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Detection of Paralytic Shellfish Toxins by a Solid-Phase Inhibition Immunoassay Using a Microsphere-Flow Cytometry System

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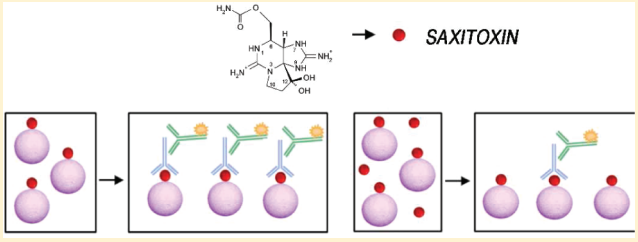
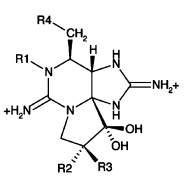
ABSTRACT: Paralytic shellfish poisoning is a toxic syndrome described in humans following the ingestion of seafood contaminated with saxitoxin and/or its derivatives. The presence of these toxins in shellfish is considered an important health threat and their levels in seafood destined to human consumption are regulated in many countries, as well as the levels of other chemically unrelated toxins. We studied the feasibility of immunodetection of saxitoxin and its analogs using a solid-phase microsphere assay coupled to flow cytometry detection in a Luminex 200 system. The technique consists of a competition assay where the toxins in solution compete with bead-bound saxitoxin for binding to an antigonyautoxin 2/3 monoclonal antibody (GT-13A). The assay allowed the detection of saxitoxin both in buffer and mussel extracts in the range of 2.2−19.7 ng/mL (IC_{20}−IC_{80}). Moreover, the assay cross-reactivity with other toxins of the group is similar to previously published immunoassays, with adequate detection of most analogs except N-1 hydroxy analogs. The recovery rate of the assay for saxitoxin was close to 100%. This microsphere-based immunoassay is suitable to be used as a screening method, detecting saxitoxin from 260 to 2360 μg/kg. This microsphere/flow cytometry system provided similar sensitivities to previously published immunoassays and provides a solid background for the development of easy, flexible multiplexing of toxin detection in one sample.



Saxitoxin (STX) and derivatives are a group of chemical compounds produced by marine dinoflagellates that induce paralytic shellfish poisoning (PSP) syndrome when they reach human consumers through the trophic chain.[1] Recently these toxins have been also detected in fresh water where they are produced by cyanobacteria.[2−4] Since the isolation of STX in 1957,[5] more than 20 naturally occurring analogs have been described arising from the modification of a common tetrahydropurine backbone (Figure 1).[6] PSP toxins block ion transport by voltage-dependent sodium-channels producing neurologic symptoms that can lead to death. The severe human health threat associated to the presence of this group of toxins in food has prompted the control and limitation of PSP toxin levels in shellfish destined for human consumption. In many countries the regulatory limit for PSP toxins in bivalve mollusks has been established as 800 μg of STX equivalents/kg of shellfish meat.[7] Therefore, detection methods capable of detecting this amount of PSP toxins in shellfish are essential to enforce regulations.



R1	R2	R3	R4:	R4:	R4:-OH	R4:-H
H	H	H	STX	GTX5	dcSTX	doSTX
OH	H	H	neoSTX	GTX6	dcneoSTX	-
OH	OSO_3^-	H	GTX1	C3	dcGTX1	-
H	OSO_3^-	H	GTX2	C1	dcGTX2	doGTX2
H	H	OSO_3^-	GTX3	C2	dcGTX3	doGTX3
OH	H	OSO_3^-	GTX4	C4	dcGTX4	-

Figure 1. Structure of paralytic shellfish poisoning (PSP) toxins (dc, decarbamoyl; do, deoxy).

Among a wide variety of methods that have been developed to detect PSP toxins, a mouse bioassay (MBA)[8] and high-performance liquid chromatography coupled to fluorescence detection (HPLC-FLD), also referred to as the Lawrence method,[9] are the only official, validated methods in European Countries.[10,11] The MBA has protected human health for decades, however it has a number of recognized disadvantages such as low sensitivity, being semiquantitative, producing high false positive rates, suffering from high variability, its reliance on specialized personnel and facilities, and the procedure raises ethical issues due to the use of laboratory animals.[12] HPLC-FLD is specific and sensitive, however it requires a higher

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degree of laboratory training and certified standard reference materials for each member of the PSP toxin group. Routine detection using these methods requires substantial amounts of valuable resources in terms of animals, toxin standards and laboratory personnel. Therefore the development of fast screening methods will allow a reduction in the numbers of samples to be tested by more expensive and ethically questionable methods. Multiple detection methods have been developed to detect PSP toxins including receptor-based assays, cytotoxicity assays, ELISAs or immunological biosensor assays.[13−21] None of these methods has been included as an official detection method due in many cases to a lack of validation studies. Very recently an SPR-based biosensor technique for PSP toxin immuno-detection has been prevalidated for screening purposes in single-laboratory and interlaboratory studies.[22,23] The new developments in this field focus on simultaneous screening of multiple groups of marine toxins. This trend has prompted interesting technological advances; such as a prototype of a SPR-based biosensor for multiple toxin detection in the same sample.[24]

