

Single Laboratory Validation of a Surface Plasmon Resonance Biosensor Screening method for Paralytic Shellfish Poisoning Toxins

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A research element of the European Union (EU) sixth Framework project BioCop focused on the development of a surface plasmon resonance (SPR) biosensor assay for the detection of paralytic shellfish poisoning (PSP) toxins in shellfish as an alternative to the increasingly ethically unacceptable mouse bioassay. A biosensor assay was developed using both a saxitoxin binding protein and chip surface in tandem with a highly efficient simple extraction procedure. The present report describes the single laboratory validation of this immunological screening method, for this complex group of toxins with differing toxicities, according to the European Decision 2002/657/EC in conjunction with IUPAC and AOAC single laboratory validation guidelines. The different performance characteristics (detection capability CC_β, specificity/selectivity, repeatability, reproducibility, stability, and applicability) were determined in relation to the EU regulatory limit of 800 µg of saxitoxin equivalents (STX eq) per kg of shellfish meat. The detection capability CC_β was calculated to be 120 µg/kg. Intra-assay repeatability was found to be between 2.5 and 12.3% and interassay reproducibility was between 6.1 and 15.2% for different shellfish matrices. Natural samples were also evaluated and the resultant data displayed overall agreements of 96 and 92% with that of the existing AOAC approved methods of mouse bioassay (MBA) and high performance liquid chromatography (HPLC), respectively.

Shellfish, a nutritious food source, are featured globally in different cuisines but are highly sensitive to the quality of their marine environment. Paralytic shellfish poisoning (PSP) toxins are produced by certain dinoflagellates and some cyanobacteria. Shellfish, filter feeding on these unicellular algae, accumulate and metabolize these toxins. Consequently, there are greater than 20

main analogues of saxitoxin (Figure 1), each with a different toxicity factor, responsible for PSP.¹ Transfer of the PSP toxins through the food chain occurs. Human and mammalian consumption of shellfish contaminated with PSP toxins can result in illness or death if exposed to sufficient levels. PSPs are neurotoxins and all analogues bind to voltage-dependent sodium channels, resulting in the blockage of ion transport which may lead to paralysis followed by death.

In the European Union, PSP toxins in bivalve molluscs are regulated with a regulatory limit of 800 µg of PSP toxins per kg of shellfish meat.² The CONTAM Panel of the European Food Safety Authority adopted this figure as being expressed as µg STX eq/kg shellfish meat.³ Therefore, an ideal screening method should simultaneously detect the entire family of toxins at or below the regulatory limit. The MBA⁴ is the internationally accepted method for testing of PSP toxins in shellfish. However, the method is prone to interferences and ethical considerations are of increasing concern. Analytical methods such as high performance liquid chromatography (HPLC) with fluorescence detection, using postcolumn^{5,6} and precolumn oxidation methods^{7–9} have been developed for the determination of PSP toxins with the latter being accepted by the EU as a first action method.¹⁰ However, these methods are laborious and analytical standards are difficult to obtain. Oxidation of PSP toxins is required for HPLC with fluorescence detection because they lack a chromophore. Oxida-

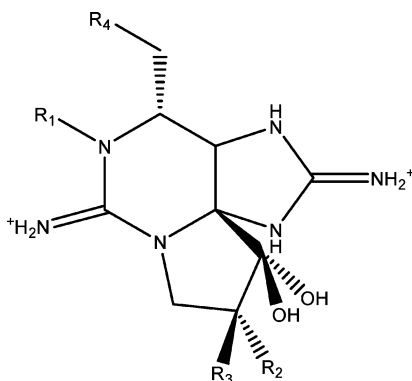
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- (3) European Food Safety Authority. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish- Saxitoxin Group. *EFSA J.* **2009**, *1019*, 1–76.
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			Carbamate Toxins	<i>N</i> -Sulfocarbamoyl toxins	Decarbamoyl toxins	Deoxydecarbamoyl toxins
R ₁	R ₂	R ₃	R ₄ - OCONH ₂	R ₄ - OCONHSO ₃ ⁻	R ₄ - OH	R ₄ - H
H	H	H	STX	B1 (GTX 5)	dc-STX	do-STX
H	H	OSO ₃ ⁻	GTX 2	C1	dc-GTX 2	do-GTX 2
H	OSO ₃ ⁻	H	GTX 3	C2	dc-GTX 3	do-GTX 3
OH	H	H	NEO	B2 (GTX 6)	dc-NEO	
OH	H	OSO ₃ ⁻	GTX 1	C3	dc-GTX 1	
OH	OSO ₃ ⁻	H	GTX 4	C4	dc-GTX 4	

Figure 1. Chemical structure of PSP toxins.

tion using both peroxide and periodate is required for the precolumn oxidation method resulting in the requirement for two analytical runs. Lack of standard material, lengthy purification procedures, and insufficient detectability has inhibited the progress in the development of other techniques such as mass spectrometry for routine monitoring. However, considering their high cost and the need for skilled scientists, physicochemical methods are more suitable for confirmatory analysis than screening methods. Laboratories required to perform screening prior to release of shellfish to markets require sensitive, cost-effective, rapid, simple methods accurate in the determination of PSP toxins. Rapid screening using immunological techniques have been developed to provide this service and automated sensors avoid the time delay and manual labor required for traditional ELISA and analytical methods.

A rapid surface plasmon resonance (SPR) biosensor assay was developed to detect PSP toxins in shellfish as a product of the EU Sixth Framework project BioCop (www.biocop.org). The Biacore Q optical biosensor uses the phenomenon of SPR to exploit the behavior of light at boundaries of different refractive indices to monitor biomolecular reactions. The technique does not require any labeling of the interacting components. Interactions are measured as they occur (real time) with an analysis time of minutes. The speed of analysis and low running costs of the biosensor make it well suited for high throughput toxin screening. The method described has been developed in support of EC Regulations No 853/2004,² 2074/2005¹¹ and 1664/2006¹² which lay down the health conditions for the production and placing on the market of live bivalve molluscs and the methods of analysis of certain marine biotoxins.

The SPR biosensor assay developed underwent single laboratory validation for the detection of PSP toxins at concentrations

suitable for regulatory monitoring of mussels and cockles. The study was designed to address the different performance characteristics (detection capability CC_β, specificity/selectivity, repeatability, reproducibility, stability, and applicability) in relation to the EU regulatory limit mainly according to the criteria required by the European Commission Decision 2002/657/EC¹³ but also considering IUPAC and AOAC guidelines for single laboratory validation.^{14,15}

MATERIALS AND METHODS

Instrumentation. A Biacore Q SPR biosensor with control software version 3.0.4, BIAevaluation software version 4.1 and CM5 sensor chips were purchased from Biacore (GE Healthcare, Uppsala, Sweden).

Chemicals and Reagents. Saxitoxin dihydrochloride (STXdi-HCl-65 μM), neosaxitoxin (NEO-65 μM), gonyautoxin 1/4 (GTX1-106 μM:GTX4-35 μM), gonyautoxin 2/3 (GTX2-118 μM:GTX3-39 μM), decarbamoyl saxitoxin (dcSTX-62 μM), decarbamoyl neosaxitoxin (dcNEO-30 μM), decarbamoyl gonyautoxin 2/3 (dcGTX2-114 μM:dcGTX3-32 μM), gonyautoxin 5 (GTX5-65 μM), and C1/C2 (C1-114 μM:C2-35 μM) as certified reference standard materials were obtained from the Institute for Marine Biosciences, National Research Council, Halifax, Canada (<http://imb-ibm.nrc-cnrc.gc.ca/crmfp/>). C3/C4 (C3-50 μM:C4-50 μM) was received as a gift from the Agri-Food and Biosciences Institute, Belfast, Northern Ireland.

