable of dispensing 50 and 100 µL.
- Paper towels or equivalent absorbent material.
- Microwell plate or strip reader with 450nm filter.
- Timer
- Wash bottle

PERFORMANCE CHARACTERISTICS

SPECIFICITY

The following table shows the % cross reactivity of Saxitoxin.

Compound	% CR
Saxitoxin dihydrochloride	**100.00 %**
Neosaxitoxin	**2.28 %**
Decarbamoyl STX	**42.30 %**
GTX 2& 3	**50.00 %**
GTX 1 & 4	**0.48 %**
Decarbamoyl GTX 2& 3	**2.36 %**
Decarbamoyl NeoSTX	**1.50 %**
B1	**16.95 %**
C1 and 2	**12.10 %**

PRECAUTIONS

- Store all kit components at 4°C to 8°C (39°F to 46°F) when not in use.
- Each reagent is optimized for use in the Beacon Saxitoxin (PSP) Microtiter Plate Test Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other Beacon Saxitoxin Plate Kits with different Lot numbers.
- Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
- Do not use reagents after expiration date.
- Reagents should be brought to room temperature, 20 – 28ºC (62 – 82ºF) prior to use. Avoid prolonged (> 24 hours) storage at room temperature.
- Saxitoxin is a toxin and should be treated with care.
- The Stop Solution is 1N hydrochloric acid. Avoid contact with skin and mucous membranes. Immediately clean up any spills and wash area with copious amounts of water. If contact should occur, immediately flush with copious amounts of water.
- Transfer of samples and reagents by pipette requires constant monitoring of technique. Pipetting errors are the major source of error in immunoassay methodology.

EXTRACTION SOLUTION AND DILUTION BUFFER

1. Prepare 70% Isopropanol by mixing 70 mL of Isopropanol with 30 mL of lab grade water.
2. Prepare 5% Acetic acid solution by mixing 5 mL of Acetic acid with 95 mL of lab grade water.
3. Mix 2 parts (100 mL) of 70% Isopropanol with 1 part (50 mL) of 5% Acetic acid. Use for sample extraction.
4. For sample dilution buffer, mix 10 mL of Methanol with 90 mL of 20 mM PBS buffer.

SAMPLE PREPARATION (MUSSELS)

1. Shellfish are shucked and rinsed with lab grade water.
2. Transfer 12 mussels to a sieve and gently shake the sieve to drain the excess liquid.
3. Put the drained tissue into a kitchen blender jar and homogenize to a soupy texture.
4. Tare a 50 mL conical tube and weigh 5 grams of homogenized tissue.
5. Add 10 mL of extraction solvent and vortex for 3 minutes.
6. Transfer approximately 1 mL of the extract into a microcentrifuge tube, and centrifuge for 5 minutes at 12,000 rpm.
7. Dilute the supernatant with 10% MeOH/20 mM PBS to 5000 folds before running in assay.

   (e.g. 0.1 mL of the supernatant + 9.9 mL of buffer, and 0.1 mL of the diluted supernatant + 4.9 mL of buffrer)

ASSAY PROCEDURE

(Note: Running calibrators and samples in duplicate will improve assay precision and accuracy.)

1. Prepare the 1X wash solution by adding the contents of the 10X wash concentrate bottle to 450 mL Lab grade water in a wash bottle.
2. Allow reagents and sample extracts to reach room temperature prior to running the test.
3. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
4. Using a pipet with disposable tips, add **50 µL enzyme conjugate** to the appropriate test wells. Be sure to use a clean pipet tip for each. Add **50 µL of Calibrators or Sample** extract to each well.
5. Dispense **50 µL of Antibody Solution** into each test well.
6. Shake the plate gently for 30 seconds and incubate the test wells for **30 minutes**.
7. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and dump. Repeat 3X for a total of four washes.
8. Following the last wash tap the inverted wells onto absorbent paper to remove the last of the wash solution.
9. Dispense **100 µL of Substrate** into each well.
10. Incubate the wells for **30 minutes**.
11. Dispense **100 µL of Stop Solution** into each test well.
12. Read and record the absorbance of the wells at 450nm using a strip or plate reader.

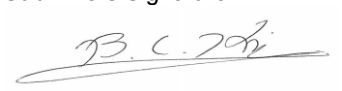
ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

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	**Saxitoxin (PSP) Microtiter Plate Test Kit**
Name of the Method Developer	**Titan Fan and Byungchul Kim**
Developer Contact Information	**Beacon Analytical Systems, Inc.** **82 Industrial Park Road** **Saco, ME 04072** **Phone: 207-571-4302** **Fax: 207-602-6502** **titan@beaconkits.com** **bkim@beaconkits.com**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Rapid and inexpensive saxitoxin (PSP) screening analysis
2. What is the intended purpose of the method?	Y	For monitoring saxitoxin levels (PSP) from shellfish (mussels, clams, steamers) as an Approved Limited Use Method.
3. Is there an acknowledged need for this method in the NSSP?	Y	Rapid screening tool prior to confirmation analysis with regulatory method
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Enzyme-Linked Immunosorbent Assay (ELISA)
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Saxitoxin (PSP) Microtiter Plate Test Kit
Method Scope	Y	For the analysis of PSP toxins in blue mussels, steamers and mahogany clams from North Atlantic Ocean as an Approved Limited Use Method.
References	Y	See Reference 1 (Page 673). See Reference 2 (Page1133). See Reference 3 (Page1649). See Reference 4 (Page 33). See Reference 5 (Page 13). Reference 1: B. J. Yakes, S. M. Prezioso, S. L. DeGrasse. (2012) Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta, 99, 668-676.

		Reference 2: G. B. Inami, C. Crandall, D. Csuti, M. Oshiro, R. Brenden. (2004) Presence/Absence Screening for Saxitoxin in Frozen Acidified Mussel and Oyster Extracts from the Coast of California with In Vitro Methods, J AOAC. Int. 87 (5), 1133-1142. Reference 3: E. Usleber, R. Dietrich, C. Burk, E. Schneider, E. Martlbauer. (2001) Immunoassay Methods for Paralytic Shellfish Poisoning Toxins. J. AOAC. Int. 84 (5), 1649-1656. Reference 4: Anderson, D.M., P. Andersen, V. M. Bricelj, J. J. Cullen, and J. EE. Rensel, 2001. Monitoring and management strategies for harmful algal blooms in coastal waters, APEC #201-MR-01.1, Asia Pacific Economic Program and Intergovernmental Oceanographic Commission Technical Series No. 59, Paris. Reference 5: F. S. Chu, T. S. L. Fan. (1985) Indirect Enzyme-Linked Immunosorbent Assay for Saxitoxin in Shellfish. J. AOAC. 68 (1), 13-16.
Principle	Y	Competitive ELISA
Any Proprietary Aspects	Y	Antibody and STX-HRP conjugate
Equipment Required	Y	Listed in the protocol (Page 3)
Reagents Required	Y	Listed in the protocol (Page 3)
Sample Collection, Preservation and Storage Requirements	Y	Described in the protocol (Page 3)
Safety Requirements	Y	Personal safety items such as safety glasses, gloves and lab coat must be required. Calibrators and enzyme conjugate are toxic materials, and must be kept in the original vials when they are not used. Since stop solution is a strong acidic solution (1 N hydrochloric acid), skin or eye contact must be avoided. Lab items with contact of toxins (sample extract and calibrator) such as pipette tips and lab wares must be soaked in 50% of house bleach solution at least 1 hour before washing or disposal.
Clear and Easy to Follow Step-by-Step Procedure	Y	Described in the protocol (Page 4)
Quality Control Steps Specific for this Method	Y	Described in the protocol (Page 5)
C. Validation Criteria		
1. Accuracy / Trueness	Y	Described in the protocol (Page 5, Table 1 - 3)
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	The precision of the method is consistent for the three sample matrices. Each sample was tested with three different levels of saxitoxin. See the details on Page 23 and Table 5 - 10.
4. Recovery	Y	Described in the protocol (Table 11 - 17)
5. Specificity	Y	Described in the protocol (Page 6, Table 18)
6. Working and Linear Ranges	Y	Described in the protocol (Page 6, Figure 1)
7. Limit of Detection	Y	Described in the protocol (Page 6, Table 19 - 21)
8. Limit of Quantitation / Sensitivity	Y	Described in the protocol (page 6, Table 19 - 21)
9. Ruggedness	Y	Described in the protocol (Page 5, Table 4)
10. Matrix Effects	Y	There is no matrix effect observed due to the high degree of dilution (15000) of sample before the assay. The potential matrix effect from any shellfish species

		could be eliminated by this high dilution.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		
D. Other Information		
1. Cost of the Method	Y	$325 per kit to test up to 44 samples (≈$7 per sample)
2. Special Technical Skills Required to Perform the Method	Y	Some ELISA experience or basic training required
3. Special Equipment Required and Associated Cost	Y	Described in the protocol (Page 3)
4. Abbreviations and Acronyms Defined	Y	Described in the protocol (Page 4)
5. Details of Turn Around Times (time involved to complete the method)	Y	One person may test 50 – 100 samples in a day including sample preparation time (shucking, grinding and extracting). Single assay can be done in 90 minutes testing up to 44 samples with duplicate.
6. Provide Brief Overview of the Quality Systems Used in the Lab		The manufacture of the kit was conducted in compliance with GMP (Good Manufacturing Practices) regulations.
Submitters Signature 	Date: June 26, 2013	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

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

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Talanta 99 (2012) 668–676

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies

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ARTICLE INFO

Article history:
Received 17 April 2012
Accepted 25 June 2012
Available online 25 July 2012

Keywords:
Surface plasmon resonance
Paralytic shellfish poisoning
Saxitoxin
Lateral flow immuno-chromatography
Immunoassay
Cross-reactivity

ABSTRACT

Paralytic shellfish toxins (PSTs) are a risk to humans upon consumption of contaminated seafood. The PST family is comprised of more than twenty congeners, with each form having a different potency. In order to adequately protect consumers yet reduce unnecessary closures of non-contaminated harvesting areas, a rapid method that allows for analysis of sample toxicity is needed. While a number of PST immunoassays exist, the outstanding challenge is linking quantitative response to sample toxicity, as no single antibody reacts to the PST congeners in a manner that correlates with potency. A novel approach, then, is to combine multiple antibodies of varying reactivity to create a screening assay. This research details our investigation of three currently available antibodies for their reactivity profiles determined using a surface plasmon resonance biosensor assay. While our study shows challenges with detection of the R1-hydroxylated PSTs, results indicate that using multiple antibodies may provide more confidence in determining overall toxicity and the toxin profile. A multiplexed approach would not only improve biosensor assays but could also be applied to lateral flow immuno-chromatographic platforms, and such a theoretical device incorporating the three antibodies is presented. These improved assays could reduce the number of animal bioassays and confirmatory analyses (e.g., LC/MS), thereby improving food safety and economic use of shellfish resources.