In the past decade multiplexing of solid-phase assays using microspheres has become a widely used technique for clinic diagnostics and research purposes.[25−29] Different classes of microspheres can be differentiated by their intrinsic properties (e.g., internal fluorescence and/or size, depending on the manufacturer) and the surface of each class of microsphere is functionalized for the detection of a certain compound. In the Luminex system, microsphere classification is based on the fluorescence of a specific mixture of two internal fluorophores that is different for each microsphere class. The quantification of the compound of interest is achieved by the fluorescence signal attached to the surface of the microsphere. Multiplexing is provided by the incubation of a sample with multiple classes of microspheres simultaneously, each class is specific for a certain analyte, and later separation of microspheres by a flow cytometry-based platform.

The aim of the present study was to optimize a microsphere-based immuno-detection system for PSP toxins to explore the capability and sensitivity of the Luminex system for marine toxin screening purposes. The evaluation of the performance of this PSP detection assay will be relevant to future development of multiplexed marine toxin detection methods.

METHODS

Materials. Certified reference standard materials of saxitoxin dihydrochloride (STXdi-HCl, 65 μM), neosaxitoxin (NEO, 65.6 μM), gonyautoxin 1/4 (GTX1, 60.4 μM; GTX4, 19.7 μM), gonyautoxin 2/3 (GTX2, 114.2 μM; GTX3, 43.4 μM), decarbamoyl saxitoxin (dcSTX, 62 μM), decarbamoyl neosaxitoxin (dcNEO, 28.9 μM), decarbamoyl gonyautoxin 2/3 (dcGTX2, 116 μM; dcGTX3, 26.1 μM), gonyautoxin 5 (GTX5, 65 μM), and C1/2 (C1, 114 μM; C2, 35 μM) were obtained from the Institute for Marine Biosciences, National Research Council (Halifax, Canada). *N*-hydroxysuccinimide (NHS), sodium tetraborate decahydrate, Jeffamine (2,2′-(ethylenedioxy)bis(ethylamine)), boric acid, sodium phosphate monobasic, ethanolamine, and Tween-20 were purchased from Sigma-Aldrich (Madrid, Spain). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, Illinois). Phycoerythin (PE) Goat Anti-Mouse Ig was purchased from Invitrogen (Eugene, Oregon). Sodium azide was purchased from Fluka (Steinheim, Germany). Mussels (*Mytilus galloprovincialis*) were purchased from the market (Lugo, Spain). Calibration and performance verification kits for Luminex 200, carboxylated microspheres (LC10038−01) and sheath fluid were from Luminex Corporation (Austin, Texas). Multiscreen 96 well filter plates (Durapore membrane), 33 mm Milex filter with 0.22 μm pore size and 0.45 μm pore size Ultrafree-MC centrifugal filters (Durapore membrane) were purchased from Millipore (Madrid, Spain). Phosphate-buffered saline solution (PBS) was 130 mM NaCl, 1.5 mM NaH_2PO_4, and 8.5 mM Na_2HPO_4, pH 7.4. PBS-BT solution was PBS supplemented with 0.1% w/v BSA and 0.02% v/v Tween-20. Buffer solutions were filtered through a 0.22 μm pore size filter before use. Hydrochloric acid, acetic acid, formaldehyde 37%, sodium acetate anhydrous, disodium hydrogen phosphate anhydrous and sodium chloride were from reagent grade commercial sources. The monoclonal GT-13A antibody was provided by the Osaka Prefectural Institute of Public Health.[17]