An amine coupling kit and HBS-EP buffer (pH 7.4, 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate) were obtained from GE Healthcare, UK.

(11) European Commission Regulation (EC) No 2074/2005. Off. J. Eur. Comm. 2005, L338, 27.

(12) European Commission Regulation (EC) No 1664/2006. Off. J. Eur. Comm. 2006, L320, 13.

(13) European Commission Decision (EC) No 2002/657/EC. Off. J. Eur. Comm. 2002, L221, 8.

(14) Thompson, M.; Ellison, S. L. R.; Wood, R. *Pure Appl. Chem.* **2002**, 74, 835-855.

(15) AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals 19/12/2002 www.aoac.org/Official_Methods/slv_guidelines.pdf.

Formaldehyde (37%), 2,2-(ethylenedioxy)bis-(ethylamine) (Jeffamine), glacial acetic acid, sodium acetate minimum 99%, hydrochloric acid solution 1 M were purchased from Sigma-Aldrich, Dorset, UK.

Sample Collection. PSP toxin free shellfish homogenates, predetermined by both the AOAC HPLC and MBA methods, for the evaluation of shellfish tissue matrix effects were obtained from the Agri-Food and Biosciences Institute, Belfast, Northern Ireland. These shellfish homogenates were obtained from samples collected at different times and from different locations. The species obtained were mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*), clams (*Veneridae* spp.), oysters (*Crassostrea gigas*), and scallops (*Pecten maximus*). Shellfish samples were collected from a number of regulatory laboratories to ensure that tissues containing variable PSP toxin profiles were included in the assessment. Shellfish samples from Europe were supplied by the UK National Reference Laboratories: the Fisheries Research Centre, Scotland, and the Agri-Food and Biosciences Institute, Belfast, Northern Ireland and the Autonomous Government Laboratory for shellfish monitoring in Andalucía, Spain.

ASSAY DEVELOPMENT

Immobilization of STX on the Sensor Chip Surface. STX was covalently immobilized to the surface of a CM5 chip by amino–amino coupling as previously described.¹⁶ However, the extension of the chemical reaction to three days provided chip surfaces with improved coverage and stability. Chip surfaces also required a 2 day wash with a flow rate of 25 $\mu\text{L}/\text{min}$ of HBS-EP buffer for conditioning prior to initial use. Each chip has four flow cells and each can be used for >300 analyses.

Antibody Production. The synthesis of the immunogen, the immunization process and antibody titer determination for the production of the polyclonal antibody (R895) were previously described.¹⁶ This antibody is herein referred to as saxitoxin binding protein (SBP).

Preparation of Assay Calibration Curve. STXdiHCl calibrants (0, 1.0, 2.5, 5.0, 7.5, and 10.0 ng/mL) were prepared in HBS-EP buffer from the NRC stock solution. These are equivalent to 0, 120, 300, 600, 900, and 1200 μg of STXdiHCl per kg shellfish based on the assay.

Extraction Method for Shellfish Samples. An extraction procedure proposed by Bates and co-workers¹⁷ was employed. Briefly, shellfish samples were removed from their shells, drained and shellfish meat (100 g) was homogenized. This homogenate was stored frozen at -20°C until required. Samples (1 g) of homogenate were weighed into centrifuge tubes and sodium acetate buffer pH5 (5 mL) was added. Each tube was vortexed for 10 s and roller mixed for 30 min. Following mixing, samples were centrifuged at 3600g for 10 min at room temperature and the supernatant was collected and diluted 1 in 20 in HBS-EP buffer (50 μL extract to 950 μL buffer).

SPR Analysis: Instrumental Parameters. Analyses were performed using the Biacore Q SPR biosensor. The SBP was diluted 1/250 in HBS-EP buffer providing a protein concentration at 280 nm of 0.2 mg/mL. The parameters were set to mix the

SBP with an equal volume of STXdiHCl calibrant (or sample) prior to injection over the STX sensor chip surface. A flow rate of 12 $\mu\text{L}/\text{min}$ for 120 s was employed. Report points were taken before (10 s) and after each injection (30 s), and the relative response units determined. The chip surface was regenerated with 8 μL injections of hydrochloric acid (50 mM) at a flow rate of 12 $\mu\text{L}/\text{min}$. Calibrants and samples were analyzed in duplicate.

VALIDATION

Specificity. Specificity is the ability of a method to distinguish between the analyte(s) being measured and other substances. The specificity of the antibody for PSP toxins compared to other predominant shellfish toxins was determined using working standards (1000 ng/mL) of domoic acid, okadaic acid and tetrodotoxin in HBS-EP buffer and calculating the percentage inhibition.

Cross-Reactivity Profile of SBP with PSP toxins in Buffer and Mussel Extract. The % cross-reactivity of the SBP to all the available STX analogues in relation to STXdiH in both buffer and mussel extract were assessed. The cross-reactivity profile for the assay was generated by producing calibration curves for each toxin based on the dose–response on the biosensor and determining the midpoint (IC_{50}) value for each curve. IC_{50} , being defined as the concentration of a PSP toxin required to reduce the response by 50% binding compared to the response when no toxin is present (100% binding). The cross-reactivity profile relative to the PSP toxin standards was calculated from the IC_{50} in the biosensor assay as follows:

$$\% \text{cross-reactivity} = (\text{IC}_{50} \text{ of STXdiH} / \text{IC}_{50} \text{ of PSP toxin}) \times 100$$

For the buffer cross-reactivity profile stock solutions of STXdiHCl, NEO, GTX1/4, GTX2/3, dcSTX, dcNEO, dcGTX2/3, GTX5, C1/C2, C3/C4 were used to prepare calibrants in HBS-EP buffer pH 7.4. The concentration range, from 0 to 10 $\mu\text{g}/\text{mL}$, was dependent on the toxin tested. The same toxins were used to evaluate the effect of the mussel matrix on the cross-reactivity profile of the SBP. Negative extracts were spiked with concentrations ranging from 0 to 50 $\mu\text{g}/\text{mL}$ depending on the toxin tested. Known negative mussels (10×1 g) were extracted; supernatants pooled and diluted 1/20 to provide aliquots of negative mussel extract for fortification. Due to the sensitivity attainable with the assay a 1/20 dilution was applied to achieve the required sensitivity for the assay based on the current EU regulatory limit. The calibration points for each of the toxin curves were prepared by fortifying 950 μL aliquots of mussel extract with 50 μL of the corresponding toxin spiking solution and then thoroughly mixed. These fortified mussel extracts were analyzed to obtain “matrix” calibration curves.

Analysis of Known Uncontaminated Mussels and Cockles for Interference Effects (Decision Limit $\text{CC}\alpha$). The decision limit ($\text{CC}\alpha$) is the limit at or above which it can be concluded with an error probability of α that a sample contains PSP toxin. Although $\text{CC}\alpha$ is not included in the mandatory performance characteristics required by Commission Decision 2002/657/EC for screening tests, the determination of $\text{CC}\alpha$ is necessary in order to evaluate the β error for the detection capability. The approach to determine the $\text{CC}\alpha$ was the duplicate analysis on three different

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Table 1. Comparison of the Toxicity Equivalent Factors for Each Toxin and the Corresponding Concentrations of Toxin at the Regulatory Limit in STXdiHCl Equivalents Based on the supplementary information from the NRC and the New EFSA Opinion

