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ARTICLE

Evaluation of Surface Plasmon Resonance Relative to High Pressure Liquid Chromatography for the Determination of Paralytic Shellfish Toxins

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A surface plasmon resonance (SPR) method, incorporating monoclonal and polyclonal antibodies, was compared to HPLC fluorescence for the determination of paralytic shellfish toxins (PSTs) in shellfish collected from different regions of Canada (n = 33) and Europe (n = 55). Cross-reactivity between saxitoxin (STX) and its structural analogues was determined for both monoclonal (GT-13A) and polyclonal (R895) antibodies. Method detection limits based on IC_{10} values, using the SPR methodology (0.55−71.3 ng/mL), in particular for GT-13A, were somewhat higher than those determined using HPLC (0.16−1.29 ng/mL). SPR analyses generally resulted in higher PST levels relative to those obtained using HPLC, although neither antibody successfully responded to the N-1-hydroxylated analogues (e.g., neosaxitoxin). Five and 10 (R895 and GT-13A, respectively) of the 88 samples tested resulted in PST concentrations above the regulatory limit (80 μg/100 g shellfish tissue as STX equivalents), although HPLC responses indicated that these samples were within acceptable levels. Two and five samples were found to have PST concentrations below the regulatory limit using the GT-13A and R895, respectively, when HPLC results exceeded the limit. SPR may be applicable as a screening technique, although improved antibody response to the N-1-hydroxylated PSTs is required prior to this method being safely used for routine testing.

KEYWORDS: Paralytic shellfish toxins; HPLC; surface plasmon resonance

INTRODUCTION

Paralytic shellfish toxins (PSTs) are produced by a variety of dinoflagellates belonging to the *Alexandrium*, *Gymnodinium*, and *Pyrodinium* genera; the primary producers of these compounds are *A. tamarense*, *A. catenella*, and *A. minutum* (*1*−*3*). During feeding, bivalve molluscs consume dinoflagellates and accumulate PSTs in their tissues including digestive glands (*4*, *5*). No known negative impacts of these compounds to shellfish occur, although consumption of contaminated shellfish by humans can lead to severe illness and death. PSTs block neuron transmission by binding to the voltage-gated sodium channels and inhibit sodium ions from entering cells, which prevents nerve cells from producing action potentials (*6*). This decrease in action potential can lead to perioral paresthesia, dizziness, paralysis, respiratory arrest, and death (*7*). PST detection in shellfish has been reported in all parts of the world, making the reliable determination of these compounds a priority for regulatory bodies responsible for food safety (*8*−*10*).

Because of the serious implications to humans upon consumption of PST contaminated shellfish, regulatory limit for these toxins is well established. The regulatory limit for PSTs is measured as saxitoxin (STX) equivalents because STX is the most potent PST analogue. Canada and the European Union both have established a regulatory limit of 80 μg STX equivalent/100 g shellfish tissue (*11*, *12*).

The mouse bioassay (MBA) is the internationally accepted method for the determination of PST levels in shellfish prior to placing shellfish products into commerce and has been an official method of AOAC International for more than 40 years (*11*, *13*). Although this assay provides reliable information on the overall toxicity of a sample, no data as to the individual STX analogues contributing to the toxicity are available using this approach (*14*, *15*). Poor sensitivity and concerns over the number of live animals that are required for testing have been cited as the major reasons for seeking a replacement for the MBA (*10*). Additionally, inaccuracies in MBA results are observed when high salt concentrations are present and zinc is accumulated in shellfish tissue (*16*). As a result of these issues, many methods to replace the MBA have been investigated (*17*−*19*). Recently, a method for the determination of PSTs in shellfish using prechromatographic oxidation followed by analysis using HPLC with fluorescence detection received first action for approval as an official method

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by AOAC International and has been identified as an alternative method to the MBA for regulatory testing by the European Union (*13*, *20*).

The search for additional methods to determine the presence of PSTs in shellfish has continued in an effort to reduce the time between sample collection and the release of shellfish to markets. These methods include postcolumn oxidation HPLC methods, LC/MS, immunochemical based methods, receptor binding assays, and insect bioassays (*3*, *9*, *10*, *15*, *20*−*24*). Because of the requirement for monitoring laboratories to handle a large number of samples, time-consuming methods are considered limited in their application, and therefore, rapid methods would be beneficial (*25*).

Surface plasmon resonance (SPR), an optical biosensor method, has been applied successfully to the detection of a number of toxins in foods including domoic acid and aflatoxin B_1 (*26*, *27*). Recently, SPR has been investigated for use in rapid screening of shellfish for PST contamination (*16*). The competitive inhibition assays tested using both a monoclonal antibody and a polyclonal antibody raised to gonyautoxin 2 and 3 (GTX 2,3) and STX, respectively, have shown promise for use as a PST screening method in shellfish tissue (*16*).

In the present study, PSTs in shellfish tissue were measured using the AOAC approved HPLC method (*28*) and an SPR method to establish whether the SPR approach would be an effective screening technique for PSTs.

MATERIALS AND METHODS

Analytical standards of STX dihydrochloride, neosaxitoxin (NEO) dihydrochloride, gonyautoxin (GTX) 1, 2, 3, and 4, decarbamoylsaxitoxin dihydrochloride (dcSTX), and *N*-sulfocarbamoylgonyautoxins (C1/C2) were obtained from the National Research Council Canada (NRC), Halifax, Canada.

Sample Collection. Samples were collected from a number of regulatory laboratories to ensure that tissues containing variable PST profiles were included in the assessment. Canadian shellfish samples (mussels, soft-shelled clams, surf clams, and an unidentified clam species) ($n = 33$) were supplied by the Canadian Food Inspection Agency (CFIA) from different regions of Canada. Shellfish samples ($n = 55$) from Europe were supplied by the UK National Reference Laboratories: the Fisheries Research Centre (FRS), Scotland, and the Agri-Food and Biosciences Institute, Belfast.

SPR Methodology. *Reagents*. HBS-EP buffer (10 mM Hepes, 150 mM sodium chloride, 3 mM EDTA, and 0.005% polysorbate 20) was obtained from GE Healthcare, UK. HPLC grade ethanol and sodium hydroxide (NaOH) solution were purchased from Sigma Chemical Co. (Oakville, ON, Canada); Milli-Q water was prepared using an in-house system. A monoclonal antibody (GT-13A) raised to GTX 2,3-keyhole limpet hemocyanin (KLH) protein conjugate and a polyclonal antibody (R895) raised to STX-bovine serum albumin were from Queen's University, Belfast.

Instrumentation. CM5 (GE Healthcare, UK) saxitoxin chips were prepared as described by Campbell and co-workers (*16*) and supplied by Queen's University, Belfast. A Biacore Q SPR biosensor system for the determination of analytes in food products, equipped with control and evaluation software (GE Healthcare (Biacore AB), Uppsala, Sweden), was used for PST determinations.