STX Immobilization on Microspheres. STX was immobilized on the surface of LC10038-01 microspheres. Luminex MicroPlex microspheres are internally dyed to differentiate microsphere classes by their unique spectral signal (class 38 was randomly selected for these experiments) and contain surface carboxyl groups for covalent attachment of ligands. The immobilization of STX was performed as described by Campbell et al.[14] for a carboxylated planar surface. Briefly, the carboxylated surface was activated by a mixture of equal volumes of 75 mg/mL EDC and 11.5 mg/mL NHS (both reagents were dissolved in water). A final volume of 150 μL of the EDC/NHS mixture was added to 2×10^6 microspheres. After 30 min of incubation, this mixture was removed and 20% Jeffamine in borate buffer (pH 8.5) was added for 1 h. After removal of the Jeffamine solution, 16.9, 36.3, or 72.5 μg of STX di-HCl in 13 μL of 0.1 M acetic acid, 22 μL of H_2O and 16 μL of 37% formaldehyde were added to 2×10^6 preactivated microspheres and allowed to react for 24 h. During this incubation the immobilization of STX is achieved by covalent bonding to Jeffamine by a formaldehyde condensation or Mannich reaction. The exact reaction pathway is not known.[30] Subsequently, free-STX was removed and the carboxyl groups that might remain active were inactivated by 1 M ethanolamine-HCl during 30 min. Finally, the microspheres were washed with PBS and stored in PBS with 0.01% sodium azide at 4 °C in the dark. The immobilization protocol was performed in an opaque, 1.2 μm filter plate with 2×10^6 microspheres per well. Removal of solutions and washing steps were performed with a vacuum manifold and vacuum/pressure pump (Millipore, Billerica, Massachusetts) using a pressure of −14 kPa. To achieve maximum specific binding capability the STX-microspheres should be let to equilibrate in PBS with sodium azide for one week after immobilization and the storage solution should be renewed at least once during this equilibration period. All incubations took place in the dark with constant shaking (700 rpm).

Inhibition Immunoassay for the Detection of PSP Toxins. The detection method was designed as a competition assay in which the STX attached to the microsphere surface competes with free STX for binding to a PSP-binding protein (monoclonal antibody GT-13A). A volume of 110 μL of calibration solution or sample was mixed with 110 μL of GT-13A antibody (1 μg/mL) in a microtube. After 1 h of incubation, 200 μL of this mixture were added to 4×10^3 STX-microspheres that had been previously placed in a well of an opaque, 1.2 μm filter plate and washed. All washing steps were performed three times with 200 μL of PBS-BT using a vacuum

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manifold and vacuum/pressure pump. After 1 h of incubation with the STX-microspheres the sample-antibody mixture was removed and the microspheres washed. A volume of 100 μL of PE-labeled antimouse antibody (0.5 μg/mL), was added to the microspheres for 1 h. Finally, the microspheres were washed and suspended in 100 μL of PBS-BT by shaking immediately before detection. The incubations took place in the dark with constant shaking (700 rpm). Calibration solutions were prepared by diluting STX stock solution in PBSBT or in shellfish extract at a concentration of 1 μM and further serial dilutions were done in PBSBT or in shellfish extract to obtain the concentrations required for the calibration curve. The same procedure was used to prepare the calibration solutions of the other PSP toxins included in the study. For PSP cross-reactivity experiments, a laboratory automation workstation Biomek 3000 (Beckman Coulter, Fullerton, California) was used for reagent and wash-solution addition.

Quantification of Binding Signal. The fluorophore attached to the surface of STX-microspheres was quantified with a Luminex 200 analyzer (LuminexCorp, Austin, Texas). Microspheres were classified with a 635 nm wavelength laser and phycoerythrin fluorescence was quantified after excitation with a 532 nm wavelength laser. Phycoerythrin emission was detected at 565−585 nm. Default values of 7500−13500 were used for doublet discriminator gating of microspheres. The acquisition volume was 75 μL and the number of minimum bead count was 100.

Shellfish Extraction Method. The extraction of PSP toxins from mussel meat was performed as previously described.[22,31] Briefly, mussel meat was removed from the shell, drained and homogenized. One gram of sample homogenate was weighed in a centrifuge tube and 5 mL of 0.2 M sodium acetate buffer, pH 5 were added. Each tube was vortexed for 10 s, roller mixed for 30 min and centrifuged at 3600 *g* during 10 min at room temperature. The supernatant was collected, diluted 1:20 (v/v) in PBS-BT and filtered through a 0.45 μm filter. The mussel samples used in this study did not contain detectable amounts of PSP toxins when tested by HPLC-FLD.[9]

Data Analysis. The results were expressed as mean ±SEM of at least 3 experiments, each performed in duplicate. The Student's *t* test for unpaired data was used for statistical analysis but multiple comparisons were performed using ANOVA test with Tukey multiple comparisons post-test ($p < 0.05$). The calibration curves were fitted using GraphPad Prism 5.0 by a four-parameter logistic equation obtained by a nonlinear regression fitting procedure: $Y = R_{hi} + (R_{lo} - R_{hi})/(1 + 10\wedge((\log IC_{50} - X) \times HillSlope))$, where R_{hi} is the bottom or the response at infinite concentration, R_{lo} is the top or the response at 0 concentration, and X is the logarithm of concentration to base 10. Calibration curves are shown in molar concentrations all through the text to provide a molar ratio comparison for cross-reactivity purposes. For easier comparison with other published studies IC_{20}, IC_{50}, IC_{80} and cross-reactivity values have been expresses both as nanomolar and nanogram/milliliter concentrations.

