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Comparison of ELISA and SPR biosensor technology for the detection of paralytic shellfish poisoning toxins

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ABSTRACT

An enzyme labeled immunosorbent assay (ELISA) and surface plasmon resonance (SPR) biosensor assay for the detection of paralytic shellfish poisoning (PSP) toxins were developed and a comparative evaluation was performed. A polyclonal antibody (BC67) used in both assay formats was raised to saxitoxin–jeffamine–BSA in New Zealand white rabbits. Each assay format was designed as an inhibition assay. Shellfish samples ($n = 54$) were evaluated by each method using two simple rapid extraction procedures and compared to the AOAC high performance liquid chromatography (HPLC) and the mouse bioassay (MBA). The results of each assay format were comparable with the HPLC and MBA methods and demonstrate that an antibody with high sensitivity and broad specificity to PSP toxins can be applied to different immunological techniques. The method of choice will depend on the end-users needs. The reduced manual labor and simplicity of operation of the SPR biosensor compared to ELISA, ease of sample extraction and superior real time semi-quantitative analysis are key features that could make this technology applicable in a high-throughput monitoring unit.

1. Introduction

Paralytic shellfish poisoning (PSP) toxins are a group of greater than 20 potent neurotoxins found in both freshwater and marine environments (Fig. 1). Bivalve molluscs become contaminated with the toxins following harmful algal blooms when they filter feed and accrue dinoflagellates such as *Alexandrium tamarense*, *Alexandrium catenella*, *Alexandrium minutum*, *Pyrodinium bahamense* and *Gymnodinium catenatum* species that are all reported producers of these toxins [1–5]. As PSP toxins are potentially fatal in mammals following their consumption in contaminated shellfish, failure to monitor and detect safe levels of the toxins would have severe implications to public health and shellfish associated industries. Therefore, worldwide monitoring for PSP toxins in shellfish is performed with the current action limit set as 80 μg of saxitoxin equivalents/100 g of shellfish meat. The dominant method is the internationally accredited AOAC biological method 959.08 [6] derived from Sommer and Meyer, 1937 [7] with only the United Kingdom using the relatively new accredited AOAC Official HPLC Method 2005.06 [8] originating from the work of Lawrence [9–11] as an alternative first action screening tool. For ethical and performance reasons [12] researchers have been encouraged to develop alternative methods that implement the three Rs of reduce, replace and refine for animal testing. This approach ensures compliance with European legislation on animal protection (Council Directive 86/609/EEC) by moving away from animal experimentation to scientifically acceptable, non-animal procedures fully validated to an international standard. Biological methods, such as receptor-based assays [13–16], cytotoxicity tests or electrophysiological assays [17–20] and analytical and spectroscopic methods, such as HPLC [8,21,22], spectroscopy [23] and mass spectrometry [24,25], have been developed and reported to detect PSP toxins in shellfish tissue. However insufficient quantities of certified PSP toxin standard reference materials have seriously inhibited significant progress in the replacement of the MBA. The availability and cost of standards, limitations in some aspects of the method and the fact that the method has not been fully validated for all shellfish species and all toxin analogues has limited the uptake of the AOAC HPLC method into monitoring programs [26]. Hence, the MBA continues to be the reference method after 40 years of accredited usage and the scope for a non-animal detection system remains.

Immunology-based assays, first introduced by Berson and Yalow in 1959 [27], could be considered as an alternative screening tool for marine biotoxins. Since the initial development of enzyme immunoassays in the 1970s, competitive ELISAs in the 96-well microtiter plate format have progressed to being the most

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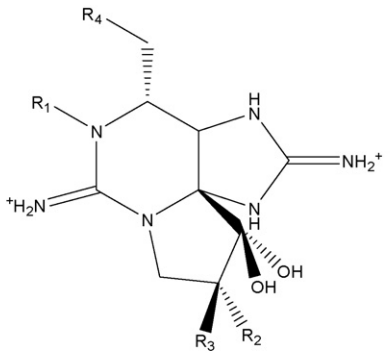

			Carbamate Toxins	*N*-Sulfocarbamoyl toxins	Decarbamoyl toxins	Deoxydecarbamoyl toxins
R_1	R_2	R_3	R_4: $OCONH_2$	R_4: $OCONHSO_3^-$	R_4: OH	R_4: H
H	H	H	STX	B1 (GTX 5)	dc-STX	do-STX
H	H	OSO_3^-	GTX 2	C1	dc-GTX 2	do-GTX 2
H	OSO_3^-	H	GTX 3	C2	dc-GTX 3	do-GTX 3
OH	H	H	NEO	B2 (GTX 6)	dc-NEO	
OH	H	OSO_3^-	GTX 1	C3	dc-GTX 1	
OH	OSO_3^-	H	GTX 4	C4	dc-GTX 4	

Fig. 1. Chemical structure of PSP toxin analogues.

recognized immunological technique in food and environmental analysis. The first reported antibody to saxitoxin was in 1964 [28] twenty years prior to the first publications for competitive immunoassays for detecting PSP toxins in shellfish. To date a number of direct and indirect ELISAs for detecting PSP toxins in shellfish have been reported [29–38] and reviewed [39]. The general consensus from these authors is that this type of assay format may be used for screening out up to 80% of samples from further analysis.