Preparation of Standards for SPR Analysis. Standards for SPR analysis ranging from 0 to 200 ng/mL of each of the STX analogues were prepared in HBS-EP buffer or as spiked matrix extracts, using the analytical PST standards obtained from the NRC for cross-reactivity and sensitivity testing. In the initial evaluation of the SPR method, standard curves for STX, GTX 1,4, NEO, C1/C2, GTX 5 (B1), dcSTX, GTX 2,3 and dcGTX 2,3 were established at concentrations ranging from 0.01−200 ng/mL in buffer. The relative response of the 0 ng/mL standard (buffer only) represented 100% binding to the STX chip surface, and therefore, standard curves were normalized to this standard.

STX standard calibration curves for the PST analysis of the unknown samples using SPR for comparison to HPLC analyses were prepared by spiking mussel tissue with known concentrations (0, 40, 80, 800, and 8000 ng/g STX).

SPR Analysis: Instrumental Parameters. Analyses were performed using Biacore Q with the parameters set to mix each antibody with an equal volume of each STX working standard prior to injection over the STX sensor chip surface. The flow rate across this chip surface was 25 μL/min, and the contact time of the antibody−standard (antibody-sample) mix with the surface was 60 s. Report points were recorded before (5 s) and after each injection (30 s), and the relative response units were determined. The chip surface was regenerated with 25 μL injections of sodium hydroxide (100 mM) at a flow rate of 25 μL/min. Standards and samples were analyzed in duplicate for both GT-13A (monoclonal) and R895 (polyclonal) antibodies during the majority of the SPR assay evaluation. Analyses performed to determine threshold limits were analyzed in triplicate.

Shellfish Extraction Protocol. For SPR analysis, the Garthwaite extraction procedure as described by Fonfria and co-workers (*7*) was used to extract the PSTs from shellfish. Shellfish samples received without prior homogenization were homogenized using a Waring variable speed laboratory blender. Samples (1 g) of homogenized shellfish tissue were weighed into centrifuge tubes, and 5 mL of 90% ethanol in water was added. Each tube was vortexed for 10 s and rolled on a rotary shaker for 30 min. Following mixing, samples were centrifuged at 4100 rpm (3300*g*) for 10 min at room temperature. The supernatant was collected, and the pellet was vortexed with an additional 3 mL of 90% ethanol and centrifuged again. The supernatants were combined and diluted to 10 mL using 90% ethanol. The samples were further diluted in HBS buffer (125 μL extract/875 μL buffer). Each sample was filtered through a 0.2 μm polytetrafluoroethylene (PFTE) filter prior to analysis.

SPR Assay Sensitivity and Specificity. The sensitivity and specificity of GT-13A and R895 antibodies to each PST in relation to STX, which was immobilized on the chip surface over the concentration range of 0 to 200 ng/mL, were evaluated and compared in the same manner as that by Campbell and co-workers (*16*). In the present study, however, different antibody dilutions and Biacore Q instrumental parameters were employed to optimize responses. Each antibody was diluted 1:200 in HBS-EP buffer in the present study rather than the 1:250 reported by Campbell and co-workers (*16*), and the contact time of the antibody with the surface was 60 s in the present study, rather than the 120 s reported previously (*16*). For this assessment, a binder to sample ratio of 1:1 and a contact time of 60 s were used, compared to 1:3 and 120 s, respectively, described by Campbell et al. (*16*). The change in the ratio of binder to sample from 1:3 to 1:1 was done to compensate for matrix effects on the binding interaction between the antibody and the surface or toxin in solution. Although this does not alter the specificity of the assay, decreased sensitivity of the assay was observed relative to the levels reported by Campbell et al. (*16*).

Evaluation of Shellfish Matrix Effects. Prior to initiating the analysis to compare the SPR to HPLC results, the impacts of different matrices on the SPR response were investigated. Homogenized samples of mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*), clams (*Veneridae* spp.), oysters (*Crassostrea gigas*), and whole scallops (*Pecten maximus*) known to be free of PSTs, on the basis of HPLC confirmation were used for this work. Each shellfish species was extracted as described, and the crude extract (1 mL) was diluted in HBS-EP buffer (7 mL). Six separate calibration curves were prepared, one in HBS-EP buffer with no matrix, as well as with each of the five different shellfish species extracts. Extracts were spiked with STX to establish calibration curves for each matrix. Each standard curve prepared using a matrix was then compared to the curve established using HBS-EP only, for both antibodies.

HPLC Methodology. *Reagents*. Reagents used in the HPLC analysis were the same as those in AOAC official method 2005.06 (*13*). Analytical standards of the available PST analogues are required for these analyses in addition to the matrix modifier prepared from PSP-free oysters (*28*). Additional reagents include doubly deionized water, glacial acetic acid, methanol, acetonitrile (ACN), ammonium formate (0.3 and 0.1 M aqueous solutions), ammonium acetate (0.01 M aqueous solution), sodium chloride (3 and 0.05 M aqueous solutions), sodium hydroxide

(1 M aqueous solution), hydrogen peroxide (10% aqueous solution, stored in refrigerator), disodium hydrogen phosphate (0.3 M aqueous solution), periodic acid solution (0.03 M aqueous solution, stored in refrigerator), and periodate oxidant.

Instrumentation. The HPLC system used in all analyses comprised a quaternary pump, equipped with an autosampler, a multiwavelength fluorescence detector, and an online degasser (Agilent 1100 Series, Missisauga, ON, Canada). Agilent Chemstation software was used for instrument control and data processing. The system was run with a C_{18}, 5 μm, 4.6 × 12.5 mm Zorbax (Agilent, Mississauga, ON, Canada) guard column prior to the analytical column, which was C_{18}, 5 μm, 4.6 × 150 mm Supelcosil (Supelco, Oakville, ON, Canada). Mobile phase A was 0.01 M aqueous ammonium formate, and mobile phase B was 0.1 M ammonium formate in 5% ACN. The mobile phases were adjusted to pH 6. The mobile phase used to elute the PST oxidation compounds was 0 to 5% mobile phase B in the first 5 min, 5−70% B for the next 4 min, and reduced to 0% B in the final 2 min. The system was flushed for 3 min with 0% B, and the flow rate was maintained at 2 mL/min for all injections. Injection volumes were 50 μL for the extract oxidized using peroxide and 100 μL when periodate oxidation was performed.

Preparation of Standards for HPLC Analysis. Standard curves used in HPLC analyses ranged in concentration from 0−232 ng/mL and were prepared in Milli-Q grade water using the analytical PST standards from the NRC.

Shellfish Extraction Protocol. The extraction procedure used to extract the PSTs from the shellfish for HPLC determination was performed as described by Lawrence and co-workers (*28*). Shellfish samples received without prior homogenization were homogenized using a Waring variable speed laboratory blender. Three milliliters of an aqueous acetic acid solution (1%) was added to 5 g of the homogenized shellfish tissue in centrifuge tubes and loosely capped. Each tube was placed in a boiling water bath, maintained at 100 °C for 5 min. Samples were then centrifuged for 10 min at 4500 rpm (3600*g*), and the supernatant was decanted into a 15 mL graduated tube. An additional 3 mL aqueous acetic acid was added to the remaining sample and recentrifuged. The supernatants were combined and diluted to 10 mL with water.