RESULTS

Optimization of STX Immobilization on Carboxylated Microphores. STX was immobilized on the surface of carboxylated microspheres using EDC/NHS chemistry. In order to achieve the maximum binding capacity, three concentrations of STX, 0.339, 0.725, and 1.451 μg/μL, were tested during the immobilization protocol described in the Methods section. A doubled reaction time (48 h) was tested for the lower STX concentration to increase the level of immobilization. During this experiment surrounding wells were filled with water to minimize evaporation of the solution; however, a loss of volume was observed in some of these wells. For later immobilizations with 0.725 and 1.451 μg/μL, the incubation time was 24 h to avoid desiccation. The performance of these three batches of STX-microspheres in the competition assay was evaluated in terms of background (signal in the absence of binding protein), maximum binding (signal in the presence of binding protein), nonspecific binding (signal in the presence of binding protein and 1 μM free STX) and maximum binding/nonspecific binding ratio. The immobilization conditions used with a STX concentration of 0.725 μg/μL yielded the best maximum/nonspecific binding ratio and a low nonspecific binding signal (Table 1). Three independent

Table 1. Binding Response of STX-Microspheres Obtained with Different Immobilization Conditions[a]

immobilization conditions				
[STX] (μg/μL)	time (h)	maximum GT-13A binding (RU)	nonspecific binding (RU)	maximum/nonspecific binding ratio
0.339	48	166	87	1.91
0.725	24	1847	92	20.08
1.451	24	679.5	230.5	2.95

[a]STX-microspheres were incubated with anti-PSP antibody GT-13A for 1 h. Nonspecific binding was evaluated by antibody binding signal in the presence of 1 μM (372.2 ng/mL) free STX. Maximum GT-13A binding and nonspecific binding signals are shown in response units (RU).

immobilizations (A, B, and C) were performed with 0.725 μg/mL STX and the homogeneity of STX-microspheres was analyzed by comparing maximum binding, nonspecific binding, and IC_{20}, IC_{50}, and IC_{80} of the STX calibration curve (Table 2). The results

Table 2. Comparison of Three Independent Immobilizations of 0.725 μg/μL STX on Carboxyl Microspheres[a]

	A	B	C
maximum binding (RU[b])	397.7 ± 59.4	326.7 ± 57.5	566.9 ± 55.6
nonspecific binding (RU)	2.2 ± 0.5	2.7 ± 0.3	3.4 ± 0.2
IC_{20} (nM)	1.3 ± 0.5	2.7 ± 0.3	4.0 ± 1.3
IC_{20} (ng/mL)	0.5 ± 0.2	1.0 ± 0.1	1.5 ± 0.5
IC_{50} (nM)	4.7 ± 1.4[c]	10.4 ± 1.3	12.3 ± 0.2
IC_{50} (ng/mL)	1.7 ± 0.5	3.8 ± 0.5	4.6 ± 0.1
IC_{80} (nM)	18.7 ± 4.9	34.4 ± 4.1	35.2 ± 0.6
IC_{80} (ng/mL)	7.0 ± 1.8	12.8 ± 1.5	13.1 ± 0.2

[a]The results were collected from calibration curves obtained independently for the three STX-microsphere batches (mean ± SEM, $n = 3$). [b]RU = response units. [c]Statistically different from the B and C immobilizations (ANOVA test with Tukey multiple comparison post-test).

obtained with the three batches of STX-microspheres were not statistically different except for the IC_{50} of the STX calibration curve that differed slightly in one batch (Table 2). Moreover, the STX-microspheres were stable when stored in the fridge for more than 6 months (maximum binding signals and IC_{50} values of calibration curves obtained in the fourth, 20th and 26th weeks after the immobilization were not statistically different, data not shown).

Optimization of the Inhibition Immunoassay for STX Detection. PSP detection was based on the competition of

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free PSP toxins and STX immobilized on the microsphere surface for binding to a specific monoclonal antibody (GT-13A). The protocol followed three basic steps starting with the interaction of the antibody with the free toxin present in a sample or calibration solution. The second and third steps consisted of binding of free antibody to STX-microspheres and subsequent detection of microsphere-bound antibody using a PE-labeled antimouse antibody. Different parameters were optimized to accomplish adequate calibration curves for the detection of STX within regulatory limits. The performance of the assay in different conditions was evaluated using the profile and sensitivity of STX calibration curves. The calibration curves were obtained following the assay protocol with calibration solutions prepared in PBS-BT from 0.01 to 1000 nM STX (HCl present in the STX stock solution did not affect the calibration curve, data not shown). Several concentrations of GT-13A antibody were tested ranging from 0.2 to 2 μg/mL. As expected, lower concentrations provided lower nonspecific binding and larger maximum/nonspecific binding ratios. An antibody concentration of 1 μg/mL was selected for the final assay format because it provided an adequate range of raw values with maximum binding signals between 300 and 500 RUs, low nonspecific binding and high sensitivity. Concentrations of PE-labeled antimouse antibody of 0.5 and 1 μg/mL were also compared with no significant effect on the STX calibration curve. Likewise, incubation times of 1 or 2 h after mixing antibody and sample did not significantly affect the sensitivity of the assay. Therefore, a final antimouse antibody concentration of 0.5 μg/mL and a 1 h incubation time were selected to provide a lower cost and shorter assay. These assay conditions provided a calibration curve (Figure 2) with an IC_{50}