Optical biosensors based on surface plasmon resonance technology (SPR) are a dynamic tool for biomedical and pharmaceutical research. These biosensor-based assays measure the competition between the interactions of a specific biological recognition element with the target analyte (e.g., toxin) immobilized onto the sensor chip surface and in the sample. In the past decade researchers have demonstrated their potential for detecting and monitoring low level chemical contaminants and toxins in food produce to ensure food safety [40–43]. Previous research has outlined that SPR biosensors displayed a strong potential as an alternative strategy for monitoring PSP toxins [44,45].

The current study compares two immunological formats; conventional ELISA and optical SPR biosensor technology using a single antibody. The methods are compared with the AOAC accredited techniques for a range of naturally contaminated shellfish species.

2. Materials and methods

2.1. Materials, reagents and sample collection

ELISA kits for the analysis of saxitoxin were developed and provided for the study by Centre d'Economie Rurale, Santés Animale et Humaine (Ref. Code: E.F.3). The contents of each kit were: a sealed microtiter plate with 8 strips x 12 wells coated with purified sheep anti-rabbit IgG; saxitoxin dihydrochloride (STXdiH) standard solutions ranging in concentration from 0 to 0.2 ng/mL; saxitoxin peroxidase conjugate (×100 concentrated); lyophilized anti-saxitoxin antibody (BC67); dilution buffer pH7.4 (×10 concentrated); rinsing buffer (×10 concentrated); substrate/chromogen solution (peroxide/TMB) and stopping solution (6N sulfuric acid solution).

Saxitoxin dihydrochloride (STXdiH-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 standard reference standard material were obtained from the Institute for Marine Biosciences, National Research Council, Halifax, Canada (http://imb-ibm.nrc-cnrc.gc.ca/crmp/). CM5 certified grade chips, ethanolamine, HBS–EP buffer (pH 7.4, 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate) and an amine coupling kit was obtained from GE Healthcare, UK. Acetic acid, acetonitrile, ammonium formate (HPLC grade), ethanol, hydrochloric acid solution, hydrogen peroxide solution, Milli-Q water, periodic acid, sodium acetate, sodium hydroxide (NaOH) solution were purchased from Sigma–Aldrich (Dorset, UK).

Samples ($n = 54$) were collected from a number of regulatory laboratories in Europe to ensure that tissues containing variable PSP toxins profiles were included in the assessment. Homogenized shellfish samples: mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*), clams (*Veneridae* spp.), oysters (*Crassostrea Gigas*) and scallops (*Pecten maximus*) were supplied from the consecutive UK National Reference Laboratories including the Fisheries Research Centre (FRS), Scotland and the Agri-food and Biosciences Institute (AFBI), Belfast, United Kingdom and the Autonomous Government Laboratory for shellfish monitoring in Andalucía, Spain.

2.2. Antibody production

For the production of the polyclonal antibody (BC67), a New Zealand White rabbit was immunized with saxitoxin–jeffamine–bovine serum albumin protein conjugate. The chemical synthesis of the immunogen and the immunization process was previously described elsewhere [44] with the exception being that the harvesting of the antibody was performed 2 months following a fifth and final booster injection. The determination of the antibody titer and the assessment of sensitivity and specificity were performed by both ELISA and biosensor.

2.3. Shellfish extraction protocols for ELISA and SPR

Two different extraction protocols were employed and compared in the study. Due to improved sensitivity of the BC67 antibody, a modification of the Garthwaite extraction [33] procedure as described by Fonfría [45] was used to prepare extracts for PSP analysis from shellfish denoted as method 1. 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 3600 g for 10 min at room temperature. The supernatant was collected and the pellet was extracted as previously described with 3 mL of 90% ethanol. The supernatants were combined and diluted to 10 mL using 90% ethanol. For SPR analysis the supernatant was further diluted 1 in 25 in HBS–EP buffer (100 μL extract: 2400 μL buffer). For ELISA analysis the supernatant was tested following a 1 in 5 dilution (as instructed by the kit) and a further dilution of 1 in 200 in diluting buffer.

A second extraction procedure denoted as method 2 originally proposed by Bates [46] was employed. Samples (1 g) of homogenized shellfish tissue were weighed into centrifuge tubes and pH5 sodium acetate buffer (5 mL) was added. Each tube was vortexed for 10 s and rolled on a rotary shaker for 30 min. Following mixing, samples were centrifuged at 3000 g for 10 min at room temperature and the supernatant was collected. For SPR analysis the supernatants were further diluted 1 in 40 in HBS–EP buffer (100 μL extract: 3900 μL buffer). For ELISA analysis the supernatant was tested following a 1 in 10 dilution and a further dilution of 1 in 200 in diluting buffer. These dilutions were selected to be comparable with method 1.