HPLC Fluorescence Method. The total toxicity of each sample was determined by correcting for relative toxicities as reported by Oshima (*29*) and totalling all STX analogues. STX concentrations were calculated as STX dihydrochloride, while other PST analogues were calculated as free base. The separation of epimeric pairs is not possible (e.g., GTX2 from GTX3, GTX1 from GTX4, and C1 from C2) when employing the precolumn oxidation method; therefore, the average of the toxicity for each of the epimeric paired analogues was used to determine the toxicity for these compounds.

Mouse Bioassay Methodology. For some samples, where data were not received from regulatory laboratories for the mouse bioassay, this assay was performed as described by the AOAC protocol AOAC 959.08 (*30*). Where MBA data were provided by the regulatory laboratory supplying shellfish tissue, these levels are reported.

Correlations. Statistical analysis to determine correlations between results obtained from each of the different methods compared were performed using SigmaStat for Windows, version 3.11 (2004) (Systat Software Inc., Richmond, CA).

RESULTS

Initial Assessment of the SPR Method for Determination of PSTs. The PST concentration required to reduce the SPR response by 10% (IC_{10}) and 90% (IC_{90}) binding compared to the response of 100% binding when no toxin is present was used to determine the dynamic range (IC_{10} to IC_{90}) for each assay format (**Table 1**; **Figure 1**). The theoretical detection limit using SPR is defined as the IC_{10}. The detection limits obtained using SPR were somewhat higher than those observed with HPLC (**Table 1**); however, the levels were below regulatory limits. HPLC detection limits were determined for the STX analogues using low level standards, on the basis of a 3:1 signal to background noise ratio, and high level standards were used to confirm the linearity of the response for these standards.

Table 1. Dynamic Range (ng/mL) Determined for Both HPLC and SPR (Mono and Polyclonal Antibodies)[a]

		SPR	
compound	HPLC	GT-13A (monoclonal)	R895 (polyclonal)
STX	1.14−291	3.50−91.1	1.40−14.8
dcSTX	0.16−40.3	2.30−79.9	1.10−9.90
GTX 2,3	1.23−314	3.80−88.3	1.55−85.2
dcGTX 2,3	0.51−130	2.53−77.8	0.85−133
GTX 5	0.97−250	5.25−87.9	1.5−70.4
C1/C2	1.27−324	2.61−87.8	2.10−128
NEO	0.91−233	36.5−>200	1.60−96.7
GTX 1,4	1.29−329	71.3−>200	0.55−>200

[a] The SPR range was determined using IC_{10} and IC_{90}.

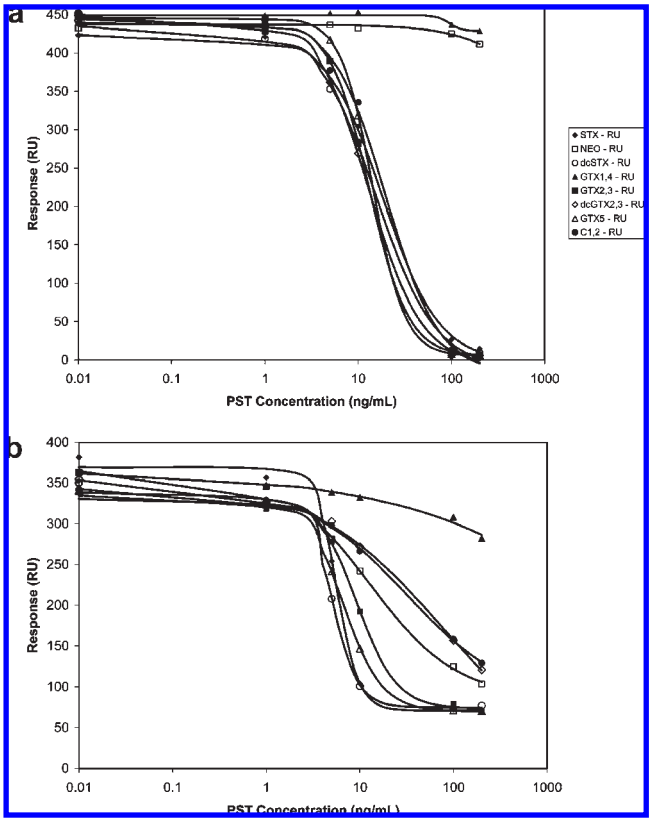

Figure 1. PST standard curves in buffer obtained by SPR (0.01−200 ng/mL) (**a**) using monoclonal antibody GT-13A and (**b**) using polyclonal antibody R895.

Cross-Reactivity. Cross-reactivity among the different STX analogues has been reported in the literature for both the GT-13A and R895 antibodies (*16*). This was confirmed in our laboratory at the outset of our work. The concentration of each STX analogue at which the response of the 0 ng/mL solution was inhibited 50% (IC_{50}) was determined and used to establish the cross-reactivity of each analogue with STX (**Table 2**) (*16*). Significant increases in the IC_{50} values were observed compared to those in the publication of Campbell et al. (*16*), but this was due mainly to differences in the ratio of antibody to standard and contact times with the surface.

Each of the antibodies displayed a different cross-reactivity profile for the PST toxins. Both antibodies responded well to most STX analogues, although neither responded well to the N-1-hydroxylated analogues (e.g., NEO and GTX 1,4), which had cross-reactivity levels of < 16% and < 40% GT-13A and R895, respectively. R895 displayed a more complex profile in terms of

Table 2. IC_{50} (ng/mL) and Cross-Reactivity of PST Analogues to STX for Both GT-13A and R895, Monoclonal and Polyclonal Antibodies, Respectively

	IC_{50} (ng/mL)		cross-reactivity (%)	
PST analogue	GT-13A	R895	GT-13A	R895
STX	33.1	6.2	100	100
dcSTX	25.7	4.9	129	127
GTX 2,3	24.3	9.3	136	66.7
dcGTX 2,3	20.1	36.6	165	16.9
GTX 5	30.7	6.9	108	89.9
C1/C2	34.4	35.7	96.2	17.4
NEO	>200	16.5	<16	37.6
GTX 1,4	>200	>200	<16	<3

specificity in relation to the structural moieties of the toxin. The N-1-hydroxylated STX analogues NEO and GTX 1,4 are toxicologically important representing 88% and 62.5% of STX toxicity, respectively, and therefore, a lack of response to these analogues may result in false negative determinations for shellfish samples (*29*).

Using GT-13A, numerous PST analogues had a greater response than observed for the benchmark STX, which resulted in cross-reactivity levels of greater than 100% (**Table 2**). Because of the overestimation of toxin concentrations using the GT-13A, some samples would be anticipated to produce results above the regulatory levels based on this antibody, relative to results from HPLC or MBA.