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1. Introduction

Paralytic shellfish poisoning (PSP) is caused by a suite of toxins, known collectively as paralytic shellfish toxins (PSTs) [1]. Saxitoxin (STX) and its congeners originate from certain dinoflagellates and some cyanobacteria [2]. Filter feeding bivalves (e.g., mussels, clams, cockles, scallops and oysters), as well as other seafood species, can accumulate and metabolize these toxins which can then lead to potentially dangerous seafood [3,4]. Human consumption of toxic seafood can result in tingling, numbness, respiratory paralysis and potentially death [5], as the PSTs bind to site 1 and block the opening of voltage gated sodium channels [6]. These small molecule toxins are also quite robust, and typical preventative food safety measures (i.e., use of heat or acid during cooking) do not destroy the PSTs [1].

Proper monitoring and implementation of harvesting bans when toxin concentrations exceed safe levels (typically 80 μg STX equivalents per 100 g tissue) have minimized PSP illnesses [1]. However, outbreaks still occur, especially in developing countries [7] and with an estimated worldwide mortality of 6% [8]. For example, a major PSP epidemic occurred in Guatemala in 1987 that claimed the lives of 26 people out of the 187 affected [7,9]. A review of PSP cases and outbreaks has been compiled by FAO, which reports PSP prevalence along coastal European nations, parts of Africa, the West Coast and Northeast region of North America, South America, and parts of Asia [10]. Within the US, the majority of illnesses and outbreaks are reported from recreational harvests among fishermen and tribal communities. For example, during May and June of 2011, 21 cases of PSP illness were reported in Southeast Alaska due to unprecedented high levels of PSTs in surrounding waters [11].

Recent reviews on PST detection have focused on improved analysis of both coastal waters and seafood [1,2,12,13]. The mouse bioassay (MBA) is one of the AOAC approved and most commonly used testing methods for PSTs [14]. While simple, this bioassay suffers performance related challenges (e.g., poor quantitation and low dynamic range, interferences to detection, low sample throughput, and lack of determination of the specific toxin associated with death) as well as ethical concerns.

A second AOAC approved method for determining PSTs is high performance liquid chromatography (HPLC) with fluorescence detection (FD) [15,16]. This method is quite effective at identifying and quantifying the toxins in a seafood sample. However, it requires a lengthy sample clean-up and pre-column oxidation procedure to

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0039-9140/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.talanta.2012.06.073

create fluorescent derivatives of the toxins for detection as well as multiple analytical runs for complete PST determination. The post-column HPLC-FD method created by Oshima [17] was refined [18] and is also now AOAC approved [19]. This post-column oxidation method has a simpler sample preparation procedure than pre-column HPLC-FD; however, multiple analytical runs under different chromatographic conditions must be conducted in order to analyze all potential PST congeners. Furthermore, both HPLC-FD approaches can be hindered by sample materials that have native fluorescence, requiring additional steps to ensure the presence of toxins [20].

Other analytical techniques that are advancing include liquid chromatography (LC) coupled with mass spectrometry (MS) [21,22], some in tandem with biosensors [23]. The major limitation of this analytical approach is matrix interference and ionization suppression, which restricts its ability to serve as a reliable, quantitative monitoring tool. Limited availability of internal reference standards (e.g., isotopically labeled toxins) currently hinders wider-spread implementation of monitoring by LC/MS.

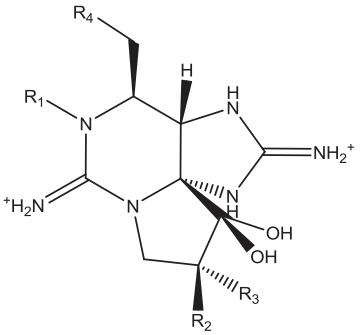
In order to overcome the challenges associated with MBA and LC methods, rapid screening techniques have been explored. These methods can be simple, cost-effective, sensitive, and accurate for high-throughput detection needs. Such methods include receptor binding assays (RBA) [24–27], lateral flow immuno-chromatography [28,29], enzyme-linked immunosorbent assays (ELISA) [30–32], and cell bioassays [33,34]. While these methods allow for high throughput and ease of use, they suffer from the use of difficult to procure radiolabeled materials for RBA, high probability of false-positive and potential for false-negative results with current immuno-chromatographic PSP tests, large amounts of manual labor and limited antibody cross-reactivity for ELISA, and nonspecific toxin recognition for the cell bioassays.

An immunological technique that has been shown to provide high throughput detection of PSTs is surface plasmon resonance (SPR) biosensors [35–38], though this method faces the same challenges with respect to antibody reactivity. SPR immunoassays are based on specific biosensor platforms that bind the molecule of interest at the surface. The change in mass due to binding is detected as a change in refractive index (RI) at the dielectric interface (i.e., gold immunoassay substrate and solution in the flow cell). This RI change causes a shift in the SPR band position that can be tracked in real-time using standard spectroscopy optics [39]. This automated technique allows for real-time analysis of PST-containing samples, requires minimal sample cleanup, no labeling of the analytes, and yields sub-ppb limits of detection in less than ten min [40,41].

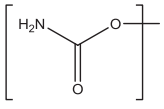
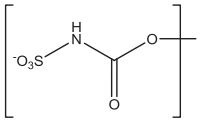
The SPR assay for the determination of PSTs currently implemented in our laboratory is robust and shows good repeatability and reproducibility; however, quantitative results do not always correlate with overall sample toxicity due to the many PST congeners having widely varying potency. The toxicities for common PSTs are shown in Table 1, and the inability to correlate results with sample toxicity when using immunological assays could lead to unsafe seafood harvested for consumers (false-negative) or destruction of safe seafood and closure of non-contaminated harvesting areas (false-positive). Clearly, there is a need for improved assays to not only protect the public but also to improve the economic viability of the industry and utilization of seafood resources. Unfortunately, a single antibody that reacts to the congeners with respect to their potency has yet to be produced. An advantage to the SPR assay is that while the response may not always correlate with toxicity, the cross-reactivity of individual congeners with an antibody can be calculated. A novel approach, then, would be to combine multiple antibodies of varying reactivity to the congeners, as screened via the SPR assay, to create a multiplexed immunoassay.

One disadvantage to SPR biosensors is the size of instrumentation and cost of materials which could prohibit routine testing in the field or dockside. Lateral flow immuno-chromatographic tests (LFIs) have been used for PST testing and could fulfill the requirements of an easy-to-use and cost-effective technique for monitoring potential toxicity of seafood when the quantitation and automation of the SPR instrumentation is not necessary.

Table 1
PST structure, congener forms, and relative toxicities [17]. Toxins used in this study are listed in bold.



Toxin	Relative Toxicity
STX	1.00
dcSTX	0.51
GTX2,3	0.36, 0.64
B1 (GTX5)	0.06
C1,2	0.01, 0.10
dcGTX2,3	0.15, 0.38
NEO	0.92
dcNEO	–
GTX1,4	0.99, 0.73

R1	R2	R3	Carbamate	Decarbamoyl	*N*-sulfocarbamoyl
H	H	H	**STX**	**dcSTX**	**B1 (GTX 5)**
OH	H	H	**NEO**	**dcNEO**	B2 (GTX 6)
OH	H	OSO_3^-	**GTX1**	dcGTX1	C3
H	H	OSO_3^-	**GTX2**	**dcGTX2**	**C1**
H	OSO_3^-	H	**GTX3**	**dcGTX3**	**C2**
OH	OSO_3^-	H	**GTX4**	dcGTX4	C4
		R4:			

The challenge with these rapid tests also lies in the inability to accurately measure sample toxicity, and the performance of such devices has been extensively studied [28,29,42]. To potentially enhance the reliability of the LFIs, multiple antibodies that have distinct reactivity patterns as determined in the SPR assay could be employed.

This manuscript details our work that evaluated three antibodies for their reactivity to nine commonly occurring PSTs. The data indicate that a multiplexed approach may not only improve SPR biosensor assays but could also be incorporated into LFI platforms for more reliable, rapid, inexpensive screening options. Such approaches could then allow for more successful assessment of overall sample toxicity and better use of confirmatory (e.g., LC-MS or MBA) techniques. The research introduced herein sets the stage for these multi-antibody devices and discusses the potential challenges when using the antibodies profiled in this study.

2. Materials and methods

2.1. Reagents

Saxitoxin (STX) dihydrochloride used in this research is the FDA reference standard, now available from the National Institute of Standards and Technology (81.0 μg/mL free base in 20% ethanol/80% water). *N*-sulfocarbamoyl-gonyautoxin-2 and -3 (C1,2, 70.8 μg/mL), decarbamoylgonyautoxin-2 and -3 (dcGTX2,3, 51.4 μg/mL), decarbamoylneosaxitoxin dihydrochloride (dcNEO, 7.93 μg/mL free base), decarbamoylsaxitoxin dihydrochloride (dcSTX, 16.0 μg/mL free base), gonyautoxin-1 and -4 (GTX1,4, 58.0 μg/mL), gonyautoxin-2 and -3 (GTX2,3, 62.1 μg/mL), gonyautoxin-5 (B1, 24.7 μg/mL), and neosaxitoxin dihydrochloride (NEO, 20.6 μg/mL free base) were purchased from NRC Certified Reference Materials Program, Institute for Marine Biosciences (Halifax, Nova Scotia, Canada).