2.4. ELISA methodology

The dilution buffer was prepared from the concentrate by diluting 1 part buffer to 9 parts distilled water. The saxitoxin peroxidase conjugate was diluted with 1 part conjugate to 99 parts dilution buffer. The lyophilized antibodies were reconstituted with 6 mL of dilution buffer producing a final antibody dilution of 1 in 32 000 from the neat sera. Micro titer wells coated with purified sheep IgG were used for the analysis of each control, standard and sample in duplicate. As a control for non-specific binding, dilution buffer (150 μL × 2) was pipetted into the appropriate wells. For the calibration curve each of the seven standards (50 μL), in duplicate, were pipetted into the appropriate wells. Each sample solution (50 μL), in duplicate was pipetted into the remaining wells. Diluted saxitoxin peroxidase conjugate (100 μL) was added to all the wells. Reconstituted saxitoxin antibody (100 μL) was added to all the wells except for the control non-specific binding wells. The micro titer plate was sealed, shaken for 1 min and incubated overnight at +4 °C. The rinsing buffer was prepared from the concentrate by diluting 1 part buffer to 9 parts distilled water. Following incubation, the micro titer wells were emptied, washed 5 times with 300 μL of rinsing buffer per well and dried by knocking on absorbent tissue. Peroxide/TMB was then added to each well (150 μL), the plate shaken and incubated for 30 minutes in the dark at room temperature. Stopping solution (50 μL) was then added to each well and the absorbance at 450 nm read within 30mins using a TECAN Safire.

2.4.1. ELISA specificity and sensitivity

For the ELISA the sensitivity and specificity of BC67 antibody for each PSP toxin over the concentration range of 0–200 ng/mL in diluting buffer were evaluated.

2.4.2. Detection limit, recovery and threshold limit

To determine the detection limit, recovery and a threshold level of the ELISA for each extraction method, known negative mussel samples were analyzed unfortified and fortified at 80 μg STXdiH/100 g of shellfish which is the current EU action limit for PSP toxins in shellfish. The threshold limit is the level that is established in a screening assay to ensure that no shellfish samples containing PSP toxins close to or at the action limit would be deemed compliant.

2.5. SPR methodology

A Biacore Q SPR biosensor system equipped with control and evaluation software purchased from Biacore AB, Uppsala, Sweden (GE Healthcare) was used in the study. The production of the CM5 saxitoxin chips was previously described elsewhere [44]. Analyses were performed using the Biacore Q with the parameters set to mix each antibody with an equal volume of each PSP toxin working standard prior to injection over the STX sensor chip surface. The BC67 antibody was diluted 1/100 in HBS–EP buffer. The flow rate across this chip surface was 12 μL/min and the contact time of the antibody-standard (sample) mix with the surface was 240 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 5 μL injections of hydrochloric acid (50 mM) at a flow rate of 12 μL/min. Standards and samples were analyzed in duplicate.

2.5.1. SPR assay sensitivity and specificity

The sensitivity and specificity of BC67 polyclonal antibody in relation to saxitoxin immobilized on the chip surface for each PSP over the concentration range of 0–10,000 ng/mL in HBS–EP buffer were evaluated.

2.5.2. Evaluation of shellfish matrix effects

PSP toxin free shellfish homogenate: Mussels (*Mytilus edulis*), cockles (*Cerastoderma edule*), clams (*Veneridae* spp.), oysters (*Crassostrea Gigas*) and scallops (*Pecten maximus*) for the evaluation of shellfish tissue matrix effects were obtained from the Agri-food and Biosciences Institute, Belfast. Each shellfish species was extracted using each of the extraction procedures described. Aliquots of HBS–EP buffer and the five different shellfish species extracts (6 × 6 × 1 mL) were spiked with STXdiH to provide calibration standards (0, 0.5, 1.0, 2.0, 3.0, 4.0 and/or 5.0 ng/mL) for each matrix curve. Each shellfish extract standard curve was compared to the HBS–EP standard curve by SPR.

2.5.3. Preparation of standards for SPR analysis

On comparison of the calibration curves prepared from extracts of the different shellfish matrices following extraction method 1 with those prepared in HBS–EP buffer and HBS–EP/ethanol, it was deemed appropriate to compare all the samples to a mussel extract curve for method 1. Known negative mussel tissue was extracted as described for method 1 and aliquots (1 mL) were spiked with STXdiH to provide 8 calibration standards (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ng/mL) for the calibration curve.

On comparison of the calibration curves prepared from extracts of the different shellfish matrices following extraction method 2 with those prepared in HBS–EP buffer, it was deemed suitable to compare all the samples to a HBS–EP calibration curve when using method 2. The STXdiH calibration standards (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 ng/mL) were prepared in HBS–EP buffer.

2.5.4. Detection limit, recovery of the assay and threshold limit

To determine the detection limit, recovery and a threshold level of the SPR assay for both extraction methods known negative mus-

Table 1
Sensitivity and specificity of ELISA Assay for each PSP toxin in buffer.

PSP toxin	ELISA		
	PSP toxin concentration (ng/mL)		
	IC_{50} (ng/mL)	% Cross-reactivity	Dynamic range IC_{20}–IC_{80} (ng/mL)
STXdiH	0.03	100	0.01–0.10
NEO	2.24	1.4	0.63–7.97
GTX 1/4	>100	<0.1	–
GTX 2/3	0.60	5.6	0.13–2.77
dcSTX	0.17	19.2	0.03–0.93
dcNEO	6.59	0.5	1.15–37.77
dcGTX 2/3	18.29	0.2	4.32–77.39
C1/C2	16.44	0.2	1.80–150.35
GTX 5	0.12	26.2	0.02–0.86
C3/C4	ND	ND	ND

sel samples were analyzed unfortified and fortified at 3 levels of 20, 40 and 80 μg STXdiH/100 g of shellfish against the corresponding standard curve.