Evaluation of Shellfish Matrix Effects. A clear difference was observed between the curves prepared using matrix, relative to that obtained when HBS-EP buffer alone was tested (**Figure 2**). These results suggest that the ethanol content (11%) remaining in the extract in addition to coextractives from the shellfish tissue may have resulted in suppression of antibodies binding to the chip surface. The linear portion of the curve resulting from extracts of all tissues was similar for both antibodies, with the exception of scallops, which appeared to further suppress the antibody binding to the chip surface (**Figure 2**). Greater suppression was observed using the R895 antibody when scallops were tested, relative to that observed using antibody GT-13A.

The implications of these results are that employing a standard curve prepared in buffer with no matrix would result in an overestimation by approximately 50% for both antibodies through the linear portion of the curve, if 100% recovery of the PST was obtained. At the lower and upper limits of the curve, this overestimation could be significantly higher. Ideally, matrix matched calibration curves should be used with each set of samples to be tested for both antibodies. Because the linear region of the curve is similar for most shellfish species (**Figure 2**), calibration curves prepared from fortified mussel extracts were considered adequate to represent all shellfish species. On the basis of the results obtained in the present study, a mussel curve would be comparable for all species using GT-13A in the linear region of the curve as a screening assay; however, the R895 antibody would be expected to overestimate PST levels at the lower concentrations of the curve, particularly for scallops.

Recovery Testing. Recovery testing was performed by fortifying mussel tissues (n = 10) with STX prior to extraction and comparing the results to curves prepared by (i) extracting tissues and fortifying the extracts with known concentrations of STX and (ii) fortifying tissues prior to extraction. These studies were performed using homogenized mussels known to be free of PSTs. Some tissues were retained with no fortification, while others were fortified with the STX regulatory limit (80 μg STX/ 100 g tissue). The samples were extracted and analyzed in triplicate to determine the recovery of the assay with both antibodies. Low STX

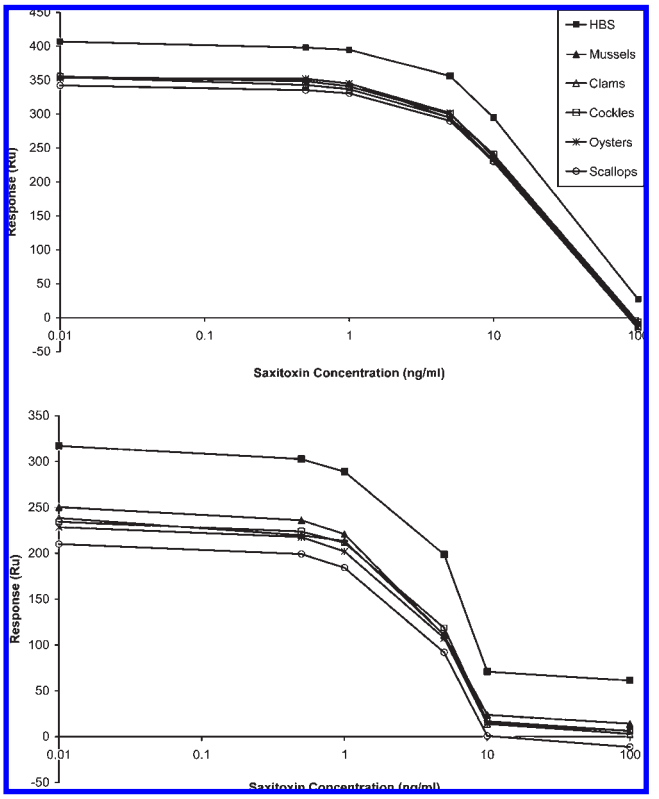

Figure 2. Comparison of STX standard curves in HBS-EP buffer and shellfish matrices obtained by SPR (0.01−100 ng/mL) (**a**) using monoclonal antibody GT-13A and (**b**) using polyclonal antibody R895.

concentrations were detected in some of the negative samples using both GT-13A and R895, indicating that there was some interference in the assay because of variability in some of the mussel samples (**Tables 3** and **4**).

PST recoveries from the extracts were consistently low (47.7% ± 5.7% and 49.4% ± 2.1%, for GT-13A and R895, respectively) when measured against calibration curves prepared using spiked extracts (**Table 3**). Dramatically improved recoveries, however, were obtained when extracts were compared to calibration curves prepared using tissues that had been fortified prior to extraction (92.0% ± 11.8% and 90.4% ± 8.0% GT-13A and R895, respectively) (**Table 4**). Calibration curves prepared by initially extracting tissue, followed by fortification, led to decreased recoveries indicating that losses were occurring during sample preparation. However, this could be overcome by preparing standard curves in the same manner as that used for unknown samples. This suggests that improvements to the extraction method used for SPR analyses to increase recoveries would aid in the efficiency of the application.

Repeatability for both antibodies resulted in coefficients of variation of less than 15%, with the R895 antibody displaying better repeatability than the GT-13A.

Detection Capability (Cut-off Point for the Assay (CCβ). Within the European Union, the detection capability is defined as the smallest concentration of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β based on Decision 2002/657/EC (*31*, *32*). For toxins with an established action limit, this means that the detection capability is the concentration at which the method is able to detect the toxin at this action limit with a statistical certainty of 1-β (*32*). To comply with this Decision for the implementation of council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, a greater

Table 3. Concentration (μg/100g Tissue) and Recovery Data (%) for Mussel Samples Known to Contain No PST (with and without Fortification at 80 μg STX/100 g) Using Calibration Curves Prepared from Spiked Extracts

	GT-13A						R895					
	unfortified tissue			fortified at 80 μg/ 100 g			unfortified tissue			fortified at 80 μg/ 100 g		
mussel sample	trial 1	trial 2	trial 3	trial 1	trial 2	trial 3	trial 1	trial 2	trial 3	trial 1	trial 2	trial 3
1	0.1	3.9	4.9	29.9	47.3	43.0	0.1	0.1	0.1	43.6	41.8	42.0
2	3.1	0.6	5.1	42.9	39.9	40.2	0.1	0.1	0.1	40.0	39.9	41.1
3	0.1	4.4	5.0	43.3	33.4	42.0	0.1	0.1	0.1	41.1	39.7	39.9
4	2.3	0.8	4.9	40.8	34.3	39.5	0.1	0.1	0.1	39.5	40.2	40.2
5	5.2	2.1	4.3	40.3	41.7	41.5	0.1	0.1	0.1	40.5	40.9	40.2
6	5.2	4.6	4.3	30.1	36.9	35.4	0.1	0.1	14.7	37.3	38.2	37.3
7	4.7	4.0	3.9	41.3	39.8	40.5	0.1	0.1	0.1	39.6	40.0	40.3
8	5.4	8.0	8.2	41.3	35.5	36.8	0.1	0.1	0.1	39.9	38.9	38.5
9	6.4	3.7	5.9	34.1	40.1	35.9	0.1	0.1	0.1	37.3	36.8	37.7
10	3.7	0.1	2.8	34.1	31.8	30.5	0.1	0.1	0.1	37.5	37.3	38.7
average	3.6	3.2	4.9	37.8	38.1	38.5	0.1	0.1	1.5	39.6	39.4	39.6
standard deviation	2.2	2.4	1.4	5.2	4.6	3.8	0.0	0.0	4.6	2.0	1.6	1.5
% CV				13.8	12.1	10.0				4.9	4.0	3.8
recovery				47.3	47.6	48.1				49.5	49.2	49.5