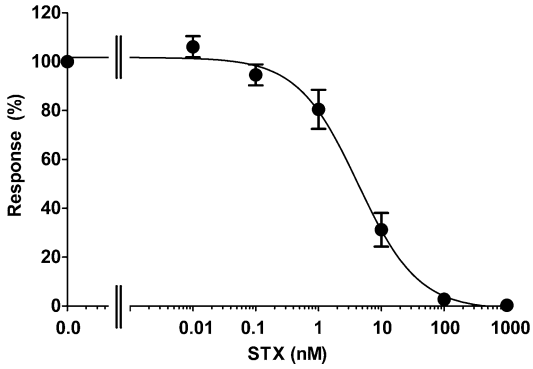

Figure 2. Saxitoxin (STX) calibration curve obtained with the STX-microsphere inhibition immunoassay in buffer. STX was diluted at concentrations of 0.01, 0.1, 1, 10, 100, and 1000 nM. Calibration solutions were mixed with an equal volume of 1 μg/mL GT-13A antibody. After 1 h this mixture was incubated for another hour with STX-microspheres. Finally a PE antimouse antibody was added to the microspheres for 1 h. Data are expressed as percentage of GT-13A binding to the STX-microspheres in the absence of free STX. IC_{50} = 4.7 ± 1.4 nM or IC_{50} = 1.7 ± 0.5 ng/mL (mean ± SEM, n = 3).

of 4.7 ± 1.4 nM (1.7 ± 0.5 ng/mL) and a limit of detection (LOD) evaluated as IC_{20} (previously used to estimate the LOD of immunoassays[22,30]) of 1.25 ± 0.51 nM (0.47 ± 0.19 ng/mL).

PSP Toxin Cross-Reactivity Profile of the Microsphere Inhibition Immunoassay. The detection capability of this inhibition assay for different PSP analogues was evaluated from the calibration curves obtained for STX, dcSTX, NEO, dcNEO, GTX2/3, GTX1/4, dcGTX2/3, GTX5, and C1/2 using certified standard solutions. Calibration solutions at concentrations of 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 100, and 1000 nM were prepared for the 9 toxins. Subsequently the calibration solutions were assayed in duplicate following the protocol optimized for STX. All the toxins were tested in the same day using the same antibody solutions. This immunoassay can detect STX, dcSTX, GTX2/3, dcGTX2/3, GTX5, and C1/2 with high sensitivity (Figure 3 and Table 3). However, the cross-reactivity of the

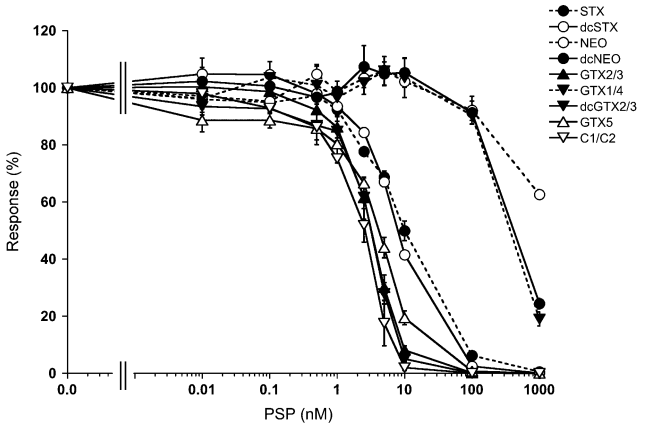

Figure 3. Calibration curves of several PSP toxins obtained with the STX-microsphere inhibition immunoassay in buffer. Each PSP toxin stock solution was diluted at concentrations of 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 100, and 1000 nM in PBS-BT. The calibration solutions were mixed with GT-13A antibody and the immunoassay was performed as described in Methods. Data are expressed as percentage of GT-13A binding to STX-microspheres in the absence of free STX (mean ± SEM, n = 3).

assay for NEO, dcNEO, and GTX 1/4 performs less well (Figure 3 and Table 3).