2.6. Sample analysis and comparison

Samples ($n = 54$) including mussels, cockles, clams, scallops and oysters were analyzed, where sample size allowed, by SPR and ELISA, using the two different extraction procedures. For the ELISA two final dilutions of the extract supernatant for each method were investigated to illustrate different applications of this ELISA format.

For comparison, samples were also analyzed using the MBA AOAC method 959.08 [6] and HPLC AOAC official method 2005.06 [8,11] using a Supelcosil LC-18 reversed phase column (15 cm × 4.6 mm and 5 μm particle size) linked to an Agilent 1100 separations module, equipped with a mobile-phase degasser and a multi λ fluorescence detector.

3. Results

3.1. Sensitivity and specificity profile for ELISA and SPR

In this study the approach used to determine the sensitivity of competitive immunoassays, was the determination of the toxin concentration that resulted in 50% binding inhibition (IC_{50}) of the antibody to antigen. The % cross-reactivities relative to STXdiH for each toxin using the ELISA are displayed in Table 1. The cross-reactivity data demonstrates that this assay format is extremely sensitive (in the picogram per mL range) and highly specific for saxitoxin with marginal cross-reactivity to GTX 5, dcSTX and GTX2/3 and no significant cross-reactivity for all the other PSP toxins analyzed. However, with the exception of GTX1/4 the IC_{50}s for the remaining toxins range from 0.03 to 18.3 ng/mL and the IC_{20}s (20% inhibitory concentration) which is defined as the detection limit [39,47] ranged from 0.1 to 4.3 ng/mL for the remaining toxins.

For SPR analysis the % cross-reactivity in relation to STXdiH was more varied compared to the ELISA (Table 2) but with similar trends. Toxins with modifications in the R4 position (Fig. 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 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 <0.1% and 0.6% for ELISA and SPR respectively. Quantification by MBA is toxicity based therefore for comparative purposes Fig. 2 displays the SPR cross-reactivity profile for the PSP toxins as both ng/mL and ng/mL of STXdiH equivalents of toxin (which corrects the concentration for the relative toxicity of the analogue with respect to STXdiH). Where toxins were available only in combination, the higher toxicity factor was used (e.g., GTX2/3). On the plot of STXdiH equivalents it can be observed that those toxins whose curves fall to the left of STXdiH will overestimate in relation to the MBA if present in samples. This includes GTX5, dcSTX and C1/C2. In contrast, those toxin curves to the right of STX will underestimate in relation to the MBA. The most significant is GTX1/4. With the exception of GTX1/4 the IC_{50}s for the remaining toxins range from 1.5 to 45 ng/mL and the IC_{20}s ranged from 0.7 to 10.3 ng/mL for all remaining toxins. The SPR showed similar trends in specificity to the ELISA but the sensitivity of the ELISA was greater for all the toxins analyzed.

3.2. Comparison of matrix curves for each extraction method by SPR

The use of 90% ethanol as the extraction solvent resulted in observable differences between the shellfish matrix curves and the HBS–EP buffer curve. The use of a HBS–EP calibration curve with this extraction method would result in an approximate over-estimation of 25% of the toxin concentration in the sample. The addition of ethanol (4%) to the HBS–EP buffer, which is comparable to the extraction protocol, reduced the observed differences between the matrix curves and the modified buffer curve.

Using the sodium acetate extraction protocol, with the exception of scallop matrix, no observable differences were seen between the HBS–EP curve and the matrix curves. The scallop matrix curve produced a slight approximate over-estimation of the results of 5% when compared to a HBS–EP buffer curve. Following this evaluation it was deemed appropriate to compare all the samples to a mussel extract curve for the ethanol extraction method (Method 1) and to a HBS–EP curve for the sodium acetate extraction proce-

Table 2
Sensitivity and specificity of SPR Assay for each PSP toxin in buffer reported as individual toxin concentrations and STXdiH equivalents.

PSP toxin	Relative toxicity factor	Surface plasmon resonance					
		PSP toxin concentration (ng/mL)			PSP toxin concentration as STXdiH equivalents (ng/mL)		
		IC_{50} (ng/mL)	% Cross-reactivity	Dynamic range IC_{20}–IC_{80} (ng/mL)	IC_{50} (ng/mL)	% Cross-reactivity	Dynamic range IC_{20}–IC_{80} (ng/mL)
STXdiH	1.0000	1.8	100	0.9–3.3	1.8	100	0.9–3.3
NEO	1.0911	12.2	14.8	1.9–66.9	13.3	13.5	2.1–73.0
GTX 1/4	0.8993	323.8	0.6	89.7–1024.8	291.2	0.6	80.7–921.6
GTX 2/3	0.6005	4.6	39.1	1.7–12.8	2.7	66.7	1.0–7.7
dcSTX	0.7451	1.5	120.0	0.7–3.1	1.1	163.6	0.5–2.3
dcNEO	0.7013	43.2	4.2	10.3–160.7	30.3	5.9	7.2–112.7
dcGTX 2/3	0.3978	26.9	6.7	5.5–118.2	10.7	16.8	2.2–47.0
C1/C2	0.0754	8.9	20.2	2.2–37.7	0.7	257.1	0.2–2.8
GTX 5	0.0632	2.4	75.0	1.2–5.0	0.2	900	0.1–0.3
C3/C4	0.0430	>43.1	<4.2	ND	>1.9	<94.7	ND