Table 4. Concentration (μg/100g Tissue) and Recovery Data (%) for Mussel Samples Known to Contain No PST (with and without Fortification at 80 μg STX/100 g) Using Calibration Curves Prepared from Tissue Spiked Prior to Extraction

	GT-13A						R895					
	unfortified tissue			fortified at 80 μg/ 100 g			unfortified tissue			fortified at 80 μg/ 100 g		
mussel sample	trial 1	trial 2	trial 3	trial 1	trial 2	trial 3	trial 1	trial 2	trial 3	trial 1	trial 2	trial 3
1	0.1	0.1	0.1	56.6	92.7	83.6	0.1	0.1	0.1	88.1	81.1	81.9
2	0.1	0.1	0.1	83.4	77.3	77.8	0.1	0.1	0.1	74.0	73.8	78.1
3	0.1	0.1	0.1	84.4	63.7	81.7	0.1	0.1	0.1	78.1	72.8	73.5
4	0.1	0.1	0.1	79.2	65.6	76.4	0.1	0.1	0.1	72.3	74.7	74.7
5	0.1	0.1	0.1	78.1	81.1	80.5	0.1	0.1	0.1	76.0	77.5	74.9
6	0.1	0.1	0.1	57.0	71.0	67.9	0.1	0.1	0.1	63.9	67.1	64.0
7	0.1	0.1	0.1	80.1	77.0	78.5	0.1	0.1	0.1	72.3	73.8	75.1
8	1.7	9.3	9.8	80.1	68.1	70.8	0.1	0.1	0.1	73.8	69.8	68.5
9	4.7	0.1	3.1	65.3	77.6	69.0	0.1	0.1	0.1	63.8	62.2	65.3
10	0.1	0.1	0.1	65.2	60.5	57.8	0.1	0.1	0.1	64.7	63.8	69.1
average	0.7	1.0	1.4	72.9	73.5	74.4	0.1	0.1	0.1	72.7	71.7	72.5
standard deviation	1.5	2.9	3.1	10.8	9.6	7.9	0.0	0.0	0.0	7.5	5.9	5.7
% CV				14.8	13.1	10.7				10.3	8.3	7.8
recovery				91.2	91.8	93.0				90.9	89.6	90.6

than 95% certainty limit must be established for screening assays (*31*). The detection capability or cutoff is the concentration whereby $<5\%$ of the results would be false negatives in relation to the action limit (*32*). If one or fewer samples among 20 samples spiked at the action limit is determined to be negative, the detection capability is less than or equal to the action limit (*32*).

Because the antibodies were raised to the STX−protein conjugate, STX was immobilized onto the chip surface. The current reference methods relate all other toxin analogues to STX equivalents; therefore, the detection capability was determined using STX as the reference PST. The results of the analyses of the blank and spiked mussel samples at the action limit are presented in **Tables 3** and **4**. Analysis of variance (ANOVA) was performed on the calculated mean STX concentration for blank and spiked saxitoxin samples, and the means were found to be statistically different ($p < 0.001$). Because of the potential health risk of this toxin group, the cutoff level was calculated as the mean concentration value determined from the fortified samples at the regulatory limit minus 3 standard deviations of this mean value (*33*). This was established from the statistical 3 sigma rule of a normal distribution to ensure that there was a 99.7% certainty that samples above this concentration level would be noncompliant or contain PSTs close to the regulatory limit (80 μg/ 100 g of shellfish tissue). This also displays an assurance that the false compliant rate is $<5\%$.

The cutoff (CCβ) values for the STX calibration curve determined in this study using 10 spiked extracts were 24.5 and 31.8 μg STX/100 g of shellfish for GT-13A and R895, respectively. These results indicate that with the use of the assay with spiked extracts, there is a greater than 95% certainty that a sample will contain PSTs close to or at the regulatory limit. At this spiking level, the cutoff values illustrate that because of the sensitivity needed for this assay format, there is marginal scope for the regulatory limit to be lowered, as some regulators have proposed (*34*). In contrast, cutoff values obtained using a standard curve from extracted spiked tissues at the regulatory limit were 45.3 and 53.2 μg STX/ 100 g of shellfish for GT-13A and R895. Increased sensitivity can be achieved using the assay under these conditions and would allow for this application to be used should the regulatory limit be lowered.

PSTs in Shellfish Tissue (HPLC vs SPR Response). Analysis of PSTs in a variety of shellfish samples was performed to compare the results obtained using HPLC with those obtained using SPR. Mussels (36), soft-shelled clams (18), surf clams (1), other clam species (5), oysters (1), cockles (15), and scallops (12) were initially tested using HPLC. HPLC concentrations for individual PST analogues and epimeric pairs (e.g., GTX2,3; GTX 1,4; and

C1,C2) were determined to establish total PST concentrations. By multiplying each PST analogue concentration by its toxicity relative to STX, the concentrations were standardized to STX equivalent units. The STX equivalence approach was adopted to allow for comparison between the HPLC results and those obtained using SPR, which are based on binding and cross-reactivity to STX.

Where detected (61 of 88 samples tested; 69%), each sample had a distinct PST profile. Total PST concentrations ranged from below detectable levels to 2970 μg/100 g (STX equivalent units) tissue using HPLC (**Table 5**). Of the 88 samples tested, 27 (31%) were found to be free of all PST analogues measured. The negative samples were obtained from each of the shellfish classes including mussels (11), blue mussel (1), oyster (1), clam (4), cockles (7), and scallops (3), rather than one type of shellfish. In general, the SPR assay results corresponded to those obtained using HPLC, although SPR resulted in one clam and four cockles having low but detectable levels of PSTs using the GT-13A antibody (7.7, 7.5, 9.3, 9.9, and 19.5 μg/ 100 g, clam and cockles, respectively), where negative by HPLC. Similarly, the R895 assay resulted in detectable levels of PSTs in two cockle samples (12.8 and 18.7 μg/ 100 g) and one scallop sample (14.2 μg/ 100 g), where HPLC results were negative. Of the 61 samples with detectable levels of PSTs, 32 (52%) were found to contain no trace of NEO; similarly, 28 (46%) samples contained no GTX 1,4 (**Table 5**).