Interference of Mussel Extracts with the STX-Microsphere Inhibition Immunoassay and STX Recovery Rates. Matrix interference with the inhibition assay was studied using an acetate buffer extraction of mussel meat homogenates.[22,31] To evaluate the assay performance in mussel matrix, maximum binding signal, nonspecific binding and calibration curves in buffer and mussel extract were compared. Calibration solutions of STX at concentrations of 0.01, 0.1, 1, 3, 10, 30, 100, and 1000 nM were prepared in buffer or extract and tested in duplicate following the assay protocol. The calibration curves obtained for STX in buffer and extract were very similar (Figure 4A). Moreover, maximum binding, nonspecific binding, IC_{50} and dynamic range of the assay in buffer or extract were not statistically different (Figure 4B).

STX recovery with this extraction method was also studied by spiking the mussel meat before extraction. Mussel meat homogenate was contaminated with 800 μg of STX/kg (944 μg of STX·2HCl/kg) and then extracted with acetate buffer. The extract was tested with the inhibition assay and the amount of toxin was quantified using the extract and buffer calibration curves obtained in the same experiment (Table 4). The volume of the extract for the calculation of STX content in 1 g of shellfish homogenate was considered 6 mL (5 mL of acetate buffer plus 1 mL of shellfish homogenate) × 20 (dilution factor). The percentage of STX recovery when using an extract calibration curve was 100.8 ± 7.1%. The recovery rate using a buffer calibration curve was lower, however it was not statistically different from the recovery rate achieved using

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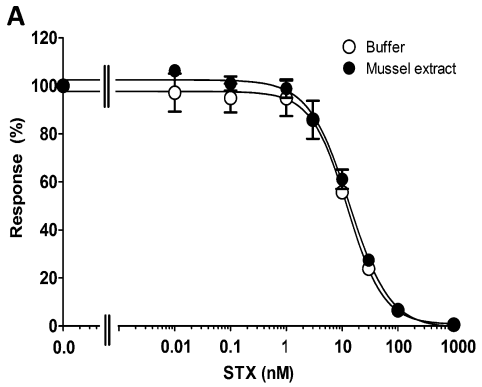
Table 3. PSP Cross-Reactivity of the GT-13A/STX-Microsphere Immunoassay[a]

toxins	IC_{50}	IC_{20}–IC_{80}	% cross-reactivity	TEF
STX	10.0 ± 1.0 nM	2.7 ± 0.3–34.4 ± 4.1 nM	100 nM	1.00
	3.7 ± 0.4 ng/mL	*1.0 ± 0.1 - 12.8 ± 1.5 ng/mL*	*100 ng/mL*	
dcSTX	8.0 ± 0.3 nM	3.0 ± 0.1–22.2 ± 1.3 nM	124.1 nM	0.51
	2.6 ± 0.1 ng/mL	*1.0 ± 0.04 - 7.3 ± 0.4 ng/mL*	*142.7 ng/mL*	
NEO	NA	NA	NA	0.92
dcNEO	199 ± 50.7 nM[b]	120 ± 3.8 – 1100 nM[b]	5.0[b] nM	0.4[d]
	68.9 ± 17.5 ng/mL[b]	*41.5 ± 1.3 - 378 ng/mL*[b]	*5.4*[b] ng/mL	
GTX 2/3	3.1 ± 0.4 nM	1.4 ± 0.4–6.7 ± 0.7 nM	320.2 nM	0.44[c]
	1.2 ± 0.2 ng/mL	*0.6 ± 0.1 - 2.6 ± 0.3 ng/mL*	*309.2 ng/mL*	
GTX 1/4	294 ± 136 nM[b]	145 ± 29.1–601 ± 348 nM[b]	3.4[b] nM	0.93[c]
	121 ± 56 ng/mL[b]	*59.8 ± 12 - 247 ± 143 ng/mL*[b]	*3.1*[b] ng/mL	
dcGTX 2/3	3.1 ± 0.2 nM	1.4 ± 0.1–6.2 ± 0.5 nM	321.4 nM	0.19[c]
	1.1 ± 0.1 ng/mL	*0.5 ± 0.03 - 2.2 ± 0.2 ng/mL*	*337.3 ng/mL*	
GTX5	4.0 ± 0.4 nM	1.2 ± 0.3–11.0 ± 1.1 nM	248.1 nM	0.06
	1.5 ± 0.1 ng/mL	*0.5 ± 0.1 - 4.2 ± 0.4 ng/mL*	*247.4 ng/mL*	
C1/2	2.3 ± 0.5 nM	5.3 ± 1.6 – 8.48 ± 0.01 nM	429.9 nM	0.03[c]
	1.1 ± 0.2 ng/mL	*0.4 ± 0.004 - 2.5 ± 0.8 ng/mL*	*337.3 ng/mL*	