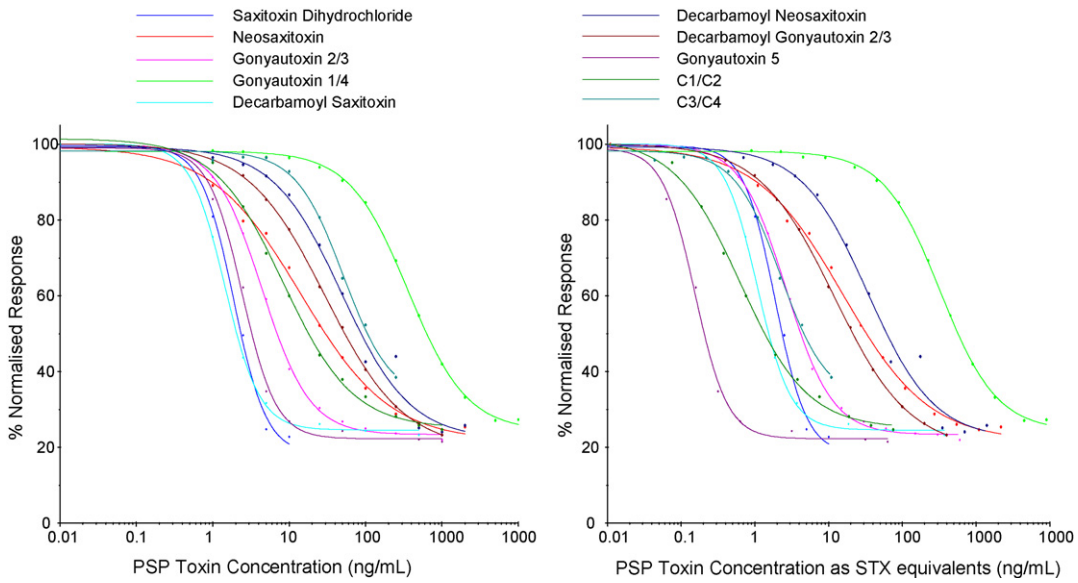

Fig. 2. SPR cross-reactivity profile for BC67.

dure (Method 2). Method 2 is advantageous in that known negative mussel tissue is not required for the SPR assay.

3.3. Limits of detection

The ELISA was evaluated at two dilutions of the supernatant for each extraction method. At the first dilution the assay is extremely sensitive in relation to the action limit of 80 μg STXdiH/100 g of shellfish and may be used to qualitatively determine if PSP is present in the shellfish at low levels of 1 μg STXdiH/100 g. The second further dilution of 1 in 200 allows for the ELISA to be semi-quantitative in relation to the other methods at the action limit.

Usleber et al. [39] reported that the absolute detection limit of an ELISA standard curve for toxin detection is usually 1/2–1/4 of the IC_{50} concentration (or 75–80% binding). This can also be applied to the SPR assay format and was denoted as the IC_{20}. Based on the STXdiH curve and the 90% ethanol extraction this would be set at as 8.0 and 22.5 μg/100 g and for the sodium acetate extraction protocol as 9.6 and 21.6 μg/100 g for ELISA (1 in 200 dilution) and SPR respectively. This method of estimating the limit of detection provides a more conservative value on which to discriminate between samples which may contain saxitoxin from samples that do not.

Alternatively the limits of detection can be determined in a more practical manner from the variability in known negative samples. The limit of detection can be expressed as the concentration value determined from the mean response value minus three standard deviations for known negative samples analyzed using each assay format. For the 90% ethanol the limit of detection for STXdiH was 9.0 μg/100 g and for the sodium acetate extraction protocol as 6.8 μg/100 g. The variability in response for the negative samples (Table 3) indicated that there was some interference to the assay due to matrix differences in the known negative mussel samples. Matrix effects were more observable in samples extracted with 90% ethanol and analyzed by ELISA with the negative mussel samples ranging from 0 to 36 μg/100 g. This was reduced when sodium acetate buffer was used as the extraction solvent.

3.4. Recovery of the assay format

Known PSP toxin free mussel samples were fortified with 80 μg STXdiH/100 g of tissue, extracted using each method and analyzed by ELISA and SPR to determine the recovery of each assay format (Table 3). The % recovery was higher when extraction method 2 was employed for both ELISA and SPR. The ethanol procedure provided a much lower recovery for STXdiH than the sodium acetate procedure and in terms of repeatability, the % CV for the sodium acetate protocol was lower. For the sodium acetate protocol by SPR analysis, the recovery at STXdiH levels of 20 and 40 μg/100 g was 90.8 and 85.0% respectively.

Table 3
Recovery of the assay with each extraction for ELISA and SPR.