Standard laboratory reagents were procured from Sigma-Aldrich (St. Louis, MO), Pharmaco AAPER (Shelbyville, KY), and J.T. Baker (Phillipsburg, NJ). Millipore Milli-Q 18.2 MΩ cm water (Billerica, MA) was used to prepare buffers. Sensor chips, amine coupling kit (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine), and buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20 buffer (HBS-EP+)) were obtained from GE Healthcare, Biacore (Piscataway, NJ).

2.2. Clam matrix

Control clam extract was prepared following a standard shellfish extraction procedure for PSTs (see [15] for procedure). A total of 100 g of clam was homogenized, from which a 5.0 g ($\pm$0.1 g) aliquot was thoroughly mixed with 3.0 mL of 1% acetic acid in water. This was heated at 95 °C for 5 min. The sample was then removed and placed on ice until cool enough to handle. The sample was vortexed and then centrifuged at 3600*g* for 10 min. The supernatant was collected into a 15 mL glass centrifuge tube. Another 3.0 mL, 1% acetic acid aliquot was added to the homogenate; the solution was vortexed and centrifuged at 3600*g* for 10 min. Following collection of the supernatant in the same 15 mL centrifuge tube, the extract volume was brought up to 10 mL with 0.1 N HCl with a final pH of 4.0. The clam extract was then filtered through a Supelco Supelclean LC-18, 3 mL solid phase extraction (SPE) cartridge (Sigma-Aldrich). The cartridge was conditioned with 6 mL methanol followed by 6 mL of water. Clam extract (1 mL) was added to the cartridge followed by 2 mL of water, and the cartridge was run dry. This extraction procedure produced 5 g of clam tissue per 40 mL and at the action level (i.e., 80 μg STX equivalents per 100 g tissue), equates to 100 ng STX eq./mL in the SPR biosensor assay. Standards were prepared by spiking the control clam matrix with the PSTs followed by serial dilution of these stock concentrations using the control clam matrix. Blank solutions containing no PSTs (0 ng/mL) were performed with control clam matrix for each PST calibration curve.

2.3. Mixed PST standards

Stock solutions of 90% STX with 10% NEO, 77% B1 with 23% STX, and 80% GTX1,4 with 20% STX were prepared and then serially diluted in clam matrix. The stock solutions were designed to have overall toxicity of 160 μg STX eq./100 g tissue for those standards containing NEO and GTX1,4, while the B1 standard had a stock concentration of 80 μg STX eq./100 g.

2.4. Antibodies

Two antibodies used in this research are commercially available in ELISA kits. The first antibody (Ab1) was used as received from the kit (Ridascreen Fast PSP SC, R-Biopharm AG, Darmstadt, Germany). Standard antibody dilutions were run on the STX chip; the 1:5 dilution in HBS-EP+ used throughout these studies had a response similar to that obtained from the 8 μg/mL burro anti-STX also used in this study as described below (see [41]).

The second antibody (polyclonal anti-STX, Ab2) was generously supplied in a purified form (ammonium sulfate precipitation followed by dialysis against 10 mM PBS, pH 7.3) from Beacon Analytical Systems (Saco, ME). The concentration that gave the same signal ($\sim$150 RU) as 8 μg/mL burro anti-STX was 25 μg/mL, and this concentration was used for all PST immunoassays.

The third antibody (polyclonal, protein G purified burro anti-STX, Ab3) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD). Antibody dilutions were run to determine the appropriate concentration for immunoassay, and 8 μg/mL was used for all studies herein.

2.5. Instrumentation

A Biacore T100 (GE Healthcare) surface plasmon resonance biosensor was used for all SPR immunoassays. The instrument was run via the Biacore T100 Controller Software v. 2.0, and data evaluation was performed with the Biacore T100 Evaluation Software v. 2.0. The instrument and sensor chips were normalized following the manufacturer procedures prior to performing the PST immunoassays. The SPR response (Resonance Unit, RU) is a measure of the angle of minimum reflected intensity that occurs upon changes in refractive index where 1 RU corresponds to a 10^{-6} change in refractive index ($\sim 10^{-4}$ degree angle shift).

2.6. Sensor chip

The STX biosensor surface was prepared on a Series S CM5 sensor chip and has been previously described [41]. Briefly, all flow cells were activated to succinimidyl esters using the instrument amine immobilization wizard and EDC/NHS from the amine coupling kit. Flow cell one was then deactivated with ethanolamine to create a reference surface, while flow cells two through four were activated with jeffamine, and unreacted sites were blocked with ethanolamine. The chip was removed from the instrument, and STX was conjugated to the chip surface via 15 h, 37 °C reaction with formaldehyde in 100 mM phosphate buffer. The chip was then rinsed with water, dried with $N_{2(g)}$, and docked into the SPR instrument. The fluidics and sensor chip were primed with HBS-EP+, and three startup cycles with 50 mM NaOH were performed prior to running the standards.

2.7. Immunoassay

Immunoassays were performed using the Biacore T100 optical biosensor with HBS-EP+ as the running buffer. The sample compartment temperature was set at 10 °C while the analysis temperature was held constant at 25 °C. The antibodies were diluted as noted above and then mixed in the instrument autosampler (90% Ab to 10% standard, 600 s mix time) prior to injection. This mixture was injected over the STX sensor chip at a flow rate of 20 μL/min for 120 s followed by a 60 s dissociation period and then a 240 s regeneration with 50 mM NaOH. STX controls (0.3, 3, 30, and 300 ng/mL in HBS-EP+ with 8 μg/mL Ab3) were run with every cycle to ensure chip stability.

2.8. Data processing

The response (RU_{PST}) for each sample was obtained by subtracting the baseline (10 s prior to injection) from the stability point (15 s after sample injection completion). These values were then normalized to the blank (0 ng/mL PST for associated antibody, RU_0), and the results multiplied by 100 to achieve percent binding for each antibody/PST combination: % binding = $(RU_{PST}/RU_0) \times 100$.

Each flow cell was normalized separately, and the data from flow cells 2, 3, and 4 were averaged together. Due to nonspecific binding and bulk effects for Ab1, the background from the blank measurement (0.45 normalized response) was subtracted from each data point for this antibody. The average response was then plotted versus the original solution concentration for each PST in ng/mL. Curve fitting was performed using a variable slope, four parameter model for log(inhibitor) vs. response in GraphPad Prism (v. 5.02, La Jolla, CA). GraphPad Prism was then used to calculate the values at inhibitory concentrations (IC): IC_{20} (80% binding), IC_{50} (50% binding), and IC_{80} (20% binding). Additionally, the response at 100 ng/mL for each PST based on the generated curves was determined. For concentrations where full inhibition was not seen (e.g., C1,2 with Ab3 where the highest standard gave 49.8% binding without flattening of the curve), the software extrapolates the curve fit to determine the 20% binding point.

Cross-reactivities (CR) for each PST were calculated from the IC_{50} values of each toxin with respect to the IC_{50} of STX: % CR = $(IC_{50,\ STX}/IC_{50,\ PST}) \times 100$. The 100 ng/mL level is defined as the response at the action level (RAL) and is equivalent to a sample containing only STX at 80 μg/100 g tissue based on extraction dilution.

For the theoretical lateral flow immuno-chromatography, the reactivity for each antibody-PST congener was translated to a band in the device. For Fig. 3, the RALs (100 ng/mL from Table 3) were used, while in Fig. 4 the response at 80 μg STX eq./100 g tissue were translated to band patterns. To account for differing sensor chips and nonspecific binding in Fig. 3, the values were corrected for each antibody by subtracting the background (Ab1 = 12.45, Ab2 = 8.60, Ab3 = 3.43) from each response. Using PowerPoint, the color of the band was defined by the RGB parameters 128 red, 0 green, 0 blue with the transparency of the band equal to (100—Response).

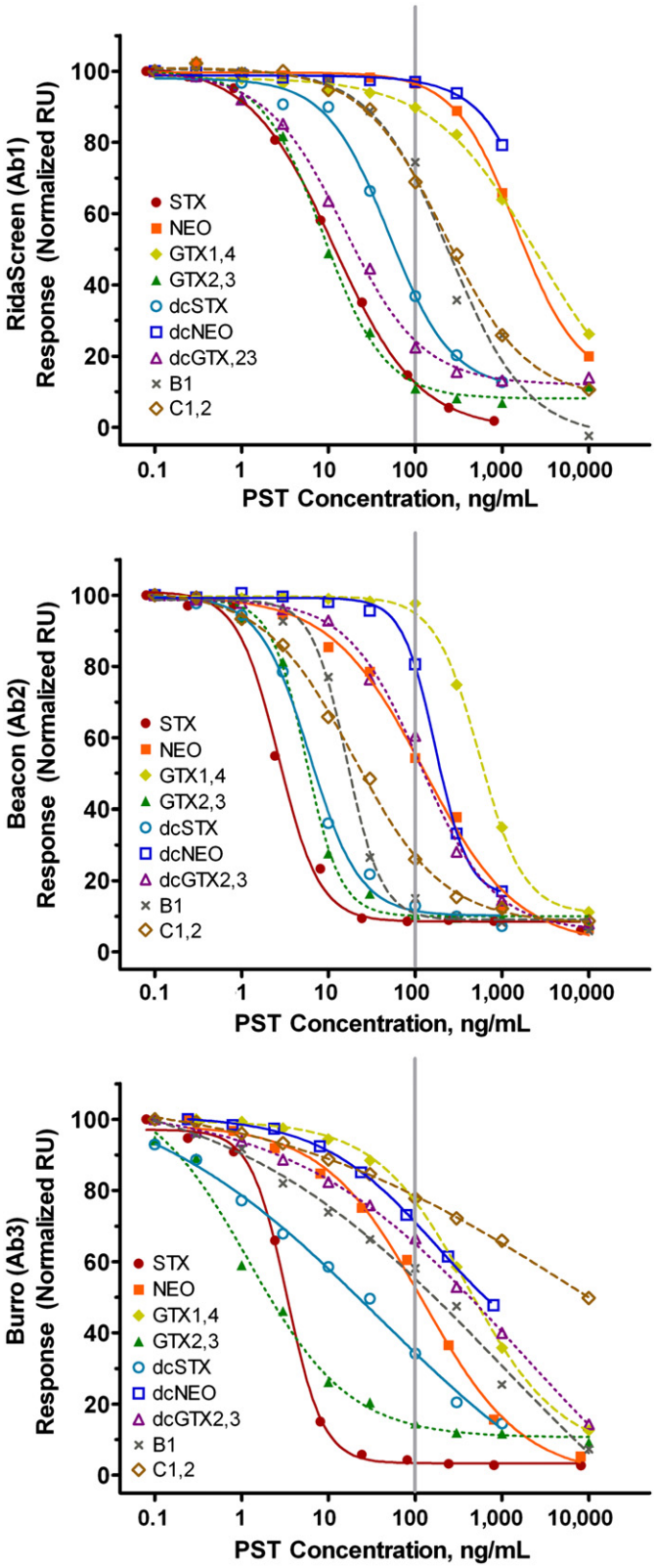


Fig. 1. Antibody reactivity with nine common PSTs. $N = 3$ with error bars smaller than the data points. The vertical grey line at 100 ng/mL represents the action level for a sample containing only STX (80 μg STX/100 g tissue).