[a]Half maximal inhibitory concentration (IC_{50}), dynamic range (IC_{20}–IC_{80}) and percentage of cross-reactivity obtained for STX, dcSTX, NEO, dcNEO, GTX2/3, GTX1/4, dcGTX2/3, GTX5, and C1/2 detected by the inhibition immunoassay. The percentage of cross-reactivity was calculated from the IC_{50} values of the calibration curve of each toxin versus the IC_{50} of the STX calibration curve. Toxicity equivalency factors (TEFs) are based on the intraperitoneal (i.p.) toxicity data by Oshima[37] unless noted (mean ± SEM, n = 3). Concentrations are shown in nanomolar, with the equivalent in ng/mL in italics. The cross-reactivity has also been calculated for nM and ng/mL (italics) concentrations. NA: not applicable. The data obtained with the NEO calibration solutions did not yield a four-parameter fit for the calibration curve and therefore an IC_{50}, IC_{20} and IC_{80} could not be calculated. Therefore cross-reactivity could not be calculated either and it should be inferred that it is lower than 3.1% (calculated in ng/mL). [b]Four-parameter fit reported by GraphPad software as ambiguous. [c]Relative toxicity proportional to the amount of GTX 2 and GTX3, GTX1 and GTX4, dcGTX2, and dcGTX3 or C1 and C2 in the NRC standard solutions. [d]Relative toxicity proposed by CONTAM Panel.[38]



B

	Buffer	Extract
Maximum binding (RU)	566.9 ± 55.6	570.5 ± 42.2
Non-specific binding (RU)	3.4 ± 0.2	3.7 ± 0.4
IC_{50} (nM)	12.3 ± 0.2	13.6 ±1.6
IC_{50} (ng/mL)	*4.6 ± 0.1*	*5.1 ± 0.6*
IC_{20} – IC_{80} (nM)	5.0 ± 1.7 – 43.7 ± 0.8	5.9 ± 0.6 - 52.9 ± 7.1
IC_{20} – IC_{80} (ng/mL)	*1.9 ± 0.6 - 16.3 ± 0.3*	*2.2 ± 0.2 - 19.7 ± 2.6*

Figure 4. Performance of STX-microsphere assay in mussel extract. (A) Calibration curves of STX obtained with the STX-microsphere inhibition immunoassay in buffer and mussel extract. STX stock solution was diluted at concentrations of 0.01, 0.1, 1, 3, 10, 30, 100, and 1000 nM in PBS-BT or mussel extract. The calibration solutions were mixed with GT-13A antibody and the immunoassay was performed as described in Methods. Data are expressed as percentage of GT-13A binding to STX-microspheres in the absence of free STX. (B) Matrix effect on GT-13A antibody binding to STX-microspheres. Maximum and nonspecific binding raw values (RU) and half maximal inhibitory concentration (IC_{50}) and dynamic range (IC_{20}–IC_{80}) for the STX calibration curve in buffer and extract (mean ± SEM, n = 3). Concentrations are shown in nM, with the equivalent in ng/mL in italics.

the extract calibration curve. The repeatability of the assay for contaminated extracts was deemed to be good with CVs lower than 12.5% (n = 3). Considering a 100% recovery of STX with this extraction protocol, the dilution of the toxin during the extraction protocol and the IC_{20} and IC_{80} values of the calibration curve in extract, the calculated detection range in shellfish meat was 260–2360 μg/kg.

DISCUSSION

The microsphere solid-phase immunodetection assay for PSP toxins performs similarly to previously published assays that use the same PSP binding protein, the monoclonal GT-13A antibody,[16,17] both in terms of sensitivity and cross-reactivity. The IC_{50} values for STX calibration curves were 3.7 ng/mL for this microsphere assay, 4.73 ng/mL for the SPR-based biosensor assay[14,16] and 4.06 ng/mL for the ELISA assay.[17] The advantage offered by the optimized microsphere assay is the ease and flexibility of end-user multiplexing by combining in the same sample several types of microspheres and suitable binding proteins, each microsphere/binding protein set being specific for the detection of a different group of toxins. Multiplexing has also been developed for SPR-based biosensors detection. Very recent technological developments both for SPR detection equipment and chip surface preparation allow detection of up to 16 analytes (or groups of toxins) in the same sample.[24] The SPR-based system has been tested for four

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Table 4. Recovery of Saxitoxin (STX) Using Acetate Extraction Followed by Detection with the GT-13A/STX-Microsphere Immunoassay in Spiked Mussel Homogenates[a]

	toxin measurement	coefficient of variation (%)	recovery (%)
binding signal	201.3 ± 9.1 RU	7.86	
toxin concentration with extract calibration curve	806.4 ± 56.7 μg/kg	12.2	100.8 ± 7.1
toxin concentration with buffer calibration curve	684.3 ± 38.5 μg/kg	9.74	85.5 ± 4.8

[a]Data showed in response units (RU) and μg of STX/kg of mussel meat (mean ± SEM, n = 3).

different groups of toxins with assay characteristics for each toxin group similar to the single assays. However the detection by SPR based biosensors is currently limited in terms of number of analytes, considering that there are multiple microsphere-based assays commercially available that include more than 20 analytes. Actually, Luminex multiplex technology can distinguish 100 classes of color-coded microspheres, which generates the possibility to detect a high number of analytes simultaneously. More than fifteen groups of marine and freshwater toxins have been already described and the number has grown considerably in recent years. Another advantage of microsphere-based assays is the flexibility of multiplexing by creating customized panels attending to user needs.