PSP toxin	relative molecular mass	NRC toxicity equivalent factors ($\mu\text{g/kg}$)	relative toxin concentration at regulatory limit ($\mu\text{g/kg}$) in free base form related to STXdiHCl based on NRC	EFSA toxicity equivalent factors ($\mu\text{g/kg}$)	relative toxin concentration at regulatory limit ($\mu\text{g/kg}$) in free base form related to STXdiHCl based on EFSA
STXdiHCl	372.2	1.0000	800	1.0	800
NEO	315.3	1.0911	733	1.18	678
GTX 1	411.4	0.8993	890	0.90	889
GTX 2	395.4	0.3382	2366	0.38	2105
GTX 3	395.4	0.6005	1332	0.56	1429
GTX 4	411.4	0.6569	1218	0.63	1270
GTX 5 (B1)	379.4	0.0632	12655	0.10	8000
GTX 6 (B2)	395.4	0.0607	13189	0.09	8889
C1	475.4	0.0047	169145		
C2	475.4	0.0754	10616	0.08	10000
C3	491.4	0.0101	79472		
C4	491.4	0.0436	18340	0.08	10000
dcSTX	256.3	0.7451	1074	1.45	552
dcNEO	272.3	0.7013	1141	0.55	1455
dcGTX 2	352.3	0.1625	4922	0.21	3810
dcGTX 3	352.3	0.3978	2011	0.42	1905
for combinations of toxins, the toxin with the greatest toxicity is used for calculative purposes					
GTX1/4 combined	411.4	0.8993	890	0.9	889
GTX2/3 combined	395.4	0.6005	1332	0.56	1429
dcGTX2/3 combined	352.3	0.3978	2011	0.42	1905
C1/C2 combined	475.4	0.0754	10616	0.08	10000
C3/C4 combined	491.4	0.0436	18340	0.08	10000

days (10 samples per day of known non-contaminated mussel ($n = 30$) and cockle ($n = 30$) samples) previously analyzed by HPLC and mouse bioassay. CC α was determined as the concentration value from the mean of the measured response units of known negative samples ($n = 30$) minus 2.33 times the standard deviation of this response to provide a 99% confidence level.

Applicability. The applicability of the method to different species of shellfish was investigated. Aliquots (6×1 g) of PSP free mussels, cockles, clams, oysters, and scallops were weighed into plastic universals and extracted using the procedure described. Aliquots of HBS-EP buffer (6×1 mL) and the five different shellfish species extracts (6×1 mL) were spiked with STXdiHCl to provide six calibrants (0, 1.0, 2.5, 5.0, 7.5, 10 ng/mL) for each matrix curve. These calibrants prepared in HBS-EP buffer and each shellfish extract were analyzed to obtain the relative response to the STXdiHCl concentration to produce calibration curves.

Recovery of the Assay. To measure the recovery of the toxins under assay conditions from known negative homogenized mussel tissue with this extraction procedure, aliquots (1 g) of shellfish were spiked with each toxin at half the regulatory limit and at the regulatory limit (where possible due to limited supply of some toxins) in STXdiHCl equivalents. Currently, the toxicity equivalence factors (TEFs) provided as supplementary information from the National Research Council (NRC) based on the data from Oshima⁵ are used for the conversion of the individual toxin amounts to STXdiHCl equivalents. However, the EFSA working group on saxitoxin have published new TEFs that will be implemented later in 2010.³ The corresponding levels of each toxin at the regulatory limit based on the existing and newly recommended TEFs are listed in Table 1. For this study the existing

NRC TEFs are used as these are in accordance with existing regulations.

For STXdiHCl, dcSTX, and NEO samples ($n = 10$) were analyzed in duplicate on three separate days and for the remaining available toxins samples ($n = 3$) were analyzed in duplicate once. The concentration at both levels was determined from the saxitoxin dihydrochloride curve to obtain the recovery of the assay for each toxin. The recovery of the assay was used to show the apparent concentration of PSP toxin at either half or the regulatory limit when compared to STXdiHCl curve.

A similar recovery experiment for cockles was performed. However, due to insufficient quantities of known PSP free cockle samples and toxins, the study was based mostly on the recovery of STXdiHCl (with limited results for dc-STX and NEO). Aliquots (1 g) of cockles were spiked with STXdiHCl at the regulatory limit ($n = 10$) and half the regulatory limit ($n = 10$) and analyzed in duplicate on three separate occasions (dc-STX and NEO spiked samples ($n = 10$) were run in duplicate once).

Detection Capability (CC β). The detection capability (CC β) is defined (2002/657/EC) as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β . Known non-contaminated mussel samples ($n = 30$) fortified at 200 $\mu\text{g/kg}$ were analyzed in duplicate on three different days (10 samples per day). This was performed for STX, NEO, and dcSTX as STXdiHCl equivalents. C1/C2 toxins were also evaluated based on availability. Therefore all structural classifications for PSP toxins were included in the study and it was replicated for cockles. The CC β was determined as the concentration at which less than 5% of false compliant results were identified.

Threshold Level for the Assay. PSP toxins have one regulatory limit for a sum of toxin analogues with varying toxicities currently based on the NRC TEFs. In order for the method to perform as an effective screening tool threshold levels are established to offer a guarantee that no false noncompliant samples above or close to the regulatory limit are reported as compliant. To determine the threshold level for the assay samples fortified at 400 $\mu\text{g}/\text{kg}$ (30) and samples fortified at the regulatory limit of 800 $\mu\text{g}/\text{kg}$ (30) in STXdiHCl equivalents were analyzed in duplicate on three different days (10 samples per day). This was performed for STX, NEO, and dcSTX and C1/C2 toxin as previously described. This study was also repeated for cockles with the exception of C1/C2 (due to lack of standards).

Precision (Repeatability and Reproducibility Conditions). The STXdiHCl calibration curve and fortified samples at three different concentrations of STXdiHCl (200, 400, and 800 $\mu\text{g}/\text{kg}$) were prepared in mussels and cockles. The fortified samples were analyzed in 10 replicates during 3 days. One analysis for each was performed by a second scientist. The concentration of each fortified sample was calculated from the response value as were the mean concentrations, the standard deviations (SD), and the coefficient of variation (CVs) at each concentration.

Stability. *Stability of STXdiHCl in HBS-EP Solution.* Fresh working calibrants of STXdiHCl were prepared. Aliquots were stored at 4–8 °C and at –20 °C over 1, 2, 4, and 9 weeks. At these time periods the standards were compared with freshly prepared calibrants from the STXdiHCl stock solution.

Stability of the Antibody at 4–8 °C. The SBP dilution (1/250) was stored at 4–8 °C over 9 weeks. The analysis of STXdiHCl calibration curve was performed with this SBP solution at days 0 and 3, and after 1 and 2, 4, and 9 weeks.

Stability of Shellfish Extracts. Mussel samples were spiked at the regulatory limit using STXdiHCl stock solution, extracted and assayed. The same extracts were then reanalyzed 3 days, 1 week, 2 weeks, 4 weeks and 9 weeks later after storage at 4–8 and –20 °C.

Analysis of Naturally Contaminated Samples. PSP contaminated samples ($n = 25$) including mussels, cockles and scallops were analyzed where possible using SPR, the AOAC HPLC, and MBA methods. The samples were analyzed in duplicate with the SPR method for the detection of PSP toxins on two different occasions by different scientists using different batches of STX sensor chips.

RESULTS AND DISCUSSION

The biosensor protocol used for the determination of PSP toxins from different shellfish species was based on previous procedures.¹⁶ However, a number of parameters were further optimized, for example, flow rate, antibody to sample ratio and regeneration solution. The duration of the sample analysis including regeneration was 6 min. Figure 2a illustrates an overlay of the typical sensorgrams obtained for the STXdiHCl calibration curve. Figure 2b illustrates a response versus STXdiHCl concentration calibration curve obtained in HBS-EP buffer and different shellfish matrices. The various shellfish matrices had little effect on the calibration curve parameters, thereby allowing use of the HBS-EP curve for quantification in all samples analyzed and therefore the results for samples are calculated with reference to the HBS-EP buffer calibration curve.