3. Results and discussion

3.1. Cross-reactivity (CR) of antibodies

Previous research has focused on developing rapid, sensitive SPR assays that are capable of detecting PSTs in buffer [41] and common shellfish matrices [36]. The challenge to correctly estimating sample potency lies in the current inability of antibodies to react with the congeners according to their toxicities (Table 1). The PSTs can be classified into three major categories with their toxicities generally following the R4 substituent groupings: carbamate > decarbamoyl > *N*-sulfocarbamoyl. It has been shown that

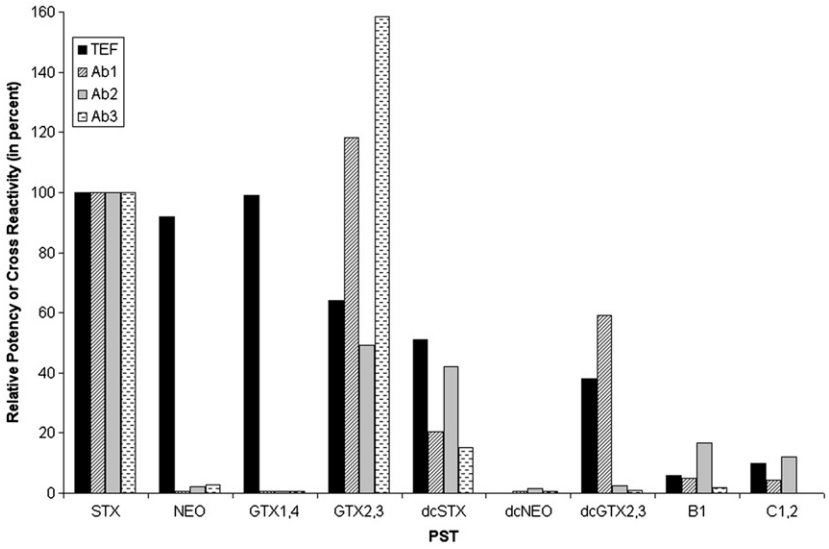


Fig. 2. Cross-reactivity for each antibody (from Table 3) in comparison to the toxin equivalency factors (from [17]) for each PST. For PST congeners where epimers are reported in pairs (e.g., GTX1,4) the value of the epimer with the higher TEF is used. No TEF value for dcNEO is reported by Oshima.

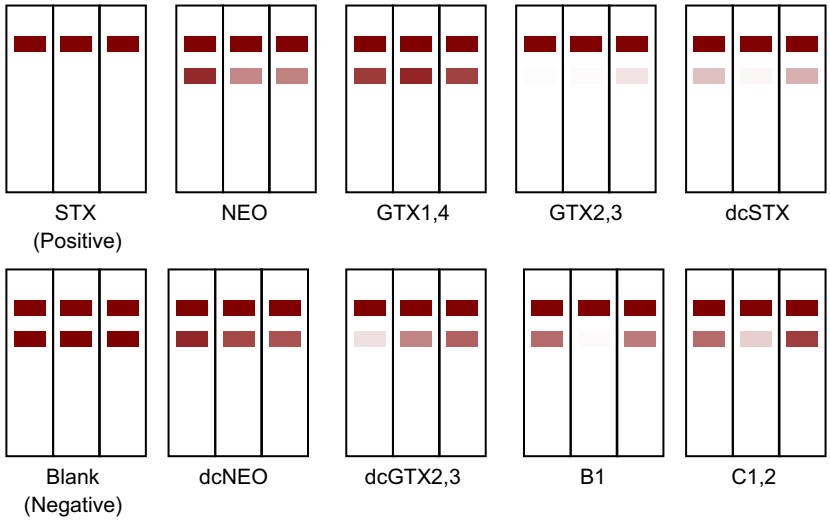


Fig. 3. Conceptual lateral flow immunochromatographic devices for samples containing a single PST at 100 ng/mL. Each strip corresponds to an individual antibody (left to right: Ab1, Ab2, Ab3) in which a competition assay is performed with the analyte.

Table 2
IC_{50} and dynamic range (DR = IC_{20} to IC_{80}) for each antibody. All values in ng/mL.

PST	Ab1: RidaScreen		Ab2: Beacon		Ab3: Burro	
	IC_{50}	DR	IC_{50}	DR	IC_{50}	DR
STX	11.9	2.7–54.3	3.0	1.4–7.7	3.4	1.6–6.9
NEO	1834.9	530.5–9986.0	131.7	23.1–793.1	116.0	17.5–726.4
GTX1,4	2346.7	324.9–16197.1	630.3	260.9–1955.7	470.3	79.2–3438.4
GTX2,3	10.1	3.1–41.4	6.0	3.0–14.3	2.1	0.4–24.6
dcSTX	58.6	16.2–290.7	7.1	2.6–24.7	22.0	0.8–442.4
dcNEO	2369.6	964.5–3724.2	199.3	100.5–593.0	642.5	43.2–39711.6
dcGTX2,3	20.1	4.2–165.9	126.9	30.7–606.1	426.6	15.9–5851.5
B1	236.4	58.1–920.7	17.7	8.8–40.8	167.4	5.5–2673.5
C1,2	266.4	56.1–1716.3	24.8	4.6–190.7	9983.5	75.1–2091542

different antibodies can have different cross-reactivities to the PST congeners [43]. To take advantage of this characteristic, the cross-reactivity of three available antibodies are examined herein using SPR biosensor evaluation to determine the feasibility and merits of using a multi-antibody approach for both SPR biosensors and LFIs.

Fig. 1 shows the cross-reactivity curves of the binding of each antibody with STX, NEO, GTX1,4, GTX2,3, dcSTX, dcNEO, dcGTX2,3, B1 and C1,2. These assays were designed to have nearly-complete inhibition with STX at the action level (indicated by a vertical grey line in the graphs). Qualitatively, it is clear that

Table 3
SPR-determined antibody cross-reactivities (CR in %), reported ELISA cross-reactivities, and responses at the action level (RAL in normalized RU, at 100 ng STX/mL).

PST	Ab1			Ab2			Ab3	
	CR	ELISA[a]	RAL	CR	ELISA[a]	RAL	CR	RAL
STX	100.0	100	12.5	100.0	100.0	8.6	100.0	3.4
NEO	0.7	12	96.5	2.3	0.8	55.4	2.9	52.8
GTX1,4	0.5		89.6	0.5	< 0.1	94.9	0.7	76.9
GTX2,3	118.3	70	12.5	49.4	12.0	10.2	158.5	14.1
dcSTX	20.4	20	37.0	42.0	18.0	11.5	15.2	34.4
dcNEO	0.5		97.1	1.5	0.7	80.2	0.5	71.1
dcGTX2,3	59.2		24.3	2.3	0.4	55.7	0.8	65.0
B1	5.0		70.1	16.8		10.8	2.0	55.3
C1,2	4.5		70.2	12.0		26.9	0.03	78.5

[a] Values for the ELISA cross-reactivities obtained from pamphlet information contained in the kits.

the antibodies have distinct reactivity with the nine PST congeners, as the calibration curve patterns for each antibody are dissimilar. This can be further seen in the individual congener plots (Supplementary materials A) where it is visually easy to compare the similarities (e.g., the three antibody curves for STX are similar with Ab2 and Ab3 responses nearly overlapping) and differences (e.g., the curve shapes for the three antibodies reacting with B1 are quite dissimilar). The data can also be quantitatively evaluated via IC_{50} and dynamic range (Table 2) as well as percent cross-reactivities and responses at the action level (RAL) (Table 3). When these results are considered together, it is clear that no antibody profile accurately reflects potency; however, each antibody has a distinct profile with advantages and disadvantages as discussed below.

For RidaScreen (Ab1), high reactivity (> 100% CR) with STX and GTX2,3 is seen while low reactivity (< 5% CR) with many congeners is observed. When used in an assay alone, Ab1 would be expected to yield false-negative results when the highly toxic NEO and GTX1,4 are present. Beacon (Ab2) has high reactivity with STX but low reactivity with NEO, GTX1,4, dcNEO, and dcGTX2,3. In this case, samples high in GTX1,4 and/or NEO may also result in false-negatives. Additionally, the moderate reactivity (~ 15% CR) with low potency B1 and C1,2 may result in false-positive results if these toxins dominated the profile. Finally, the Burro (Ab3) has high reactivity with STX and GTX2,3 and to a lesser extent with all congeners tested. In this scenario, a sample with a toxin profile dominated by GTX1,4, and/or NEO may be screened as negative when toxin levels may be above the action level, whereas a false-positive may occur if GTX2,3 dominated the sample.

When comparing the profiles based on substitution groups (R1 to R4 as shown in Table 1, Supplementary materials B1–B3), all antibodies show limited reactivity with OH modification at the R1 group potentially due to steric hindrance, charge, or hydrophilicity imparted by this group. Indeed, weak reactivity with the R1-hydroxylated PSTs is frequently found with antibodies [43]. Antibody cross-reactivity for the non-hydroxylated compounds is mainly driven by the R4 functionality with highest reactivity seen in the carbamate modified PSTs followed by decarbamoyl PST forms and *N*-sulfocarbamoyl conjugations.