This first approach to toxin detection by a microsphere-based system demonstrates that this technology would allow detection with similar sensitivity and cross-reactivity to previously published immunoassays when using the same antibodies and surface chemistry. The sensitivity of the present immunoassay could probably be increased by changing the binding protein to a more sensitive antibody, such as the anti-STX polyclonal antibodies used in other immunoassays, which have been demonstrated to perform better in terms of sensitivity and cross-reactivity than the monoclonal GT-13A antibody, or modulating the assay conditions.[14,15,22] Unfortunately, polyclonal antibodies are limited resources and these antibodies were not available for this study. Although the sensitivity of the assay might be increased, it would implicate an increase of the cost of the assay in terms of reagents and/or time, which is not advisible for routine screening methods when an alternative exists to detect STX within current regulatory limits with a more cost-effective assay. Moreover, the toxin immobilization conditions can be reproduced in different microsphere batches with similar performance of the assay and toxin-bound microspheres can be stored for long periods of time. Therefore, transferring of immunoassays that have been developed for toxin detection to microsphere-based platforms seems to be a feasible option for multiplexing. However, the multiplexed assay must be optimized and validated since unexpected interactions may appear when combining several competition assays.[32] The most difficult part of multiplexing for marine and freshwater toxin detection will in all likelihood be to optimize an extraction protocol suitable for all toxin groups.

After optimization, the assay allows the detection of saxitoxin in mussel extracts at and below the regulatory limit established by current regulations. In the present experiments the extraction protocol yielded excellent recovery rates of around 100%. The mussel extracts did not interfere significantly with the assay, because the calibration curves in buffer and extract of three independent assays overlapped almost completely and antibody binding was not statistically different in both conditions. The recovery rate of the extract seems lower when using a buffer calibration curve for the calculation of toxin concentrations. This unexpected difference in view of the similarity of the calibration curves obtained in buffer and extract (Figure 4, mean of three experiments) is due to slight variations of the calibration curves in each independent experiment. However, although the recovery seems lower for calibrations in buffer, the results were not statistically different from the results obtained using calibration curves in extract. These data suggest that calibration curves of STX in buffer could be used for sample screening purposes. Previous studies of matrix interference with PSP-detection immunoassays have demonstrated that most immunoassays can be used with several shellfish matrices.[16,22] Although more matrix interference studies would be needed to confirm the lack of matrix effects, the aim of this work was to explore the capabilities of microsphere-based immunoassays with the objective of multiplexing. Considering that the introduction of more toxin groups in the assay will certainly require a modification of the extraction protocol to recover also lipophilic toxins, the compatibility with diverse shellfish matrices will be explored with a multiplexed assay. The main limitation of the current assay comes from the antibody cross-reactivity, since the detection of Neo, dcNeo and GTX1/4, toxins with a hydroxyl moiety in N1 position (Figure 1), is not very efficient, and the toxicity of these compounds is comparable to that of STX toxicity (Table 3). Most anti-PSP antibodies have poor cross-reactivity with N1 hydroxyl toxins and several groups have been working to develop good antibodies for these PSP analogs.[30,33−35] While these antibodies were not available during the present study, it appeared to be feasible to include a parallel assay for the detection of N1 hydroxyl toxins in this multiplexing platform. None of these toxins has been demonstrated to occur in isolation during toxic blooms, however there have been reports of toxic blooms with a significant fraction of total toxicity due to GTX1/4.[36] Therefore adequate protection of human health should guarantee the detection of Neo, dcNeo, and GTX1/4 along with the other highly toxic congeners.

In summary, this study has demonstrated, for the first time, that a microsphere-based immunodetection method for detecting PSP toxins can provide similar performance characteristics to other immunoassay techniques. This microsphere-based assay can be used to detect STX in acetate extracts of mussels with sensitivity in the range of 260 to 2360 μg/kg (IC_{20}−IC_{80}). This detection system when optimized can be multiplexed to allow the simultaneous detection in the same sample of other groups of toxins or saxitoxin analogs with poor cross-reactivity with this monoclonal antibody.

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Notes

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