	ELISA			
	90% Ethanol extraction		NaAc extraction	
	Negative (μg/100 g)	Positive @ 80 (μg/100 g)	Negative (μg/100 g)	Positive @ 80 (μg/100 g)
Average (n = 10)	12.0 ± 13.3	68.8 ± 11.6	0.3 ± 0.3	78.2 ± 8.8
% CV	N/A	16.8	N/A	11.3
% Recovery	N/A	86 ± 14.5	N/A	97.7 ± 11.0

	SPR					
	90% Ethanol Extraction		NaAc Extraction			
	Negative (μg/100 g)	Positive @ 80 (μg/100 g)	Negative (μg/100 g)	Positive @ 20 (μg/100 g)	Positive @ 40 (μg/100 g)	Positive @ 80 (μg/100 g)
Average (n = 10)	3.7 ± 2.3	54.9 ± 6.1	<0.24	18.1 ± 1.3	33.9 ± 2.3	77.4 ± 2.4
% CV	N/A	11.06	N/A	7.1	6.8	3.1
% Recovery	N/A	68.7 ± 7.6	N/A	90.8 ± 6.4	85.0 ± 5.8	96.8 ± 3.0

Table 4
PSP toxin profiles for shellfish samples tested as determined by HPLC with fluorescence detection using the pre-chromatographic oxidation method (μg/100 g).

Lab number	Sample type	STX	NEO	dcSTX	GTX1/4	GTX2/3	dcGTX2/3	GTX5	C1/C2	Total PSP toxin	STX equiv
1–10	Mussels	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	Oysters	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12–15	Clams	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16–23	Cockles	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24–29	Scallops	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
30	Mussels	5.6	16.6	ND	ND	29.2	ND	ND	ND	51.4	41.2
31	Mussels	8.8	15	ND	ND	20.3	ND	ND	ND	44.1	37.3
32	Mussels	14.9	ND	ND	ND	75.4	ND	ND	36.1	126.4	62.9
33	Scallops	8.2	ND	ND	ND	5.4	ND	ND	ND	13.6	11.4
34	Scallops	11	15.1	ND	76.1	24.9	ND	ND	ND	127.1	110.9
35	Scallops	20.9	14.2	ND	35.2	29	ND	ND	ND	99.3	85.4
36	Mussels	19.7	27.7	ND	277.4	84.1	ND	ND	49.1	458	353.6
37	Scallops	14.9	ND	ND	ND	8.6	ND	ND	ND	23.5	20.1
38	Scallops	11.7	ND	ND	ND	14.2	ND	ND	ND	25.9	20.3
39	Cockles	5.1	ND	ND	ND	ND	ND	ND	ND	5.1	5.1
40	Scallops	10.6	ND	ND	ND	16.8	ND	ND	ND	27.4	20.6
41	Scallops	12.6	ND	ND	ND	14.5	ND	ND	ND	27.1	21.3
42	Scallops	13	ND	ND	ND	12.4	ND	ND	ND	25.4	20.4
43	Scallops	6.3	ND	ND	ND	6.4	ND	ND	ND	12.7	10.1
44	Mussels	16.6	12.8	ND	148.7	47.6	ND	ND	85.8	311.5	199.3
45	Mussels	8.3	ND	ND	43.7	30.2	ND	ND	ND	82.2	65.7
46	Mussels	13.4	9.6	ND	187.1	113.2	ND	ND	39.4	362.7	263
47	Mussels	17.9	11.2	ND	41.7	26.8	ND	ND	ND	97.6	83.8
48	Cockles*	61.2	ND	127.9	ND	13.6	35	906	437	1580.7	268.9
49	Cockles*	87.5	ND	126	71.9	14.5	35.2	954	494	1783.1	366.4
50	Cockles*	64.5	ND	74	30.8	10.6	16.2	601	181.7	979.2	211.87
51	Cockles*	57.4	ND	113.9	33.4	13.3	18.2	849	94	1178.8	248.3
52	Cockles*	77.1	ND	66.3	12.3	8.2	10.7	560	74	808.6	187.7
53	Cockles*	52.6	ND	91.2	20.9	8.5	12.7	580	35	800.9	188.9
54	Cockles*	55.1	ND	38.9	ND	4.1	4.4	316	17.8	436.5	109.63

ND: not detected.
* C3/C4, dcNEO and GTX6 were also present but not quantified due to lack of standard material.

3.5. Threshold limit for each assay format

The threshold limit of the assay is the limit set that will provide a 95% certainty that all samples with levels determined above this limit will be non-compliant or contain PSP toxins above or close to the action limit of 80 μg/100 g of shellfish tissue. The threshold limit for a screening assay may be calculated as the mean concentration value determined from the fortified samples at the action limit (80 μg/100 g) minus three standard deviations of this mean value [40,48]. The subtraction of three standard deviations, based on the statistical three sigma rule, was performed to take into consideration any errors associated with the assay measurements, from weighing of the sample through to analysis, to ensure that no false compliant results would be reported. For each extraction procedure for ELISA (based on 1/200 dilution) and SPR, the threshold values were 34.0 and 36.6 μg STXdiH/100 g of shellfish for method 1 and 51.8 and 70.2 μg STXdiH/100 g of shellfish for method 2. Based on these threshold values for each extraction procedure the ethanol extraction displayed marginal scope if the action limit was to be lowered as some regulators propose [49].

3.6. Sample analysis

Analysis of PSP toxins in shellfish samples ($n = 54$) encompassing five species was performed using each of the four analytical procedures and for SPR and ELISA using the two different extraction procedures for a comparative evaluation. For some samples, MBA data was unavailable. The data generated corresponded well between procedures for most samples tested.