Specificity. Specificity investigations demonstrated that the SBP did not cross-react with domoic acid, okadaic acid, or tetrodotoxin. As these toxins can co-occur in certain seafood products and may be extracted along with the PSP toxins from contaminated shellfish, in particular domoic acid as it is also hydrophilic, it is essential that the SBP eliminates them as an interfering factor from the assay. As tetrodotoxin has a similarity in structure to STX, binds to the same target and acts in the same way as STX, producing symptoms virtually identical to PSP it was important to assess this toxin for cross-reactivity in the assay.

Cross-Reactivity Profile of SBP with PSP toxins in Buffer and Mussel Extract. Calibration curves of normalized response units relative to the toxin concentration for each toxin in buffer and mussel extract were produced and compared using a 4-parameter fit function with BIAevaluation software (version 4.1). This software was also used to determine the % binding at each toxin concentration, the midpoint (IC_{50}) of each curve and the dynamic range (IC_{20} – IC_{80}) values for each curve. The dynamic range (theoretical lower and upper detection limits) for the assay are the 20% inhibitory concentration (IC_{20}) to the 80% inhibitory concentration (IC_{80}). IC_{20} and IC_{80} are defined as the concentrations of a PSP toxin required to reduce the SPR response by 20 and 80% binding within the curve, respectively, compared to the response of 100% binding when no toxin is present.

Table 2 shows that the cross-reactivity profile for the SBP in buffer and mussel extract is fairly similar. For the commercially available PSP toxins 11 out of the 13 tested displayed some cross-reactivity. For comparison, since the MBA is toxicity based each table displays the SPR cross-reactivity profile for the PSP toxins as ng/mL of toxin and ng/mL of STXdiHCl equivalents of toxin taking into consideration the NRC TEFs for each toxin. The higher TEF was used when only combinations of toxins are available such as in the case of gonyautoxin (GTX) 2/3.

Toxins with modifications in the R4 position (Figure 1) displayed the highest % cross-reactivity followed by those with modifications to the R2 and R3 position and then those toxins that are hydroxylated in the R1 position. Combinations of modifications to the R groups compared to STX showed an additive decrease in % cross-reactivity with the outcome for GTX1/4, which is modified at R1, R2, and R3 positions, displaying the lowest % cross-reactivity at <1%.

In summary those toxins with IC_{50}s lower than that of STXdiHCl will overestimate in relation to the MBA if present in samples including GTX5, dcSTX and C1/C2. However, those toxins with IC_{50}s greater than that of STXdiHCl will be underestimated in relation to the MBA, for example GTX1/4. Though GTX1/4 are potent toxins these have not been reported to occur in isolation but have been demonstrated to occur in Alaskan shellfish contributing up to 50% of the total amount of toxins.¹⁸

Several teams have worked on the production of antibodies for ELISA based assays.^{19–28} Although PSP toxins have a common core in their structure, the production of a generic antibody for

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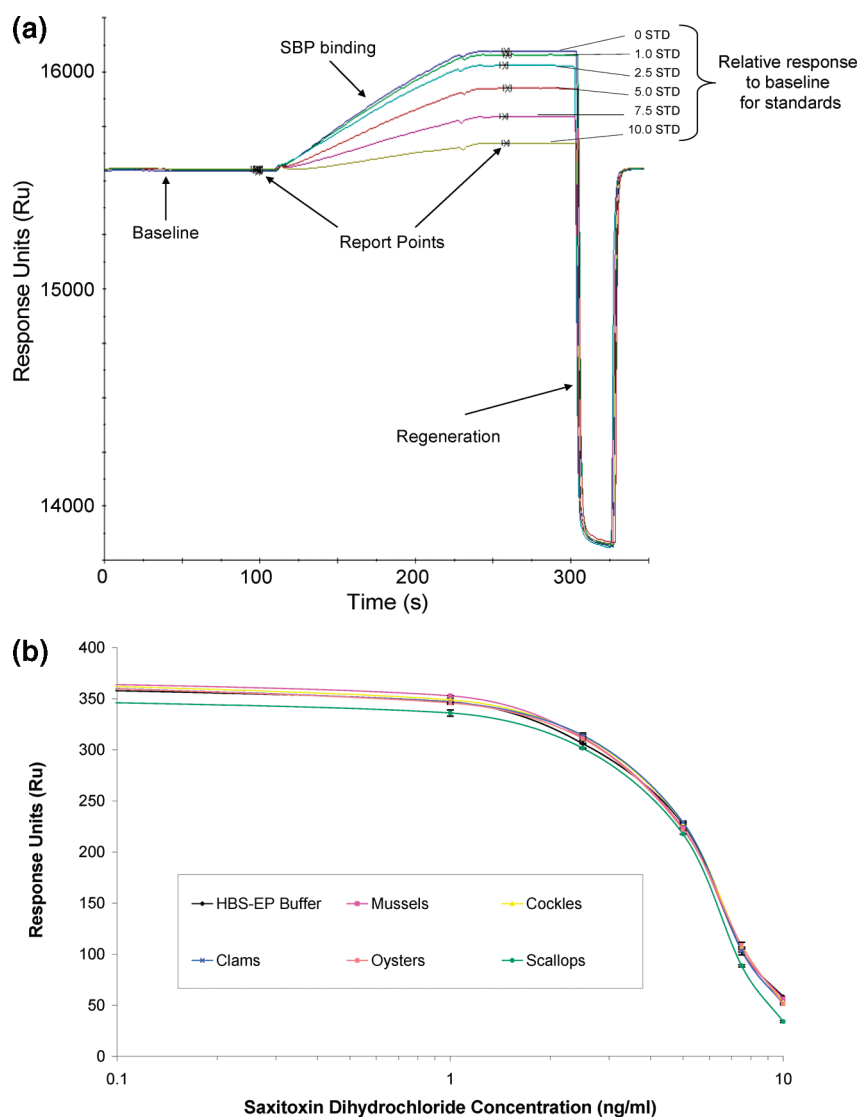


Figure 2. (a) Real time response curves for each STXdiHCl calibration standard. (b) STXdiHCl toxin concentration vs response calibration curves illustrating the comparison of the matrix effects for different shellfish species.

all toxins has not as yet been successful with the consensus opinion being that separate antibodies are required for hydroxylated and nonhydroxylated toxins. In addition, the fact that antibody responses do not directly correspond to the toxicity profile of the toxins is the main preventive factor in the acceptability of a wide screening immunological method for the detection of PSP toxins.

Limit of Detection (LOD). The limit of detection has been defined as the lowest concentration of an analyte in a sample that

can be detected but not necessarily quantified under the stated conditions of the test.²⁹ Different approaches have been used to determine the sensitivity of immunoassays with the preferred method being the IC_{50} determination which was used for determining the cross-reactivity. Usleber et al.³⁰ quotes that the absolute detection limit of an enzyme immunoassay standard curve is usually $1/2-1/4$ of the IC_{50} concentration (or 75–80% binding). This can also be applied to the present assay format in the form of the IC_{20} . This conservative approach is reported to provide a more realistic estimate than other calculations and reduces the frequency of false positive results. The IC_{20} values for all available toxins are reported in buffer and mussel extract (Table 2). From these values dcNEO and GTX1/4 with detection limits of 9.4 and 112 ng/mL corresponding to 1128 and 13450 $\mu\text{g/kg}$ in relation to their toxicity respectively that both may not be detected at the regulatory limit in the assay if they occur in isolation though there is little likelihood of this.