The two antibodies used for this work that were obtained from commercial ELISA kits have been previously evaluated for cross-reactivities (Table 3). For Ab1, the published cross-reactivity order (STX > GTX2,3 > dcSTX > NEO) is similar to the SPR results (GTX2,3 > STX > dcGTX2,3 > dcSTX > B1 > C1,2 > NEO > GTX1,4 = dcNEO) except for the exchange of STX with GTX2,3. This discrepancy may not be significant due to the very similar reactivity of the antibody to these PSTs as seen in the SPR curves in Fig. 1. For Ab2, the published results are STX > dcSTX > GTX2,3 > NEO > dcNEO > dcGTX2,3 > GTX1,4; however, the SPR analysis showed STX > GTX2,3 > dcSTX > B1 > C1,2 > NEO = dcGTX2,3 > dcNEO > GTX1,4. The order differences of GTX2,3/dcSTX as well as NEO/dcNEO/dcGT2,3 could be expected due to the very similar curve shapes and, with the error in measurements, these values may not be significantly different.

One further and important consideration is how cross-reactivity corresponds to toxin-equivalency factors (TEFs) for each PST (Fig. 2). The values for STX are set at 100%, based on the definitions for TEF and CR, and therefore show no differences between potency and cross-reactivity with each antibody. Ideally, the best antibody profile would have CRs to each congener that match their TEFs. However, it is clear that antibodies perform poorly at matching the TEF values, especially for R1-hydroxylated toxins (e.g., NEO and GTX1,4) and with varied success for the non-hydroxylated congeners. For example, Ab2 cross-reactivities correspond reasonably well with the TEF for GTX2,3, dcSTX and C1,2, while Ab1 and Ab3 cross-reactivities correlate closely with the TEF for B1. These distinctions in reactivity between the antibodies could be exploited for use in a multiplexed format to create an assay that would yield more information regarding the toxin profile and thus more confidence in sample potency.

3.2. Theoretical lateral flow immuno-chromatography

In general, the format of the LFI used to detect PSTs is a competitive displacement assay. In this assay, sample extract is added to the sample well of the pad and is drawn up through the membrane. The toxin first interacts with the conjugate pad containing antitoxin-coated gold particles. The antibody and toxin interact and remaining antitoxin-coated gold particles bind to the test line coated with toxin conjugated protein. The higher the toxin concentration in solution the fainter the red line at the test line position. A control line is also present and should always yield a strong red response. In this way, two red bands indicate that a sample contains little to no toxin, whereas a single band at the control position indicates the sample contains toxin.

By using the antibodies screened via SPR, combinations of antibodies that create a unique pattern could be incorporated into an LFI for higher confidence in sample toxicity. Prior to undertaking extensive studies in incorporating multiple antibodies into an LFI, a theoretical model was designed and is shown in Fig. 3 with each strip in the three-strip system containing a single antibody-gold colloid. In envisioning the LFI functionality, only single toxin solutions at 100 ng/mL (e.g., equivalent to the action level for a toxin containing only STX, per extraction procedure used) are applied to each three-strip system. As can be seen in the STX LFI and Blank LFI, a positive sample would have only the three control bands while a negative result would show control bands as well as three strong bands at the test line position below each control band.

The theoretical LFIs for the other PST congeners show that, indeed, more confidence in sample toxicity could be gained by using multiple antibodies. For example, the pattern for B1 (which has low toxicity) with only Ab2 indicates an unsafe sample (a false-positive result), but when used in combination with the information from the Ab1 and Ab3 strips the pattern and strength of the bands could allow a user to realize the sample is safe for harvest and consumption. Unfortunately, false-negative results were still not eliminated. This is demonstrated in the NEO and GTX1,4 conceptual tests in which these two LFIs demonstrate very little discernible difference from the blank (negative), yet have concentrations equivalent to toxicity near the action level.

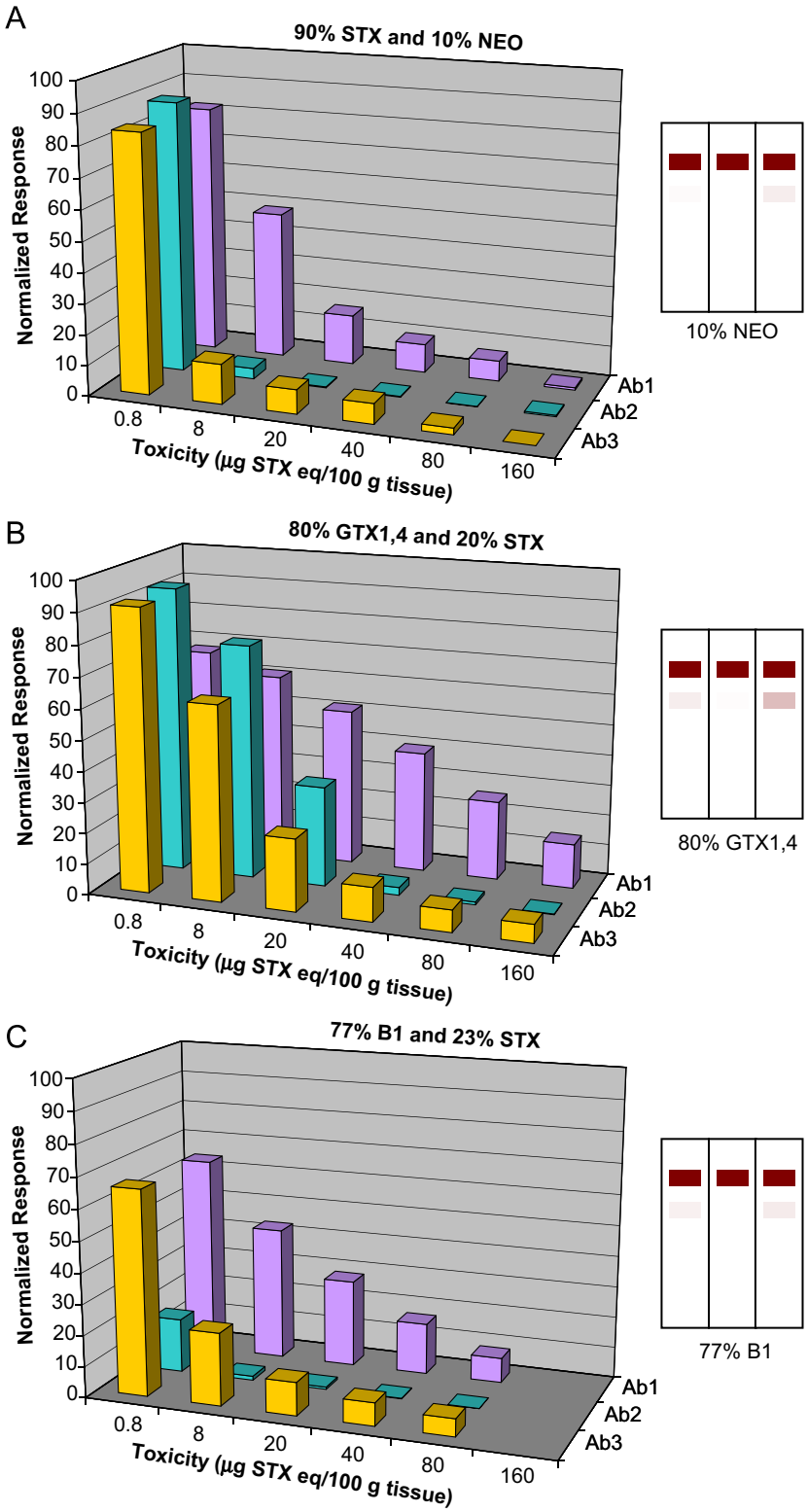


Fig. 4. Reactivity of antibodies to PST mixtures using the SPR biosensor and corresponding theoretical LFI device for mixtures containing 80 µg STX eq. per 100 g tissue for (A) 90% STX and 10% NEO, (B) 80% GTX1,4 and 20% STX, and (C) 77% B1 and 23% STX.

3.3. *PST mixtures*

PST congeners commonly exist as mixtures in naturally contaminated shellfish which could complicate such a simplistic "pattern matching" approach of the LFIs. To investigate this, experiments were performed with the antibodies and mixtures of PST compounds. Differing toxicity PST mixtures were tested to determine if the antibodies would perform well in situations that challenged high and low cross-reactors that do not correlate with toxicity (i.e., the potential for false-positive or false-negative results, respectively). The selected mixtures represented examples encountered in natural waters: 90% STX with 10% NEO

(e.g., *Alexandrium* sp. and shellfish in Alaska [anticipated accurate test performance]), 80% GTX1,4 with 20% STX (e.g., shellfish in Scotland [anticipated potential false-negative]), and 77% B1 with 23% STX (e.g., *Pyrodinium* sp. in Florida [anticipated potential false-positive]). Results from these SPR assays, and corresponding theoretical LFIs, are shown in Fig. 4 where the toxicity at 80 μg STX eq. per 100 g tissue is the action level in the United States.

For all mixtures, the antibodies respond well for samples in which there is high toxicity, with all SPR biosensor data showing the desired inhibition at the 80 μg STX eq./100 g tissue action level. If these were naturally contaminated samples, the SPR screening technique would indicate that further, confirmatory testing is required. With the mixture of 90% STX with 10% NEO, the curves nearly follow that predicted from a sample of STX alone, thus yielding a representative, accurate test. From this, an arbitrary cut-off level of all antibodies having an SPR response below 50 could be set for the level at which further confirmatory testing would be required. For the 90% STX with 10% NEO, this level corresponds to samples of approx. 8 μg STX eq./100 g tissue and would allow for adequate screening without over burdening confirmatory testing techniques. As expected, the potential false-negative system (80% GTX1,4 with 20% STX) would only have further testing indicated for samples containing more than 20 μg STX eq./100 g tissue. While this is below the action level, any potential systematic errors could lead to a false-negative result. Finally, for the potential false-positive mixture of 77% B1 with 23% STX, samples very low in toxicity (<8 μg STX eq./100 g tissue) would be indicated for further testing. In this case, false-positive results could still be common.