The ELISA was tested at two dilutions of the extract for each extraction method. At the first dilution when no PSP toxin was determined in the samples by HPLC and MBA, the optical densities obtained fell within the range of the standards equivalent to <1 μg STXdiH/100 g. However, when PSP toxin was present, even at the lowest concentrations, the optical densities obtained fell outside the standard range of the curve, indicating PSP toxin was present >1 μg STXdiH/100 g. Due to the sensitivity of this test, at this first dilution of the extract the test could distinguish between relatively non-toxic and toxic samples for those samples tested by each extraction procedure. As such this format has the potential to be used as a qualitative early warning tool. The second further dilution of extract of 1 in 200, allowed for by the high sensitivity of this assay format, was used to semi-quantify the amount of toxin present in the sample relative to the action limit of 80 μg STXdiH/100 g.

Mussels (18), cockles (15), clams (4), oysters (1) and scallops (15) were initially tested using HPLC. Concentrations for individual PSP toxin analogues were determined by HPLC to establish total PSP toxin concentrations. By multiplying each PSP toxin analogue concentration by its toxicity relative to STXdiH, the concentrations were standardized to STXdiH equivalent units. The STXdiH equivalence approach was adopted to allow for comparison between the HPLC and MBA results and those obtained using ELISA and SPR, which are based on binding and cross-reactivity to STXdiH.

Each toxin-contaminated sample had a distinct PSP toxin profile (Table 4). Total PSP toxin concentrations ranged from below detectable levels to 366.4 μg/100 g (STXdiH equivalent units) tissue using HPLC. Of the 54 samples tested and compared with each method (Table 5), 29 were found to be free of all PSP toxin analogues measured by HPLC and MBA. The negative samples were obtained from each of the shellfish classes including mussels (10), cockles (8), clams (4), oyster (1) and scallops (6). Generally, for non-PSP toxin containing samples obtained using HPLC and MBA, the corresponding ELISA and SPR results displayed undetectable or low levels of toxins. For these negative samples (1–29) the ethanol extraction displayed more non-specific binding of the antibody to sample matrix with both ELISA and SPR. By ELISA (1/200 dilution) three negative samples displayed levels greater than 20 μg

Table 5
Comparison of total STXdiH equivalent PSP toxin concentrations (μg/100 g) in shellfish samples as determined by HPLC, MBA (where data available), SPR and ELISA (further antibody dilution 1:200).

Lab number	Sample type	ELISA		SPR		HPLC	MBA
		Method 1 (90% ethanol)	Method 2 (NaAc)	Method 1 (90% ethanol)	Method 2 (NaAc)		
1	Mussels	2.9	0.4	ND	ND	ND	ND
2	Mussels	13.1	0.6	1.2	ND	ND	ND
3	Mussels	7.3	ND	2.1	6.0	ND	ND
4	Mussels	35.9	ND	ND	ND	ND	ND
5	Mussels	8.7	0.2	7.1	ND	ND	ND
6	Mussels	32.3	0.5	ND	ND	ND	ND
7	Mussels	20.0	ND	ND	ND	ND	ND
8	Mussels	ND	0.7	4.3	ND	ND	ND
9	Mussels	ND	0.4	ND	ND	ND	ND
10	Mussels	ND	0.2	ND	ND	ND	ND
11	Oysters	1.8	0.9	ND	ND	ND	ND
12	Clams	3.4	ND	ND	ND	ND	ND
13	Clams	5.5	0.2	ND	ND	ND	ND
14	Clams	3.8	0.3	ND	ND	ND	ND
15	Clams	2.8	ND	ND	ND	ND	ND
16	Cockles	3.2	0.1	ND	ND	ND	ND
17	Cockles	3.3	ND	ND	ND	ND	ND
18	Cockles	4.2	0.2	7.1	ND	ND	ND
19	Cockles	2.6	ND	1.2	ND	ND	ND
20	Cockles	4.5	0.5	ND	ND	ND	ND
21	Cockles	5.2	0.4	ND	ND	ND	ND
22	Cockles	4.3	0.3	ND	ND	ND	ND
23	Cockles	2.5	ND	ND	ND	ND	ND
24	Scallops	2.1	0.8	12.3	11.0	ND	ND
25	Scallops	0.6	ND	ND	ND	ND	ND
26	Scallops	4.9	0.2	ND	ND	ND	ND
27	Scallops	1.5	0.3	ND	ND	ND	ND
28	Scallops	2.0	0.2	ND	ND	ND	ND
29	Scallops	5.3	0.4	ND	ND	ND	ND
30	Mussels	26.7	28.5	31.0	54.9	41.2	48
31	Mussels	18.3	29.9	22.6	37.3	37.3	37
32	Mussels	42.3	62.7	53.4	80.0	62.9	109
33	Scallops	15.8	31.0	27.6	47.5	11.4	42
34	Scallops	17.9	32.9	30.5	41.3	110.9	65
35	Scallops	46.7	72.6	52.5	91.6	85.4	64
36	Mussels	101.2	124.9	83.1	106.6	353.6	–
37	Scallops	13.4	50.6	24.3	54.5	20.1	–
38	Scallops	10.0	33.9	21.1	41.5	20.3	–
39	Cockles	6.6	9.0	8.3	14.1	5.1	–
40	Scallops	8.8	40.5	27.7	49.3	20.6	–
41	Scallops	14.6	34.5	24.5	39.4	21.3	–
42	Scallops	15.5	50.9	27.3	45.9	20.4	–
43	Scallops	8.6	27.5	22.5	37.2	10.1	–
44	Mussels	73.0	137.4	81.8	122.0	199.3	86
45	Mussels	25.7	60.1	34.4	47.8	65.7	44
46	Mussels	60.2	170.3	69.4	111.6	263	115
47	Mussels	95.1	176.6	71.6	115.9	83.8	81
48	Cockles	227.6	>240	167.1	>120	268.9	223.8
49	Cockles	>200	>240	205.5	>120	366.4	271
50	Cockles	>200	>240	163.1	>120	211.87	115
51	Cockles	225.1	>240	165.5	>120	248.3	215
52	Cockles	190.4	>240	166.0	>120	187.7	99
53	Cockles	238.0	>240	187.9	>120	188.9	125
54	Cockles	101.1	>240	150.2	>120	109.6	96