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Table 2. Cross-Reactivity in Buffer and Mussel Extract

PSP toxin	HBS-EP Buffer					Mussel Extract				
	PSP toxin concentration as saxitoxin equivalents (ng/mL)					PSP toxin concentration as saxitoxin equivalents (ng/mL)				
	dynamic range					dynamic range				
	relative toxicity factor	IC ₅₀	IC ₂₀ -IC ₈₀	% cross-reactivity	IC ₅₀	IC ₂₀ -IC ₈₀	% cross-reactivity	IC ₅₀	IC ₂₀ -IC ₈₀	% cross-reactivity
STXdiHCl	1.000	4.7	2.8-7.8	100	4.7	2.8-7.8	100	5.2	3.3-7.9	100
NEO	1.091	14.4	2.4-84	33	15.7	2.6-92	30	17.8	3.1-96	29
GTX 1/4	0.899	>722	182-2396	<0.7	>649	164-2155	<0.7	>475	112->1120	<1.1
GTX 2/3	0.601	12.1	4.2-35	39	7.2	2.5-21	65	12.1	2.5-22	72
dcSTX	0.745	3.3	1.6-6.5	142	2.4	1.2-4.9	196	3.1	1.2-4.3	226
dcNEO	0.701	80.4	23-266	6	56.4	16.0-187	8	>60.8	9.4->106	<12
dcGTX 2/3	0.398	37.0	7.5-163	13	14.7	64.7-134	32	15.1	2.8-69	34
C1/C2	0.075	23.0	5.3-98	20	1.7	0.4-7.4	277	2.4	0.5-11.1	217
GTX5	0.063	6.2	3.4-11.6	76	0.4	0.2-0.7	1175	0.4	0.2-0.8	1300
C3/C4	0.043	>68.8	ND ^a	<7	>3.0	ND ^a	<157	ND ^a	ND ^a	ND ^a

^a ND = not determined due to lack of toxin.

Analysis of Known Uncontaminated Mussels and Cockles for Interference Effects (Decision Limit CC_α). The effect on the assay of possible interfering compounds from known non-contaminated mussel and cockle tissue was examined. CC_α values of 0.72 and 0.47 ng/mL corresponding to 86.4 and 56.4 μg STXdiHCl per kg of tissue were obtained for mussel and cockle samples respectively. These concentrations represent the noise level of the assay and concentrations above the CC_α denote a 99% chance that PSP is present in the sample.

Applicability. The applicability of the method to different species of bivalve mollusc was assessed. Standards prepared in HBS-EP buffer and each shellfish extract were analyzed to obtain the relative response to the STXdiHCl concentration to produce calibration curves. Figure 2b illustrates these matrix curves in comparison to the HBS-EP buffer curve. Apart from scallops, which showed marginally lower signals all curves overlay the HBS-EP buffer curve.

Recoveries. The assay was not designed to distinguish between PSP toxins but rather indicate the presence or absence of toxins. Table 3a shows the % recovery of the assay when the mussel sample is spiked with half the regulatory limit and the regulatory limit of the available PSP toxins in STX equivalents. This % recovery of the assay is linked to the calibrant used, the cross-reactivity of the assay and the recovery of the analyte. The cross-reactivity profile for GTX1/4 relative to STXdiHCl meant this toxin could not be detected at these two levels. It was observed that the % recoveries of the assay tended to increase with lower contamination values due to different binding affinities of the antibody to each of the toxins relative to the STXdiHCl calibration curve.

The recoveries of STX, dcSTX and NEO from fortified cockle samples at levels of 400 and 800 μg/kg STXdiHCl equivalents are shown in Table 3b. The recoveries from cockles were found to be comparable to those obtained for mussels.

Detection Capability (CC_β). The detection capability (CC_β) was determined for STXdiHCl, NEO, dcSTX and C1/C2 toxins. These were selected as structural representatives from each toxin group. STXdiHCl was chosen as the reference molecule as STXdiHCl is the reference PSP toxin to which all other analogues are reported as STXdiHCl equivalents originating from the MBA toxicity factors. In addition, the SBP was produced against a STX protein conjugate and a STX surface was developed and characterized due to the availability of this toxin. The determination of cross-reactivities with other PSP toxins allowed the evaluation of the overall detection capabilities. CC_β is established in a practical manner from the analysis of known negative samples and these samples fortified at defined levels. Within the literature different constant factors of the standard deviation have been applied 1.64, 2, 2.33, and 3 to ensure different levels of certainty. A constant factor of 2.33 times the standard deviation representing 99% certainty was selected for the assay. The results of the analyses of the uncontaminated mussel and cockle samples (*n* = 30) was 0.0 ± 0.01 μg/kg. The results of the same mussel and cockle samples fortified at 200 μg/kg of STXdiHCl equivalents of STXdiHCl, NEO, dcSTX, and for C1/C2 as μg/kg are presented in Table 4. The variability of blank samples was much lower than that of the fortified samples at 200 μg STXdiHCl eqs/kg for each toxin analyzed and it was always possible to discriminate between

Table 3a. % Recovery of STXdiHCl, NEO, and dcSTX (all *n* = 30) and Other Toxins (*n* = 3) from Mussels

PSP toxin	relative toxin concentration at regulatory limit ($\mu\text{g/kg}$) in free base form related to STXdiHCl	% recovery of assay			
		half regulatory limit at 400 μg STXeqs/kg	regulatory limit at 800 μg STXeqs/kg	half regulatory limit at 400 μg PSP/kg	regulatory limit at 800 μg PSP/kg
STXdiHCl	800	98.6 $\pm$ 6.4	107.9 $\pm$ 8.1	98.6 $\pm$ 6.4	107.9 $\pm$ 8.1
NEO	733	81.6 $\pm$ 5.2	64.4 $\pm$ 3.0	89.0 $\pm$ 5.7	70.2 $\pm$ 3.3
dcSTX	1074	202.1 $\pm$ 21.0	143.1 $\pm$ 7.3	150.6 $\pm$ 15.6	106.6 $\pm$ 5.4
GTX 1/4	890	3.0 $\pm$ 0	2.0 $\pm$ 0	3.0 $\pm$ 0	2.0 $\pm$ 0
GTX 2/3	1332	120.9 $\pm$ 1.3	80.1 $\pm$ 0.4	72.7 $\pm$ 0.8	48.1 $\pm$ 0.2
dcNEO	1141	62.1 $\pm$ 3.9	ND ^a	43.5 $\pm$ 2.7	ND ^a
dcGTX 2/3	2011	93.6 $\pm$ 0.7	56.9 $\pm$ 0.4	37.3 $\pm$ 0.3	22.6 $\pm$ 0.2
C1/C2	10616	161.3 $\pm$ 0.6	ND ^a	12.2 $\pm$ 0	ND ^a
GTX 5	12655	419.1 $\pm$ 6.9	ND ^a	26.4 $\pm$ 0.3	ND ^a

^a ND = not determined due to lack of toxin.