In general, PST levels determined using the monoclonal antibody GT-13A were higher relative to HPLC measurements (**Table 6**). Using the monoclonal antibody, 10 of the 88 samples tested (11%) were found to be above the guideline level for total PSTs (>80 μg/ 100 g), when the results for the samples using HPLC determination were within regulatory limits (concentrations ranged from 37.9 μg/100 g tissue to 75.7 μg/100 g tissue). More importantly, SPR analysis resulted in a single soft-shell clam and scallops having total PST concentrations below the regulatory limit, while concentrations exceeded the guideline level based on HPLC determination (**Table 6**). The PST profile of the clam sample contained most STX analogues at low levels; however, the major contributor to total PST levels was from NEO (76% of total STX equivalent) (**Table 6**). GTX 1,4 (60% of total STX equivalent) was the greatest contributor to PST levels in the scallop sample, with low levels of the other PSTs detected in this sample. Although PST profiles with elevated N-1-hydroxylated PST analogues and low concentrations of all non-N-1-analogues are unusual, on the basis of the observations of this study, they do occur, and therefore, the method must be robust enough to respond to these samples.

Most samples with PST concentrations exceeding guideline levels based on HPLC were also determined as exceeding acceptable limits using SPR with the polyclonal R895. PST levels were above regulatory limits by SPR using the polyclonal antibody for 5 of the 88 samples tested, while they were below the limit using HPLC (**Table 6**). PST levels were found to be below the regulatory limit using the R895 polyclonal antibody in five samples that were found to have levels exceeding the limit using HPLC (**Table 6**). All of the samples determined to have PST levels below the regulatory limit where HPLC indicated levels above the limit had concentrations close to the regulatory level (87.3 μg/100 g$-$114 μg/100 g) based on HPLC results. Two of these samples, when compared to the available mouse bioassay data, were correctly below the regulatory level indicating that the HPLC overestimated PST concentrations for these samples. The SPR data for the remaining three samples showed PST levels greater than 70% of the action level. The single sample found to have PST levels below the regulatory limit using the monoclonal antibody was found to contain PSTs at concentrations above the level using both HPLC and the R895 antibody. In each of the samples determined to have low concentrations relative to those of HPLC, significant contributions to total PST levels from NEO and GTX 1,4 were observed (**Tables 5** and **6**).

Approximately half of the samples tested using SPR had concentrations $>130\%$ of the concentrations obtained using HPLC regardless of the antibody tested (**Table 6**). The elevated response for the majority of samples tested may be attributed to the high specificity of the antibodies to some of the PST analogues with substantially lower toxicity factors relative to STX (**Figure 1**), particularly GTX5.

The data obtained using both HPLC and SPR were compared to the MBA results, where available (**Table 6**). The general trends corresponded well regardless of the method used for most samples tested. Because of very high PST concentrations in two of the samples, only approximate PST concentration estimates were possible using MBA because of the rapid onset of symptoms in mice (**Table 6**). Although the monoclonal antibody result, which generally is overestimated, compared to the polyclonal result was consistent with HPLC levels for a soft-shelled clam sample, the polyclonal antibody more accurately reflected PST levels based on the MBA. Similarly, the HPLC response underestimated PST levels in a mussel sample (62.9 μg/100 g) relative to that of MBA (109 μg/100 g), and SPR determined this sample had total PSTs present in excess of the 80 μg/100 g regulatory limit using both antibodies. This indicates that the HPLC method can also result in over- and underestimation of PST levels in certain samples, particularly if the PST levels are close to the regulatory limit of 80 μg/100 g tissue.

Correlations. Although MBA data were not available for all samples tested using HPLC, we felt it to be beneficial to compare the results of both AOAC approved methods where possible, to confirm their comparability. A number of samples resulted in MBA reports of <40 μg/100 g; therefore, a correlation was calculated using only samples with clearly definable concentrations for this comparison. A linear correlation was found to exist between MBA and HPLC concentrations regardless of whether the comparisons included samples with extremely high PST levels using the approximate concentrations reported for the MBA or when these high concentrations were removed ($r^2 = 0.851$, 0.902; no high concentrations, high concentrations included, respectively) (**Figure 3a**). HPLC results were then compared with the results obtained using SPR with both monoclonal and polyclonal antibodies. Although a strong linear relationship was found to exist between the HPLC and the R895 (polyclonal antibody) ($r^2 = 0.836$) (**Figure 3c**), a weaker relationship was obtained using the monoclonal antibody (GT-13A) ($r^2 = 0.679$) (**Figure 3b**). These results suggest that the polyclonal antibody would correspond more closely to the HPLC data. The polyclonal antibody, however, resulted in an increased number of false negative results over the monoclonal antibody in this study, and therefore, using it exclusively for PST determination may result in inaccurate results.

DISCUSSION

The samples found to have PST levels below the regulatory limit using SPR, while the HPLC results indicated concentrations above the limit in the present study, contained greater than 70% of the action level of PSTs. This suggests that the use of threshold values with the assay using either antibody should compensate and ensure that this does not occur. The impact of the use of the threshold values could result in increasing the number of samples with PST concentrations exceeding the 80 μg/100 g tissue

Table 5. PST Profiles for Shellfish Samples Tested as Determined by HPLC with Fluorescence Detection Using the Pre-Chromatographic Oxidation Method (μg/100g)