With regard to the theoretical LFI devices for these mixtures, all conceptual LFIs with the three antibody system show patterns which indicate further testing would be required based on the faint-red response of the test lines for all three antibodies. In the case of the 80% GTX1,4 with 20% STX, the red line from Ab3 is clearly visible and a faint line from Ab1 can be seen. If a pattern matching approach was used, the pattern for this toxic sample could mimic that of the nontoxic B1 sample in Fig. 3. Thus, while the pattern matching and intensity approach could enhance reliability, the current antibody combination does not fully alleviate the challenges associated with false-negative and false-positive results.

While this three antibody system could increase the knowledge of a sample composition and potential toxicity, the antibodies and LFI format used herein do not allow for full resolution of the false-negative and false-positive challenges that currently plague PST immunoassays. Furthermore, the SPR results will also suffer from similar challenges but could allow for more confidence in results due to the quantitative nature of the immunoassay versus reliance on visual readout of minor hue variations from the LFI device. Our research studies continue to focus on the generation and screening of antibodies with differing cross-reactivities, especially with improved reactivity to the hydroxylated toxins, and to determine if a mixture of multiple antibodies [44] or spatially separate antibodies (as shown in the conceptual LFIs) will be best for rapid tests.

4. Conclusions

Rapidly screening seafood samples for potential contamination by PSTs remains an analytical challenge. Sensitive, real-time SPR assays for PSTs have been developed and were used to evaluate the reactivity of three antibodies. The results show that each antibody has a unique reactivity for the PST congeners. This highlights the potential for developing antibodies that could have a higher correlation of response with sample potency. While the production of such antibodies continues, techniques employing the current antibodies for higher-confidence screening were evaluated and a conceptual model was created. This model indicated that while false-positive and false-negative results were not completely eliminated, there is potential to improve immunoassays and reduce the use of MBA and confirmatory analytical tests. Current research is focusing on the development of PST binders (i.e., antibodies, aptamers, receptors) that have better cross-reactivity with the congeners. Once candidate binders are evaluated by the SPR biosensor, they will be incorporated into a multi-binder, rapid test to fully realize the potential of a potency-based screening technique.

Acknowledgments

The authors express their gratitude to Mark Poli and his colleagues at USAMRIID for providing the burro anti-STX used in this work. We also thank Titan Fan from Beacon Analytical for providing purified anti-STX. Thank you to Katrina Campbell, Christopher Elliott, and Simon Haughey for valuable discussions and their contributions to the field of PST biosensors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.06.073.

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SEAFOOD TOXINS

Indirect Enzyme-Linked Immunosorbent Assay for Saxitoxin in Shellfish

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An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of saxitoxin (STX). Antibodies against STX were demonstrated in rabbits 5 weeks after immunizing with STX-bovine serum albumin (STX-HCHO-BSA). In the ELISA, STX-HCHO-BSA or polylysine-STX was coated onto the microtiter plate, followed by incubation with standard toxin and anti-STX antibody. The amount of antibody bound to the solid phase was determined by incubation with goat anti-rabbit IgG peroxidase conjugate and a reaction with chromogenic substrate. Competitive indirect ELISA revealed that the antiserum did not cross-react with either carbamoyl-neo-STX-sulfate or tetrodotoxin. The antibodies for STX cross-reacted with decarbamoyl-STX and neo-STX about 56% and 16% as much as they did with STX, respectively. The lower detection limits for STX, decarbamoyl-STX, and neo-STX in this sytem were about 25, 45, and 156 pg per assay, respectively. When STX added to clams or mussels was assayed, the detection limit for STX was about 50–100 ppb, and recoveries were in the range of 86.8–107%.

Saxitoxin is one of the major and most potent in a group of toxins involved in paralytic shellfish poisoning (PSP) (1–3). The toxin is produced predominantly by the dinoflagellate *Gonyaulax catenalla* (4) and is primarily isolated from toxic mussels, clams (2), and other marine animals in waters inhabited by *Gonyaulax*. The poison has no adverse effect on the shellfish using the dinoflagellate as a food source. Human ingestion of toxin-contaminated shellfish has resulted, however, in paralytic poisoning and occasionally death (2). The amount of PSP poison that causes death in humans is estimated to be about 0.5–4 mg (2). The Food and Drug Administration has set the maximum acceptable level for paralytic poison in fresh, frozen, or canned shellfish at no more than 400 mouse units (MU) or about 80 μg/100 g edible portion (2).

Because of the potential health hazard, a quick, sensitive, and specific method is needed to determine the presence of toxins in shellfish. Due to its simplicity, the mouse bioassay (5) has been adopted as an official method for monitoring the poison; however, this method is neither specific nor sensitive, and also requires a continuous supply of mice. About 0.18 μg STX is required to kill a 20 g mouse in 15 min. Other methods, including fluorometric assay (6) and colorimetric techniques (7), also have sensitivity and specificity problems. More recently, a liquid chromatographic method was developed by Sullivan and Iwaoka (8). Although this method can detect low levels of PSP (0.5–25 ng/assay), it requires expensive equipment, and samples must be analyzed one at a time (8, 9).

Because of the highly specific antigen-antibody interaction, several laboratories have attempted to develop an immunoassay for PSP. As early as 1964, Johnson et al. (10) reported an immunoassay involving a hemoagglutination reaction; however, the sensitivity was not high enough for practical application of the immunoassay. Most recently, Carlson and Guire (11) demonstrated a radioimmunoassay system that permitted detection of less than 1 ng STX, but the antiserum did not react with neo-STX, and, therefore, the antiserum has only limited use. In our laboratory, we have attempted to produce specific antibodies against STX. Among several approaches tested, we found that the antibody produced in rabbits after immunizing with STX conjugated to bovine serum albumin was useful for STX assay. Subsequently, we developed an indirect enzyme-linked immunosorbent assay (ELISA), and details for production, characterization, and the ELISA protocols for determination of STX in clams and mussels are described here.

Received May 24, 1984. Accepted August 20, 1984.
Presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

Experimental

Materials

Purified STX was provided by E. J. Schantz and R. W. Wannemacher, Jr. Decarbamoyl STX was prepared according to the method of Ghazarossian et al. (12, 13). Neo-STX and tetrodotoxin were supplied by R. E. Carlson, and carbamoyl-neo-STX sulfate was provided by H. Schnoes. Bovine serum albumin (BSA, RIA grade), polylysine (mol. wt. 60,000), Tween 20, 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), 30% hydrogen peroxide, and goat anti-rabbit IgG peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO). Complete and incomplete Freunds's adjuvant were obtained from Difco Laboratories (Detroit, MI). Gelatin was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Polystyrene microwell plates (NUNC product, Denmark; No. 2-69620) were obtained from Vangard International Inc. (Neptune, NJ). CF-1 mice were purchased from Harlan/Sprague-Dawley (Madison, WI). Albino rabbits, female, 5 lb size, were purchased from Klubertanz Rabbit Farm (Edgerton, WI) and were tested to be *Pasteurella*-negative before use. All chemicals and organic solvents were reagent grade or better.

The cultivated mussels *(Mytilus recurvus)* and cherrystone clams *(Mercenaria mercenaria),* harvested from the Chesapeake Bay, MD, were purchased from a local seafood store. Clams were kept refrigerated and mussels were kept in crushed ice. Both extracts were tested by mouse bioassay which showed no toxicity. The naturally contaminated scallop (Pecten grandis) samples, supplied by E. J. Schantz who collected them from the Bay of Fundy in 1980, were shucked and kept frozen at −20°C before extraction.

Preparation of Saxitoxin Antigen

The antigen (STX-HCHO-BSA) was prepared according to Johnson et al. (10) with slight modifications. In a typical experiment, 14.25 mg bovine serum albumin in 6 mL 0.1M sodium acetate buffer (pH 4.2) was reacted with 1.5 mg saxitoxin in the presence of 0.081 mL 37% formaldehyde (w/w). The reaction was carried out at room temperature for 72 h and then at 5°C for another 12 h. The reaction mixture was dialyzed against 2 L acetic acid (0.001M) at 5°C for 72 h with 2 changes of the acetic solution during this period to remove residual-free saxitoxin. The molar ratio of STX:BSA was estimated to be about 16:1 by measuring the free amino groups

before and after reaction (14). The same method was used to prepare the STX-polylysine. Succinyl-STX-BSA and decarbamoyl-STX BSA were prepared according to the procedure of Ghazarossian (15).

Immunization Schedule

Three rabbits were immunized with STX antigen, using a multiple site injection method (16). The fur along the back and the proximal limbs of the rabbits was shaved before injection. Two mL of emulsion was made by mixing 500 μg STX-HCHO-BSA in 0.5 mL sterilized saline and 1.5 mL complete Freund's adjuvant. About 30–50 μL emulsion was injected intradermally at each site over the shaved area, and 0.2–0.25 mL emulsion was injected subcutaneously on each shoulder. Each animal received about 30–40 injections. Three or four weeks after the initial immunization, the rabbits were bled on a weekly basis, and the titers were determined. Six to eight weeks after the initial injection, the animals were boosted intramuscularly with another 500 μg antigen in incomplete Freund's adjuvant emulsion prepared by emulsifying 1 volume of antigen with 1 volume of adjuvant. The same schedule (5–6 weeks interval) was used for subsequent booster injections. The antiserum collected was precipitated with ammonium sulfate to a final 33% saturation. The precipitate was redissolved in water to the same original serum volume, dialyzed against 2 L 0.1M PBS overnight, and then lyophilized. The lyophilized, purified antibody was stored at −4°C.