Table 3b. % Recovery of STXdiHCl (*n* = 30), dcSTX (*n* = 10) and NEO (*n* = 10) from Cockles

PSP toxin	relative toxin concentration at regulatory limit ($\mu\text{g/kg}$) in free base form related to STXdiHCl	% recovery of assay			
		half regulatory limit at 400 μg STXeqs/kg	regulatory limit at 800 μg STXeqs/kg	half regulatory limit at 400 μg PSP/kg	regulatory limit at 800 μg PSP/kg
STXdiHCl	800	96.0 $\pm$ 7.1	97.0 $\pm$ 5.9	96.0 $\pm$ 7.1	97.0 $\pm$ 5.9
NEO	733	66.6 $\pm$ 5.6	50.0 $\pm$ 4.0	72.6 $\pm$ 6.1	54.6 $\pm$ 4.4
dcSTX	1074	170.1 $\pm$ 6.6	130.8 $\pm$ 1.1	126.7 $\pm$ 4.9	97.4 $\pm$ 0.8

Table 4. Repeatability and Reproducibility Data for Mussels and Cockles Spiked at a Quarter of the Regulatory Level, Half the Regulatory Level and at the Regulatory Level with STXdiHCl

Repeatability within Run (<i>n</i> = 10 Samples)				
sample type		200 $\mu\text{g/kg}$	400 $\mu\text{g/kg}$	800 $\mu\text{g/kg}$
mussel	mean	156	396	886
	SD	15	10	23
	%CV	9.6	2.5	2.6
cockle	mean	243	410	815
	SD	30	34	57
	%CV	12.3	8.3	7

Reproducibility between Runs (<i>n</i> = 3 Days)				
sample type		200 $\mu\text{g/kg}$	400 $\mu\text{g/kg}$	800 $\mu\text{g/kg}$
mussel	mean	191	394	863
	SD	29	26	65
	%CV	15.2	6.6	7.5
cockle	mean	228	384	776
	SD	24	29	47
	%CV	10.5	7.5	6.1

negative and spiked samples at this level of fortification. Hence $CC\beta$ was less than the level of fortification. The detection capability $CC\beta$ for STXdiHCl in mussels was set with a 99% certainty at 120 $\mu\text{g/kg}$. Therefore, in routine use, when the calculated concentration of an unknown sample is $<120 \mu\text{g/kg}$, this sample would be considered as compliant and when calculated to be $>120 \mu\text{g/kg}$, would be considered non-compliant. Due to the similarity in results observed for mussels the detection capability $CC\beta$ for STXdiHCl in cockles was also set at 120 $\mu\text{g/kg}$. If the cutoff level is set too low there is the greater risk of obtaining false non-compliant results. The mean STXdiHCl concentrations calculated from the

analyses of different shellfish species (mussels and cockles) fortified at the three levels were found not to be substantially different. It has thus been demonstrated that the protocol could be easily extended to cover all shellfish species with limited additional validation. If the method is applied as is to other shellfish species it is recommended to test one negative sample and one positive sample spiked with STXdiHCl at the fortified level of 200 $\mu\text{g/kg}$ as quality controls (QCs) to check that detection capability is unchanged and could be applied to all samples.

Threshold Levels for the Assay. Threshold levels are set in screening methods to reduce the number of samples requiring further quantification or confirmatory analysis. Where a regulatory limit is set a level is generally established that will ensure that false compliant results are avoided and false noncompliant minimized. However, for PSP toxins due to the variability in TEFs this level can become extremely difficult to establish for an antibody based test due to the cross-reactivity profile of the antibody in relation to the toxicity.

Due to the severity of PSP toxin poisoning large margins of safety are applied when setting thresholds. To ensure no false compliant samples pass to the food chain the threshold for the present assay could be set as low as the $CC\alpha$. However this threshold would require a number of samples to be reanalysed that contain PSP toxin but less than the regulatory limit. This is how the AOAC HPLC method is currently applied for screening for PSP toxins in the UK whereby any samples that are greater than 3 times the noise level are confirmed by MBA for their toxicity. In some countries where PSP toxins are not a recurring problem this eliminates the MBA completely. The problem with such a low threshold arises in areas where low level toxic episodes occur frequently. Samples below the regulatory level would be deemed as containing PSP toxins and shellfish farms closed until

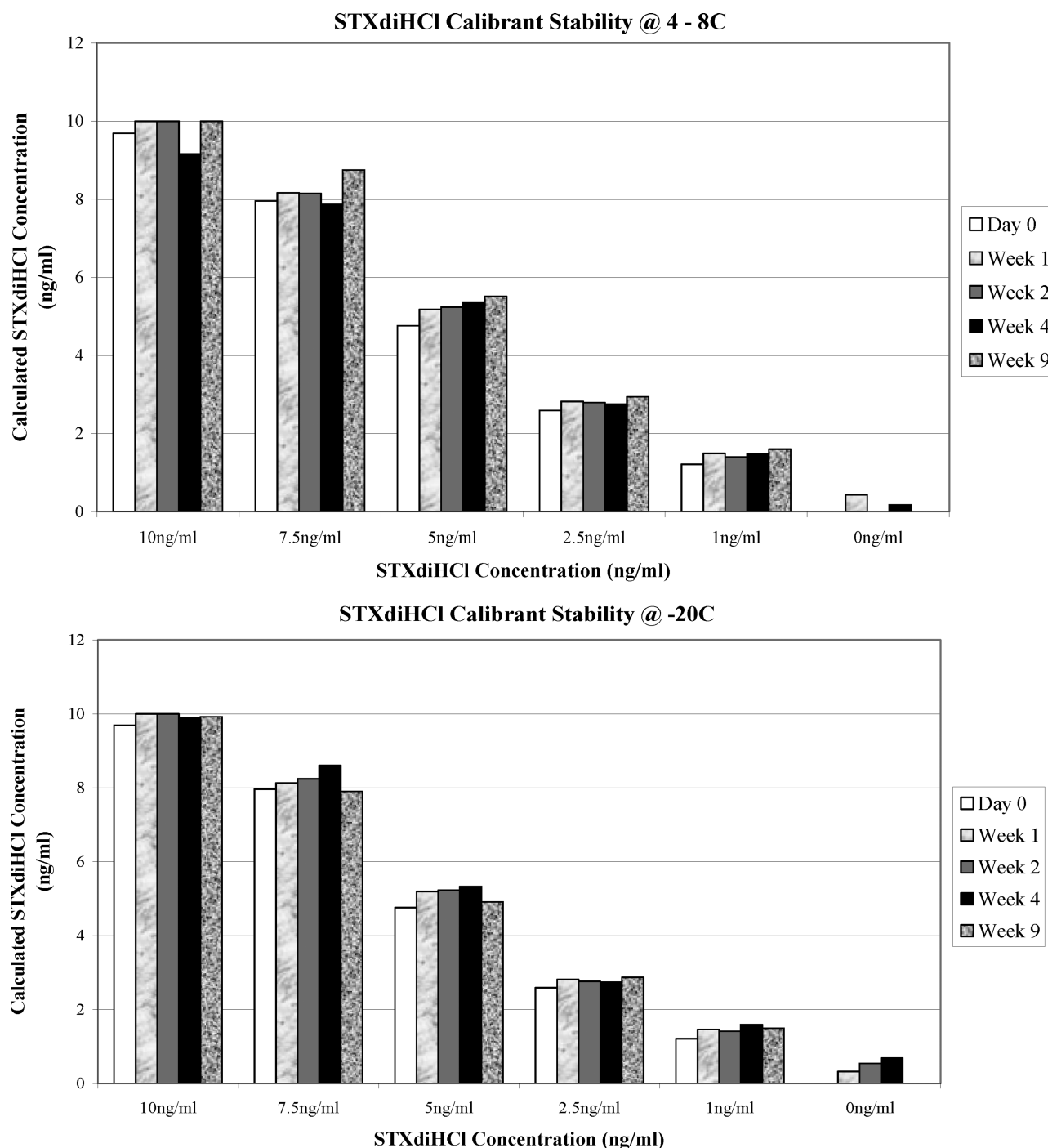


Figure 3. STXdiHCl calibrant stabilities at +4 and -20 °C over 9 weeks.

further analysis was performed. If low levels persist a screening method may be deemed unfit for purpose.

The production of an antibody or binder for the assay that correlated more effectively with the toxicity equivalent factors of the individual toxins would be a major advance with a threshold based close to the regulatory limit being applied without fear of releasing toxic material for consumption.