lab number	sample type	STX	NEO	dcSTX	GTX1/4	GTX2/3	dcGTX2/3	GTX5	C1/C2	total PST	STX equiv
1	mussel	474	1060	ND	149	41.9	ND	147	2070	3940	1590
2	mussel	109	233	ND	37.7	10.7	ND	35.4	465	941	402
3	mussel	32.2	12.0	ND	170	121	ND	2.8	42.1	380	196
4	mussel	15.0	4.6	ND	70.8	31.8	ND	0.6	6.6	129	75.7
5	mussel	28.1	15.0	ND	95.7	221	ND	2.3	49.6	412	187
6	mussel	43.0	15.0	ND	35.0	37.8	ND	3.0	11.3	145	93.0
7	surf clam	28.1	8.8	6.2	73.3	6.8	8.9	ND	ND	132	93.3
8	soft-shelled clam	879	630	6.7	1240	1980	ND	33.2	216	4980	2970
9	soft-shelled clam	35.6	229	ND	7.6	26.3	ND	0.9	2.2	302	252
10	soft-shelled clam	30.0	ND	ND	16.2	21.1	ND	ND	ND	67.3	48.1
11	blue mussel	45.1	3.1	0.4	ND	34.1	ND	4.3	ND	87.0	61.3
12	blue mussel	13.0	9.2	ND	15.7	24.7	ND	ND	3.4	66.0	40.4
13	soft-shelled clam	30.8	22.0	0.2	26.0	54.3	ND	ND	4.2	138	87.3
14	blue mussel	4.3	12.6	ND	18.5	6.0	ND	3.7	56.1	101	31.2
15	soft-shelled clam	69.4	5.1	ND	35.7	85.5	ND	1.8	6.2	204	129
16	soft-shelled clam	27.7	74.0	ND	10.4	38.2	ND	ND	ND	150	114
17	soft-shelled clam	27.7	33.2	0.3	45.3	66.2	ND	3.1	11.3	187	111
18	blue mussel	1.0	ND	0.6	ND	7.2	ND	ND	ND	8.8	4.1
19	blue mussel	148	202	ND	93.6	136	ND	19.2	144	742	441
20	blue mussel	9.9	ND	ND	54.6	49.3	ND	2.0	7.3	123	63.1
21	soft-shelled clam	22.1	7.0	0.3	ND	25.0	ND	ND	ND	54.4	37.9
22	blue mussel	51.1	ND	ND	ND	13.6	ND	ND	ND	64.7	56.3
23	soft-shelled clam	9.6	ND	0.6	ND	11.6	ND	ND	2.9	24.7	14.4
24	blue mussel	59.7	72.1	ND	583	434	ND	7.0	41.6	1200	654
25	soft-shelled clam	4.4	7.1	ND	88.5	62.7	ND	ND	6.7	169	90.0
26	soft-shelled clam	32.5	ND	0.6	ND	18.7	ND	1.1	ND	52.9	40.0
27	soft-shelled clam	5.1	ND	ND	ND	1.5	ND	1.3	ND	7.9	5.8
28	soft-shelled clam	8.8	ND	ND	ND	1.4	ND	ND	ND	10.2	9.4
29	soft-shelled clam	13.1	ND	ND	ND	9.4	ND	ND	ND	22.5	16.7
30	blue mussel	ND	ND	ND	ND	ND	ND	3.1	ND	3.1	0.2
31	soft-shelled clam	4.9	ND	ND	ND	3.4	ND	ND	ND	8.3	6.2
32	blue mussel	11.5	ND	ND	ND	3.2	ND	ND	ND	14.7	12.7
33	soft-shelled clam	4.3	ND	ND	ND	ND	ND	ND	ND	4.3	4.3
34	soft-shelled clam	10.1	ND	ND	ND	6.8	ND	ND	ND	16.9	12.7
35	soft-shelled clam	3.2	ND	ND	ND	ND	ND	ND	ND	3.2	3.2
36	clams	28.5	25.1	ND	86.2	99.1	ND	1.6	11.5	252	142
37	mussels	5.6	16.6	ND	ND	29.2	ND	ND	ND	51.4	41.2
38	mussels	8.8	15	ND	ND	20.3	ND	ND	ND	44.1	37.3
39	mussels	14.9	ND	ND	ND	75.4	ND	ND	36.1	126.4	62.9
40	scallops	8.2	ND	ND	ND	5.4	ND	ND	ND	13.6	11.4
41	scallops	11	15.1	ND	76.1	24.9	ND	ND	ND	127	111
42	scallops	20.9	14.2	ND	35.2	29	ND	ND	ND	99.3	85.4
43	mussels	19.7	27.7	ND	277	84.1	ND	ND	49.1	458	354
44	scallops	14.9	ND	ND	ND	8.6	ND	ND	ND	23.5	20.1
45	scallops	11.7	ND	ND	ND	14.2	ND	ND	ND	25.9	20.3
46	cockles	5.1	ND	ND	ND	ND	ND	ND	ND	5.1	5.1
47	scallops	10.6	ND	ND	ND	16.8	ND	ND	ND	27.4	20.6
48	scallops	12.6	ND	ND	ND	14.5	ND	ND	ND	27.1	21.3
49	scallops	13	ND	ND	ND	12.4	ND	ND	ND	25.4	20.4
50	scallops	6.3	ND	ND	ND	6.4	ND	ND	ND	12.7	10.1
51	mussels	16.6	12.8	ND	149	47.6	ND	ND	85.8	312	199
52	mussels	8.3	ND	ND	43.7	30.2	ND	ND	ND	82.2	65.7
53	mussels	13.4	9.6	ND	187	113	ND	ND	39.4	363	263
54	mussels	17.9	11.2	ND	41.7	26.8	ND	ND	ND	97.6	83.8
55	cockles[a]	61.2	ND	128	ND	13.6	35	906	437	1580	269
56	cockles[a]	87.5	ND	126	71.9	14.5	35.2	954	494	1780	366
57	cockles[a]	64.5	ND	74	30.8	10.6	16.2	601	181.7	979.2	212
58	cockles[a]	57.4	ND	114	33.4	13.3	18.2	849	94	1180	248
59	cockles[a]	77.1	ND	66.3	12.3	8.2	10.7	560	74	809	188
60	cockles[a]	52.6	ND	91.2	20.9	8.5	12.7	580	35	801	189
61	cockles[a]	55.1	ND	38.9	ND	4.1	4.4	316	17.8	437	110

[a] C3/C4, dcNEO, and GTX6 were also present but not quantified.

regulatory limit using SPR, while HPLC results indicate that PST levels are below the limit and would increase the number of samples requiring confirmatory testing. Nevertheless, this would mean that greater than 80% of the samples could be screened using SPR with either antibody without the need for further analysis. In general, the R895 antibody correlates better with the

Table 6. Comparison of Total STX Equivalent PST Concentrations (μg/ 100g) in Shellfish Samples as Determined by HPLC, SPR (Antibody Dilution 1:200), and MBA (Where Data Were Available)

			SPR		
lab number	sample type	HPLC	GT-13A	R895	MBA
1	mussel	1590	8350	4020[a]	~1000
2	mussel	402	1230	1090	
3	mussel	196	610	303	
4	mussel	75.7	118	106	
5	mussel	187	586	288	
6	mussel	93.0	217	141	
7	surf clam	93.3	121	74.0	
8	soft-shelled clam	2970	4590	3510	~5000
9	soft-shelled clam	252	62.7	131	
10	soft-shelled clam	48.1	92.4	78.2	42
11	blue mussel	61.3	175	112	
12	blue mussel	40.4	ND	ND	47
13	soft-shelled clam	87.3	106	58.5	108
14	blue mussel	31.2	76.8	72.5	
15	soft-shelled clam	129	230	116	
16	soft-shelled clam	114	134	76.1	65
17	soft-shelled clam	111	226	114	
18	blue mussel	4.1	ND	ND	<40
19	blue mussel	441	1180	864	400
20	blue mussel	63.1	153	93.6	59
21	soft-shelled clam	37.9	121	73.6	48
22	blue mussel	56.3	127	97.4	50
23	soft-shelled clam	14.4	30.3	28.9	44
24	blue mussel	654	1630	942	670
25	soft-shelled clam	90.0	103	67.1	94
26	soft-shelled clam	40.0	89.1	70.6	70
27	soft-shelled clam	5.8	ND	ND	<40
28	soft-shelled clam	9.4	ND	ND	<40
29	soft-shelled clam	16.7	ND	24.7	<40
30	blue mussel	0.2	ND	ND	<40
31	soft-shelled clam	6.2	ND	ND	<40
32	blue mussel	12.7	ND	ND	<40
33	soft-shelled clam	4.3	ND	ND	<40
34	soft-shelled clam	12.7	ND	17.4	<40
35	soft-shelled clam	3.2	ND	ND	<40
36	clams	142	182	85.6	274
37	mussels	41.2	80.5	59.4	48
38	mussels	37.3	48.4	38.8	37
39	mussels	62.9	169	90.8	109
40	scallops	11.4	39.8	41.3	42
41	scallops	111	74.0	62.6	65
42	scallops	85.4	107	84.9	64
43	mussels	354	303	141	
44	scallops	20.1	41.8	44.4	
45	scallops	20.3	40.8	42.7	
46	cockles	5.1	22.0	ND	
47	scallops	20.6	50.8	56.1	
48	scallops	21.3	40.8	41.9	
49	scallops	20.4	50.0	47.7	
50	scallops	10.1	35.3	37.7	
51	mussels	199	319	159	86
52	mussels	65.7	81.4	65.1	44
53	mussels	263	270	132	115
54	mussels	83.8	171	129	81
55	cockles	269	1290	793	224
56	cockles	366	1740	981	271
57	cockles	212	789	561	115
58	cockles	248	916	643	215
59	cockles	188	474	484	99
60	cockles	189	590	588	125
61	cockles	110	301	371	96