Enzyme-linked Immunosorbent Assay

Titration of antibody titers.—The ELISA protocols are essentially the same as those we described for the analysis of aflatoxin B_1 (17). The optimal dilution of antigen necessary for precoating microtiter plates was determined by the checkerboard test. Fifty μL antigen (STX-HCHO-BSA) at a concentration of 1.6 μg/mL in 0.5M bicarbonate buffer at pH 9.6, was added in each well and incubated overnight at 4°C. The plate was washed with various amounts of washing solution (0.1M sodium phosphate buffered saline containing 0.1% Tween 20, pH 7.5) in the following sequence: twice with 0.1 mL, twice with 0.2 mL, and 3 times with 0.32 mL each well. Three hundred μL 0.1% gelatin in PBS was added to each well to eliminate nonspecific binding by blocking the plastic surface where protein was not bound. After 30 min to 1 h incubation at 37°C, the well was washed 4 times each with 0.32 mL washing solution. Next, 50 μL of various dilutions of antibody, diluted in PBS containing 0.1% BSA, was added to each well. Following 1 h incubation at 37°C, the plate was washed again to remove the free antibody. Fifty μL goat antirabbit IgG peroxidase conjugate (1:500 dilution in PBS containing 0.1% BSA) was added to each well, and the wells were incubated another hour at 37°C. The plate was washed again. The peroxidase substrate (ABTS in citrate buffer, pH 4.0) of 0.1 mL was finally added to each well. After developing the color 20 min at 37°C, the reaction was terminated by adding 0.1 mL hydrofluoric acid–ethylenediamine tetracetic acid stopping reagent, and absorbance at 410 nm was determined by a Dynatech minireader. The antibody titer was defined as the reciprocal of the antiserum dilution that gives an absorbance at 410 nm that is 0.1 unit greater than that of the pre-immune serum.

Competitive indirect ELISA.—Protocols used for titration of antibody titers were slightly modified in this assay. One hundred μL of an appropriate dilution of antigen in 0.05M carbonate buffer, pH 9.6, was coated to the plate. In addition, 50 μL purified STX, other PSP toxins, or unknown samples were incubated together with 50 μL of appropriate dilution of antibody in each well. The amount of bound antibody was determined by adding 0.1 mL goat antirabbit IgG peroxidase conjugate.

Preparation of Samples

The AOAC acid extraction method (5) was used throughout the experiment for extraction of STX from clam and mussel before ELISA. The whole tissue of clam was used for extraction whereas only the dark gland of mussel was used. Mussels were shucked and the dark glands (hepatopancreas) were removed. About 15 mussels gave a total of 5 g dark glands. The samples (50–100 g clam or 5 g dark gland) were homogenized with an equal volume of 0.1N HCl and then boiled 5 min. After cooling to room temperature, the solution was adjusted to pH 4.0–4.5 and then centrifuged to remove the tissue. The acidic extract was adjusted to pH 7.0 and immediately used in the ELISA.

In the recovery experiment, different concentrations of STX diluted in PBS were injected into 6 groups (5 g each group) of dark gland samples. After 30 min, 5 mL 0.1N HCl was added, and the sample was extracted by the procedures described. For clam samples, toxin was added to 50 g of sample during the homogenization step.

Analysis of Samples by Competitive Indirect ELISA

In a preliminary study, the effect of the acid extracts of blank samples on the indirect ELISA was tested. However, we observed significant interference in the ELISA where more than 50 mg sample was used. The interference was minimal when 5 mg/mL of mussel dark gland or 50 mg/mL of clam meat was used. Subsequently, a phosphate-buffered saline solution (0.1M, pH 7.4) containing either blank mussel dark gland or blank clam meat acid extracts, at concentrations of 5 and 50 mg/mL, respectively, was used for preparation of STX standard and sample solutions for ELISA. A naturally contaminated scallop sample was also analyzed for STX by ELISA. A high level of STX was present in this sample, so high dilution was necessary (1 to 10,000 dilution of the acid extract). ELISA was carried out in regular phosphate buffer instead of buffer containing blank samples for this naturally contaminated sample.

Results and Discussion

In the initial studies, we tested several different STX-BSA conjugates for their ability to produce antibodies against STX, using indirect ELISA to monitor the antibody titer. We found that decarbamoyl-STX BSA, succinyl-STX BSA, and STX-HCHO-BSA all elicited antibody against STX. Because the best antibody titers were obtained from rabbits that had been immunized with STX-HCHO-BSA, our efforts were concentrated on this immunogen. Figure 1 shows a typical titration curve for the antibody titer for a rabbit immunized with this antigen. Figure 2 shows results for the average antibody titers of 3 rabbits over a period of 10 weeks. Good antibody titers were obtained from rabbits as early as the 5th week after immunization. Antiserum titer increased considerably after the first booster.

Antibody specificity was determined by a competitive indirect ELISA in which different STX derivatives were present in the assay system to compete the binding of STX coated to the solid phase with the antibody. In this assay, the STX-polylysine conjugate was coated to the plate because this conjugate gave less aggregation after repeated thawing. After incubation with the antibody and different STX derivatives, the rabbit antibody bound to the STX-polylysine solid phase

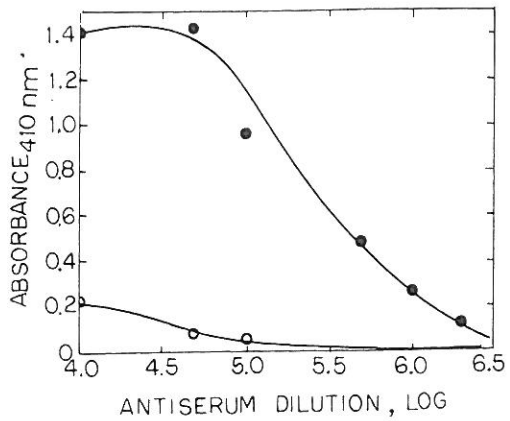

Figure 1. Titration of antibody titer by indirect ELISA. See text for description of procedure. Titer is defined as reciprocal of antiserum dilution that gives an absorbance at 410 nm that is 0.1 unit greater than that of the pre-immune serum. Antisera from rabbits immunized with STX-HCHO-BAS (●); pre-immune serum (○).

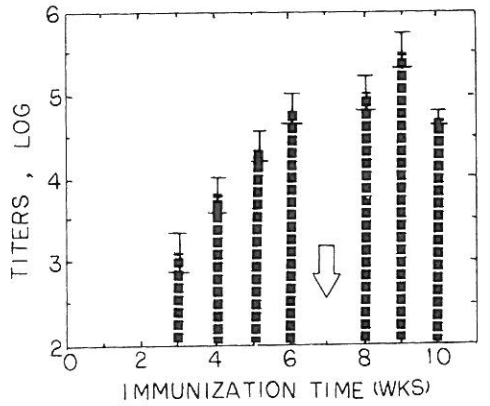

Figure 2. Average antibody titers for 3 rabbits immunized with STX-HCHO-BSA. Range of antibody titer for bleeding of 3 rabbits is represented by an error bar. Arrow indicates time of booster injection.

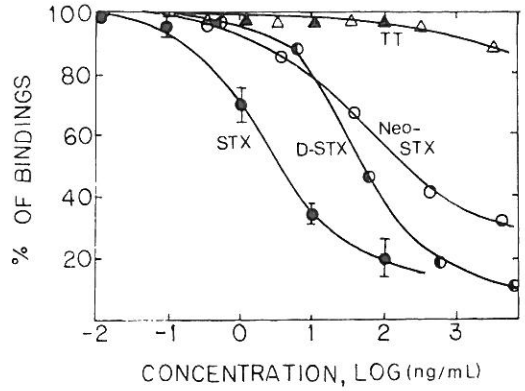

Figure 3. Competitive indirect ELISA for STX. See text for description of procedure. Saxitoxin (●), neo-saxitoxin (○), decarbamoyl-saxitoxin (◐), carbamoyl-neo-saxitoxin sulfate (△), and tetrodotoxin (▲).

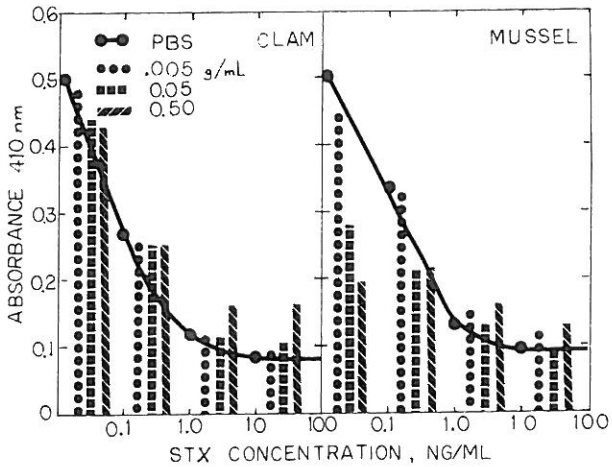

Figure 4. Effect of clam and mussel extracts on indirect ELISA of STX. Curves represent data obtained from experiments using STX in PBS buffer. Data from experiments using clam and mussel extracts are shown as bars. STX concentrations for spiking experiments are indicated to left of bars on X-axis. All data represent average of 3 measurements.

was determined by reaction with goat-antirabbit peroxidase complex and enzyme substrate. Results (Figure 3) indicate that the antibody has high affinity for STX. The concentrations that cause 50% inhibition of binding of the antibody to the solid phase antigen (i.e., STX-HCHO-polylysine) by STX, decarbamoyl-STX and neo-STX are 3.2, 50, and 180 ng per assay, respectively. Thus, the antibodies for STX cross-reacted with decarbamoyl-STX and neo-STX only about 56.2% and 16% as much as they did with STX. Carbamoyl neosaxitoxin sulfate and tetrodotoxin showed no cross-reaction with the antibody at the maximal concentration tested.

Carbamoyl neo-STX sulfate did not cross-react with the antibodies whereas neo-STX did, so modification of the structure of the C-11 position may greatly affect the conformation of STX and thus prevent these derivatives from reacting with the antibody. Because the decarbamoyl-STX cross-reacts with the antibodies somewhat, the role of the terminal amide bond in the STX in determining the antibody specificity may not be as important as the side chain in the C-11 position. Additional experiments using other derivatives such as 11-hydroxysaxitoxin sulfate, which is produced by *Gonyaulax tamarensis* and is also a major PSP along the Alantic Coast (3), are needed to v

Competitive ELI
system can detect

150 pg neo-STX (3 ng/mL) in each assay where 3 standard deviations of the blank response were 5% of binding (Figure 3). It has been reported that the reactivity of neo-STX with anti-saxitoxinol antibody, which was obtained from rabbits immunized with saxitoxinol-BSA conjugate, is less than 1% of those for STX (11). Therefore, the anti-BSA-HCHO-STX antiserum has some advantage over the anti-saxitoxinol antibody, and can be used for monitoring the neo-STX, one of the major toxins in PSP.