Precision (Repeatability and Reproducibility). The assay exhibited excellent repeatability and within-laboratory reproducibility for both mussels and cockles at various concentration levels (Table 4). The intra-assay (within day) variations (%CV) at 200, 400, and 800 μg STXdiHCl/kg for mussels were found

to be 9.6, 2.5, and 2.6%, respectively, whereas the interassay (between days) variations at these concentrations were 15.2, 6.6, and 7.5%, respectively. The intra-assay variations (%CV) at 200, 400, and 800 μg STXdiHCl/kg for cockles were found to be 12.3, 8.3, and 7.0%, respectively, whereas the interassay variations at these concentrations were 10.5, 7.5, and 6.1%, respectively.

Stability. During the nine week evaluation, the calibrants in HBS-EP buffer when evaluated with the STXdiHCl concentrations showed no substantial deterioration when stored at both temperatures (Figure 3). This is beneficial for the assay in reducing costs incurred from preparing fresh toxin calibrants

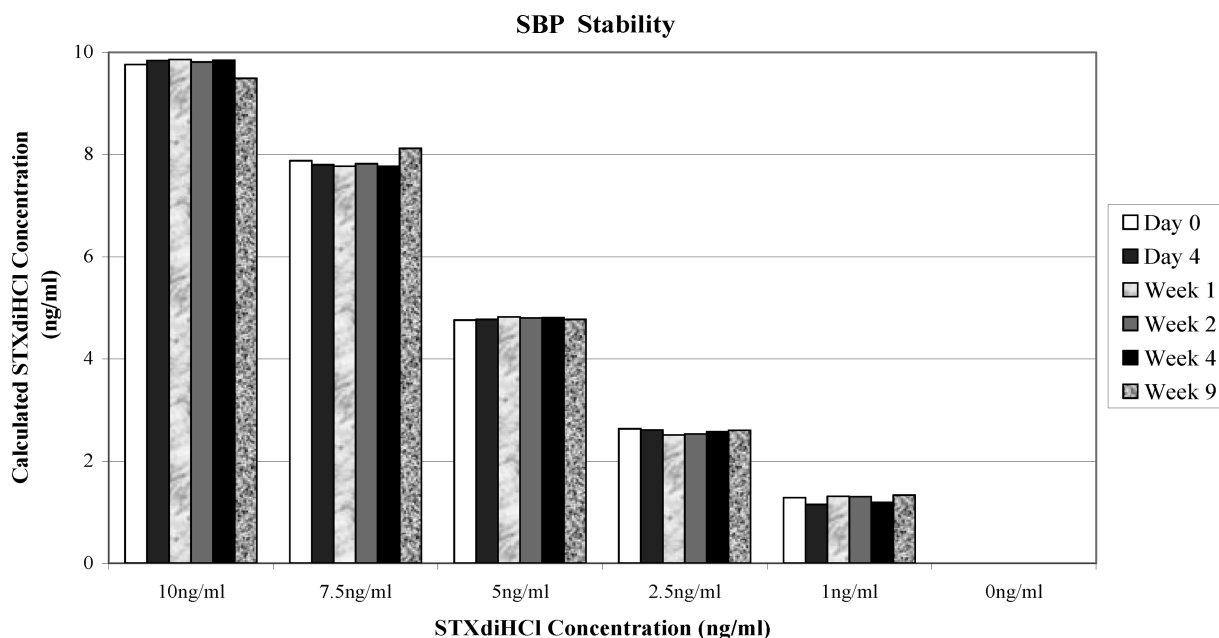


Figure 4. SBP Stability Study Over Nine Weeks.

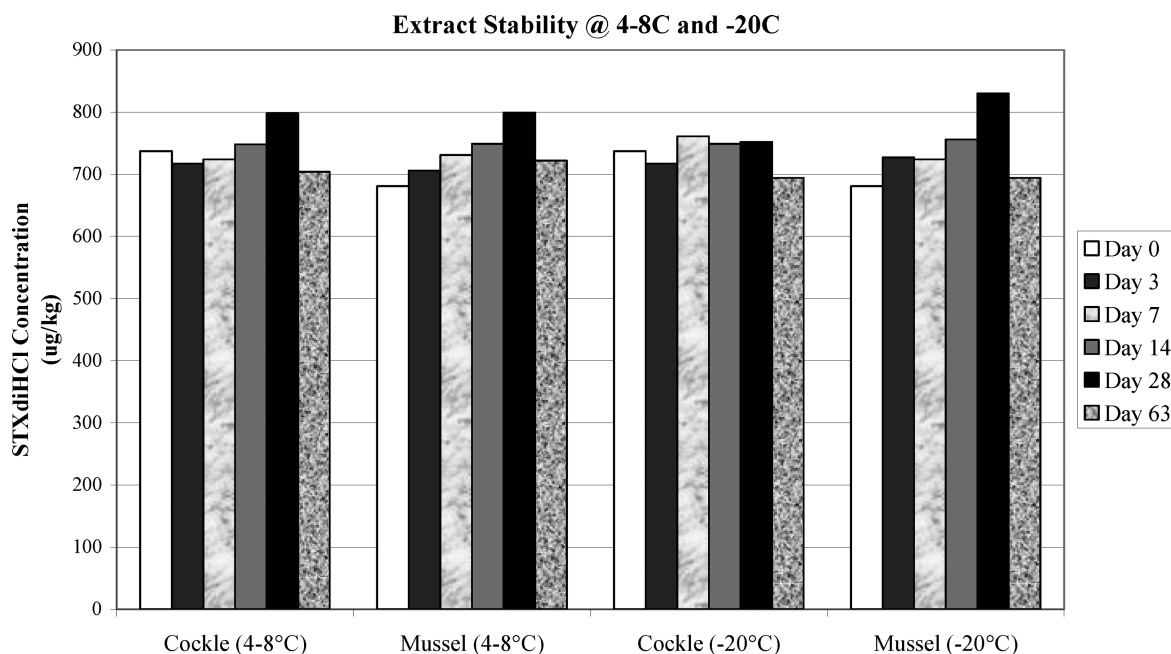


Figure 5. Mussel and cockle extract stability study at 4 and -20 °C for 9 weeks.

for every analysis. Figure 4 demonstrates that the stability of the SBP dilution (1/250) stored at 4–8 °C over this same time period is more than satisfactory when evaluated with varying STXdiHCl concentrations. At STX concentrations of 2.5–10.0 ng/mL the variability was <5% and 10% at the lower 1.0 ng/mL concentration compared to day 0. In addition, Figure 5 illustrates that the stability of the STXdiHCl at the regulatory limit in both mussel and cockle extracts (sodium acetate pH 5.0 buffer) is moderately stable when stored at both temperatures for screening purposes.

Analysis of Naturally Contaminated Samples. Tables 5a and 5b show the data for the PSP contaminated samples ($n = 25$) including mussels, cockles and scallops. The toxin compositions for each sample as identified by the AOAC HPLC method is

provided in Table 5a, and the comparative analysis by HPLC, MBA, and SPR is shown in Table 5b. Some of the MBA samples were not quantified but were determined as being above or below the regulatory limit. A 2×2 comparative analysis was performed for the real sample data between each of the detection methods based on whether the sample was determined as being above or below the regulatory limit (Table 6). For the sample set analyzed the correlation between the MBA and HPLC was 88%, between SPR and HPLC was 92%, and between SPR and MBA was 96%. The variation between analysts was also satisfactory in that at the regulatory limit there were no variations between analyst in determining the results above or below the regulatory limit. It is of major importance to the present study that the mussel samples P7, P15, P16, P17, and P18 contain a significant proportion of