[a] Average of testing undiluted and 1:10 and 1:100 dilutions. [b] Not detected.

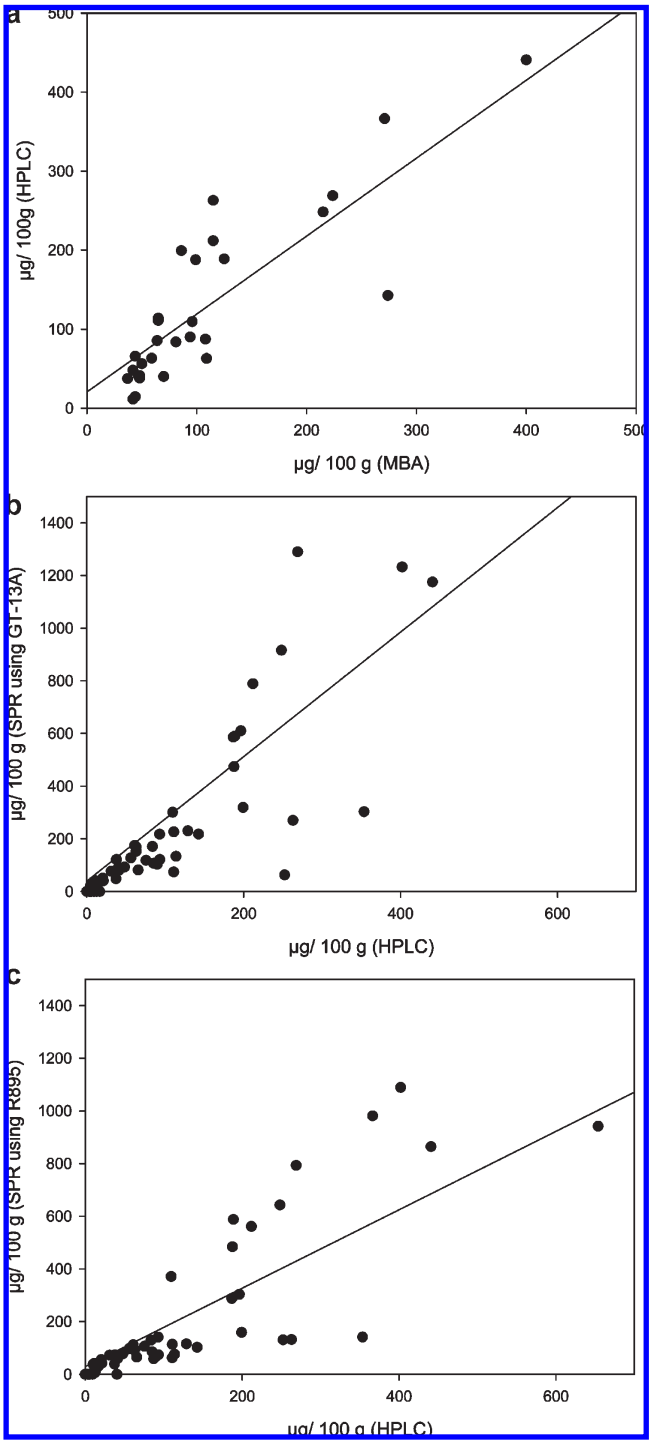

Figure 3. Correlation (**a**) between MBA data and those obtained using the HPLC method; no extremely high PST concentrations included [$y = 20.544 + 0.986x$];(**b**) between HPLC method and SPR with GT-13A [$y = 39.358 + 2.364x$] (regression determined using all data points including very high concentrations [<1000 μg/100 g tissue]); and (**c**) between the HPLC method and SPR with R895 [$y = 29.203 + 1.489x$] (regression determined using all data points including very high concentrations [<1000 μg/100 g tissue]).

HPLC data compared to the GT-13A antibody, which tends to overestimate on the PST levels because of its specificity profile and the toxicity factors of the toxins.

Worldwide, the MBA remains the method for compliance or the reference method of analysis. In the UK, the HPLC method has been implemented as a screening method, and

the samples containing PST levels above background are still referred to the MBA for confirmation. This SPR assay, with a defined cutoff level could be used as an alternative screening technique which could be followed up with confirmatory testing by HPLC or MBA.

In countries where samples are shipped extensive distances at a high expense for regulatory testing, this relatively fast and simple SPR assay could be used to substantially reduce the number of samples requiring confirmatory analyses, which would reduce costs to the industry and regulatory authorities. Additionally, the SPR assay could considerably reduce the number of animals used in the MBA for confirmatory analysis.

The SPR technique does not provide regulatory authorities with PST profiles, similar to the MBA and despite the requirement for some animals in antibody development, far fewer animals are required to perform these tests using SPR than MBA. Although the SPR technique using both the monoclonal antibody (GT-13A) and the polyclonal antibody (R895) generally do result in data that correspond to the safety guideline for most shellfish tested, some samples may indicate PST levels below the regulatory limit, when they are in fact above this level, particularly if the toxin profile contained only the N-1-hydroxylated PST analogues using this technique. Improvement in the antibody response to these structural analogues is required before this approach could be used with confidence on a routine basis for regulatory testing.

In this study, the focus for comparisons in terms of PST levels was set at the regulatory limit of 80 μg STX/100 g tissue. Although some samples were found to be below the regulatory limit, while HPLC determinations showed that they exceeded 80 μg/100 g, those samples contained greater than 70% of this PST regulatory limit. It is important to recognize that currently in Europe, those samples, if tested, by HPLC would still be referred to the MBA for confirmation.

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