Although the indirect ELISA could detect as little as 2 pg STX in each assay when the analysis was carried out with pure STX in buffered solution, high interference was observed when the food samples were analyzed. The problem was particularly serious when whole mussel meat extract was used. Results (Figure 4) indicate only a slight effect on the standard curve when an extract equivalent to 50 mg/mL (or 2.5 mg/assay) of clam meat was used; however, high interference was observed when an extract equivalent to as little as 5 mg/mL (0.25 mg/assay) of mussel meat was used. Therefore, subsequent recovery experiments, including the standards, were carried out in a buffer that contained either blank extract of clam meat (50 mg/mL) or blank mussel dark gland

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Table 1. Recovery (%) of saxitoxin from clam and mussel by ELISA[a]

Toxin added, ppb	Clam			Mussel		
	Rec., % (N)	SD[b]	CV[b]	Rec., % (N)	SD[b]	CV[b]
50	207 (12)	27.3	13.2	95.2 (12)	17.4	18.3
100	103 (20)	15.8	15.3	97.5 (20)	15.8	16.2
200	107 (24)	9.5	8.9	106.5 (12)	9.6	9.0
250	—[c]	—[c]	—[c]	89.6 (8)	8.8	9.8
500	90.8 (24)	11.8	13.0	86.8 (12)	11.4	13.1
1000	105.2 (24)	9.3	8.8	95.4 (14)	19.0	19.9

[a]Saxitoxin was added to whole clam tissue or mussel dark gland. In the assay, either whole tissue (clam) or whole dark gland (mussel) extract was used. In the final calculation, weight of whole mussel was used. In general, dark gland of mussel accounted for about 10% of whole mussel weight.
[b]SD and CV represent standard deviation (%) and coefficient of variation (%), respectively.
[c]Not determined.

that this gland is relatively easy to separate from the meat tissue. Several cleanup protocols were tested, but we felt that adding the blank sample extracts to the assay was the simplest approach in solving the interference problem. Table 1 gives results for the recovery of STX spiked in mussel dark gland and clam. Recoveries were between 86.8 and 107% in the range 50–1000 ppb (on the basis of total tissue weight) and 90.8–105% in the range of 100–1000 ppb STX added to the mussel dark gland and clam samples, respectively. Coefficients of variation for these assays are in the range 9–20%, which is in the range for most ELISAs. The detection limit appears to be in the range of 50–100 ppb where the blank response at 95% confidence was 3 standard deviations.

A naturally contaminated scallop sample was tested by mouse bioassay according to the AOAC method (5) and by the present ELISA. The sample contained 97.8 ± 7.8 μg STX/g sample by ELISA and 112.53 ± 6.53 μg/g by mouse assay. In the bioassay, a series of standard solutions were injected into the same strain of mice under the same experimental conditions where 0.363 μg STX was determined to be 1 mouse unit. Present results indicate that 86.9% of the STX determined by mouse bioassay was detected by ELISA. The lower recovery by ELISA may result from the presence of other toxins in the sample or from problems existing in both assays. Further comparative analyses for additional naturally contaminated samples by both methods as well as testing the cross-reactivity with other PSP toxins, are needed to establish the validity of the assay.

Conclusions

Results obtained from the recent study indicate that antibodies obtained from rabbits immunized with STX-HCHO-BSA are adequate for the analysis of STX in foods, according to ELISA protocols described. Although the antibodies also recognize neo-STX, the ELISA for this toxin is not as sensitive as that for STX. The antibodies, however, could not recognize STX when the OH-group at the C-11 position was esterified by sulfate, i.e., carbamoyl-neo-STX-sulfate. Additional experiments are needed to prepare other STX-protein conjugates for eliciting antibodies that will recognize other PSP toxins.

Acknowledgments

This work was supported by the College of Agricultural and Life Sciences, the University of Wisconsin-Madison, and by contract No. DAMD-80-C-2021 from the U.S. Army Medical Research and Development Command of the Department of Defense.

The authors thank E. J. Schantz and R. W. Wannemacher for supplying the saxitoxin and R. E. Carlson for providing the neosaxitoxin and tetrodotoxin in the present study; S. W. Li for his help in the preparation of saxitoxin-protein conjugate; and E. J. Schantz and N. Higley for their suggestions and discussion throughout the present study.

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Beacon Analytical Systems, Inc.
4 parameter ELISA Data Reduction Worksheet

Operator:
Date:
Assay ID:
Kit Lot#:

Section I)
Standards Data

#	ppb	ABS	% Bo
	0.00	1.449	100.0
	0.01	1.321	91.2
	0.08	0.661	45.6
	0.32	0.217	15.0

Section II)
Curve Fitting

Starting/Final values for Parameters A - D

A	B	C	D
100.00	1.22	0.07	2.61

Squares of differences between actual and calculated curves

X	0.00	0.01	0.08	0.32
Y Predicted	100.00	91.17	45.62	14.98
(Y-Y Pred.)^2	0.00	0.00	0.00	0.00

Minimize sum of square of differences

Min. (Y-Y Pred.)^2 =	**0.0**

Correlation Coeff.

R^2 =	1.000

Section III)
Sample Calculations

#	Sample	ID	ABS	% Bo	ppb
			0.501	34.6	0.119
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
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				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!

Reviewed & Accepted: ______________________ Date: __________

Checklist for Saxitoxin (PSP) Microtiter Plate Test Kit

Operator:		
Date:		
Sample:		
Assay ID:		
Kit name:		
Kit Lot#:		
Kit expiration date:		
Laboratory equipment		
The kitchen blender and its accessories are clean.	☐ YES	☐ NO
The balance provides a sensitivity of at least 0.1 g.	☐ YES	☐ NO
The balance calibration is checked monthly.	☐ YES	☐ NO
Microcentrifuge has a maximum speed of ≥ 12,000 rpm.	☐ YES	☐ NO
Single-channel (200 and 1000 µL) and multi-channel micropipette (200 µL) are calibrated every six months.	☐ YES	☐ NO
Microwell plate or strip reader has a filter for 450 nm wavelength.	☐ YES	☐ NO
Reagents and chemicals		
Kit components are stored at 4 – 8 °C and equilibrated to room temperature for 1 hr before use.	☐ YES	☐ NO
Make up water is distilled or deionized.	☐ YES	☐ NO
Isopropanol (2-Propanol) and Methanol are laboratory grade.	☐ YES	☐ NO
Acetic acid, Sodium Phosphate Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$) and Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4) are ACS grade.	☐ YES	☐ NO
<u>Extraction solution</u> 1. 70% Isopropanol – 70 mL of Isopropyl alcohol + 30 mL of water 2. 5% Acetic acid – 5 mL of Acetic acid + 95 mL of water 3. A mixture of 70% Isopropanol and 5% Acetic acid – 100 mL of 70% Isopropyl alcohol + 50 mL of 5% Acetic acid	☐ YES	☐ NO
<u>Dilution buffer solution</u> 1. 20 mM PBS (pH 7.2) – Sodium Phosphate Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$): 0.77g – Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4): 2.04g – Sodium Chloride (NaCl): 8.5g – Distilled or deionized water: 1L 2. 10% Methanol/20mM PBS – 10 mL of Methanol + 90 mL of 20 mM PBS	☐ YES	☐ NO

Sample preparation	
1. At least 12 shellfish are used per sample.	☐ YES ☐ NO
2. The inside of each shell is rinsed with laboratory grade water.	
3. Shellfish are shucked and strained through a #10 mesh sieve (or equivalent).	
4. Strained meats are blended at high speed until homogenous.	
5. 5 g of sample and 10 mL of extract solution are accurately measured in a 50 mL centrifuge tube and vortexed for 3 minutes.	
6. Approximately 1 mL of the extract is transferred to a microcentrifuge tube and centrifuged for 5 minutes at 12,000 rpm.	
7. The supernatant is diluted with dilution buffer 5000 fold. e.g. 0.1 mL of supernatant + 9.9 mL of dilution buffer -> 0.1 mL of the diluted supernatant + 4.9 mL of dilution buffer	
Assay	
1. Prepare the 1X wash solution	☐ YES ☐ NO
2. Allow reagents and sample extracts to reach room temperature prior to running the test.	
3. Dispense 50 µL enzyme conjugate to the appropriate test wells. Be sure to use a clean pipet tip for each.	
4. Add 50 µL of Calibrators or sample extract to each well.	
5. Dispense 50 µL of antibody solution into each test well.	
6. Shake the plate gently for 30 seconds and incubate the test wells for 30 minutes.	
7. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and dump. Repeat 3X for a total of four washes.	
8. Tap the inverted wells onto absorbent paper to remove the last of the wash solution.	
9. Dispense 100 µL of substrate, and incubate for 30 minutes.	
10. Dispense 100 µL of stop solution into each test well.	
11. Read and record the absorbance of the wells at 450nm.	
Calculation	
1. Use a 4-parameter curve fit for calculation.	☐ YES ☐ NO
2. When the data reduction spreadsheet is used for calculation, type in absorbance values for both calibrator and sample. Follow the instruction in the spreadsheet to calculate the concentration in ppb.	
3. Multiply the results in ppb by dilution factor (15000).	
4. The unit (ppb) of the result can be converted to µg/100 g by dividing the result by 10.	

5. Any samples below 30 µg/100 g are considered negative, and no further testing is needed. Samples falling between 30 and 100 µg/100 g are considered as suspicious, and confirmation test (MBA) is followed. Samples above 100 µg/100 g are considered positive, and may not require further tests to confirm.	

Operator Signature:_____ Date:_____

Supervisor Signature:_____ Date:_____