Table 5a. PSP Toxin Profiles for Shellfish Samples Tested As Determined by AOAC HPLC method ($\mu\text{g}/\text{kg}$)^a

lab Number	STX	NEO	dcSTX	GTX1/4	GTX2/3	dcGTX2/3	GTX5	C1/C2	total PSP toxin
P1 - mussels	56	166	ND	ND	292	ND	ND	ND	514
P2 - mussels	88	150	ND	ND	203	ND	ND	ND	441
P3 - mussels	149	ND	ND	ND	754	ND	ND	361	1264
P4 - scallops	82	ND	ND	ND	54	ND	ND	ND	136
P5 - scallops	110	151	ND	761	249	ND	ND	ND	1271
P6 - scallops	209	142	ND	352	290	ND	ND	ND	993
P7 - mussels	197	277	ND	2774	841	ND	ND	491	4580
P8 - scallops	149	ND	ND	ND	86	ND	ND	ND	235
P9 - scallops	117	ND	ND	ND	142	ND	ND	ND	259
P10 - cockles	51	ND	ND	ND	ND	ND	ND	ND	51
P11 - scallops	106	ND	ND	ND	168	ND	ND	ND	274
P12 - scallops	126	ND	ND	ND	145	ND	ND	ND	271
P13 - scallops	130	ND	ND	ND	124	ND	ND	ND	254
P14 - scallops	63	ND	ND	ND	64	ND	ND	ND	127
P15 - mussels	166	128	ND	1487	476	ND	ND	858	3115
P16 - mussels	83	ND	ND	437	302	ND	ND	ND	822
P17 - mussels	134	96	ND	1871	1132	ND	ND	394	3627
P18 - mussels	179	112	ND	417	268	ND	ND	ND	976
P19 - cockles ^b	612	ND	1279	ND	136	350	9060	4370	15807
P20 - cockles ^b	875	ND	1260	719	145	352	9540	4940	17831
P21 - cockles ^b	645	ND	740	308	106	162	6010	1817	9788
P22 - cockles ^b	574	ND	1139	334	133	182	8490	940	11792
P23 - cockles ^b	771	ND	663	123	82	107	5600	740	8086
P24 - cockles ^b	526	ND	912	209	85	127	5800	350	8009
P25 - cockles ^b	551	ND	389	ND	41	44	3160	178	4365

^a ND, not detected. ^b Denotes C3/C4, dcNEO and GTX6 were also present but not quantified due to lack of standard material.

Table 5b. Comparison of Data for Each Method with Naturally Contaminated Samples (Values above the Regulatory Limit Are in Bold Text)

sample	HPLC analysis ($\mu\text{g}/\text{kg}$ of PSP toxins)	HPLC analysis (μg STXeq/kg) based on NRC TEFs	HPLC analysis (μg STXeq/kg) based on EFSA TEFs	MBA analysis (μg STXeq/kg)	biosensor analyst 1 ($\mu\text{g}/\text{kg}$)	biosensor analyst 2 ($\mu\text{g}/\text{kg}$)
P1 - mussels	514	412	415	480	653	570
P2 - mussels	441	373	379	370	486	310
P3 - mussels	1264	629	600	1090	871	1040
P4 - scallops	136	114	112	420	545	530
P5 - scallops	1271	1109	1113	650	572	550
P6 - scallops	993	854	856	640	837	860
P7 - mussels	4580	3536	3527	above	>1200	1100
P8 - scallops	235	201	197	below	585	460
P9 - scallops	259	203	197	below	541	420
P10 - cockles	51	51	51	below	125	90
P11 - scallops	274	206	200	below	582	450
P12 - scallops	271	213	207	below	490	420
P13 - scallops	254	204	199	below	569	500
P14 - scallops	127	101	99	below	473	400
P15 - mussels	3115	1993	1993	860	>1200	1140
P16 - mussels	822	657	645	440	609	540
P17 - mussels	3627	2630	2596	1150	>1200	1090
P18 - mussels	976	838	837	810	>1200	1060
P19 - cockles ^a	15807	2689	3945	2238	>1200	>1200
P20 - cockles ^a	17831	3664	4927	2710	>1200	>1200
P21 - cockles ^a	9788	2119	2869	1150	>1200	>1200
P22 - cockles ^a	11792	2483	3601	2150	>1200	>1200
P23 - cockles ^a	8086	1877	2553	990	>1200	>1200
P24 - cockles ^a	8009	1889	2745	1250	>1200	>1200
P25 - cockles ^a	4365	1096	1487	960	>1200	>1200

^a Denotes C3/C4, dcNEO and GTX6 were also present but not quantified due to lack of standard material.

GTX1/4 but in combination with other PSP toxins. Despite the problems highlighted with the detection of these toxins by the present assay they did not result in false compliant results being generated.

For the HPLC analysis the NRC toxicity equivalent factors were used to calculate the total amount of toxin in the sample as STXdiHCl equivalents. If the results were evaluated with

the new STX toxicity equivalent factors recommended by EFSA the most significant difference would occur with samples that contain dcSTX. Samples with dcSTX present would be quantified the same with MBA but higher using HPLC with the EFSA TEFs compared to the NRC TEFs. Due to the high cross-reactivity profile of this biosensor assay with dcSTX this would be complementary.

Table 6. 2 × 2 Comparative Analysis for Real Sample Data

		HPLC analysis		
		above regulatory limit	below regulatory limit	total
MBA	above regulatory limit	11	1	12
	below regulatory limit	2	11	13
	total	13	12	25
			overall agreement	88.0%
		HPLC analysis		
		above regulatory limit	below regulatory limit	total
SPR	above regulatory limit	12	1	13
	below regulatory limit	1	11	12
	total	13	12	25
			overall agreement	92.0%
		mouse bioassay		
		above regulatory limit	below regulatory limit	total
SPR	above regulatory limit	12	1	13
	below regulatory limit	0	12	12
	total	12	13	25
			overall agreement	96.0%

CONCLUSIONS

An assay was developed and rigorously validated for the semiquantitative screening of PSP toxins in shellfish: mussels and cockles. In relation to the TEFs low cross-reactivities (<0.7 and 8.3%) were observed for GTX 1/4 and dcNEO respectively. The detection capability $CC\beta$ for saxitoxin dihydrochloride was calculated to be 120 $\mu\text{g}/\text{kg}$ in mussels which would be in line with the recommended regulatory level of the EFSA opinion. Due to the dilution factor in the sample preparation and the biosensor parameters employed the assay has also the ability to be manipulated to achieve a lower detection capability if required.

Compared to the AOAC accredited methods for detecting PSP toxins, the sample preparation, once samples were homogenized, was extremely efficient (40 samples per hour) and the biosensor allowed rapid toxin detection (5–6 min) providing the capability of a high throughput assay format. It was also demonstrated that the protocol could be extended and applied to other shellfish matrixes such as cockles, clams, oysters, and scallops with reference to the calibration curve applicability study.

However, this would need to be confirmed with naturally contaminated clams and oysters as these were unavailable for this study. The SPR biosensor technology was demonstrated to be one of the most promising tools available for the development of a fast, sensitive, and repeatable screening method for PSP toxins in shellfish matrixes.

Due to the simplicity of the assay it could be used as a first action screen at a designated site on the shellfish farm or in monitoring laboratories for the presence of PSP toxins. If a sample

is evaluated to contain PSP toxins, the AOAC HPLC method could be then applied in tandem to determine and quantify the individual PSP toxin analogues present. In some countries, this biosensor assay could eliminate greater than 90% of samples from MBA analysis. In addition, due to the lack of requirement for trained personnel to perform the assay, the shellfish industry could use the assay to conduct their own risk management regime to make decisions relating to both harvesting and transportation of shellfish. This biosensor based methodology presents itself as a real alternative screening method to the current AOAC accredited methods.

SAFETY

Saxitoxin and its analogues are responsible for incidents of PSP. Therefore, when using PSP toxin standard solutions special care should be taken. Gloves and eye protection should be worn at all times. Appropriate disposal methods should also be utilized.

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