(–): not quantified.

STXdiH/100 g. The extraction using sodium acetate buffer appeared to reduce non-specific binding to extracted matrix components.

Based on HPLC determinations, three samples had PSP toxin concentrations below 20 μg STXdiH/100 g (STXdiH equivalent units), six samples had PSP toxin concentrations between 20 and 40 μg STXdiH/100 g and three samples had concentrations ranging from 40 to 80 μg/100 g tissue. The remaining 13 samples had PSP toxin concentration levels exceeding regulatory guideline values (>80 μg STXdiH/100 g). Of the 25 samples with detectable levels of PSP toxins, twelve samples contained GTX 1/4 the toxin with the lowest cross-reactivity to the antibody. Two samples (No. 34 and 35) and 1 sample (No. 32) were determined as greater than and less than the action limit by HPLC compared to the MBA. This demonstrated that HPLC analysis can also result in over- and under-estimation of toxin levels in samples relative to the MBA, particularly if the PSP toxin levels are close to the regulatory limit.

In general, PSP toxin levels determined following the sodium acetate extraction procedure were higher compared to the ethanol extraction for both ELISA and SPR. When the ELISA method was employed nine and eleven samples were found to be greater than 80 μg STXdiH/100 g with the ethanol and sodium acetate extraction respectively. When compared to the MBA this method had three and one false negative by extraction method 1 and 2. Based on the samples tested there were no false positives. When the SPR method was employed nine and thirteen samples were found to be above 80 μg STXdiH/100 g with the ethanol and sodium acetate extraction

Table 6
Summary and comparison of the main characteristics for each method of detection for PSP toxins in shellfish employed in the study.

Main characteristics	Mouse bioassay (AOAC official method 959.08)	HPLC method (AOAC official method 2005.06)	SPR–BC67 antibody	ELISA–BC67 antibody
Spectrum of analysis possible	Mouse bioassay (MBA) method is the EU reference method	Method detects the PSP toxins well known over the last years (approximately 24). However the lack of standards to quantify all the toxins, poor recovery for certain toxins, and the presence of complex toxin profiles can lead to lower results when comparing to MBA	It can be used as screening method. Confirmation results would be by mouse bioassay Has the potential to be linked to mass spectrometry	It can be used as screening method. Confirmation results would be by mouse bioassay
Use of experimental animals	Use of 3 mice per sample	No animals used	No animals used	No animals used
Safety of method	Chemicals—low risk Extract—high risk	Chemicals—high risk Extract—low risk	Chemicals–low risk Extract–low risk	Chemicals–low risk Extract–low risk
Portability of analysis	Portability of analysis is not possible	Portability of analysis is not possible	Portability of analysis is not possible	Some scope for portability of analysis
Ease of training in method	It is difficult to train people in the method. Furthermore a special license is needed by personnel to work with animals	It is not easy to train people in the method. The person trained must have previous experience working with HPLC techniques. Personnel must be well organized and qualified to interpret complex toxin profiles	Training of personnel is relatively easy. No previous experience is required	Training of personnel is relatively easy
Ease of use	Method is tedious and long. Requires well-trained personnel to reduce the variability of results	Method is extremely laborious and long. Requires well-trained and very organized personnel. Evaluation of chromatograms and sample total toxicity calculations can be extremely complex in certain samples	Method is relatively easy to use and requires a small amount of hands-on time	Method is laborious, requires trained personnel and a significant amount of hands-on time
Benefits	The method is effective	The identification and quantification of some available PSP toxins is possible	SPR-based biosensor method is sensitive in the range of the current EC regulatory limits for PSP toxins. Improved simplicity and real time analysis The SPR-based biosensor method does not require animals, avoiding legal and ethical inconveniences. It can be used as screening assay, saving time, costs and animal lives	ELISA method is sensitive in the range of the current EC regulatory limits for PSP toxins. Improved simplicity and speed of the test This test does not require animals, avoiding legal and ethical inconveniences. It can be used as screening assay, saving time, costs and animal lives
Sensitivity	Mouse bioassay can detect 350 μg of STX equivalents/kg mollusc. Quantification of toxicity is calculated from time of death and mouse weight	HPLC method is applicable to identification and quantification but only of those PSP toxins for which standards are commercially available • STX >22 μg/kg; • GTX 2/3 together >125 μg/kg (lowest concentration tested); • GTX5 (B1) >27 μg/kg; • dcSTX >8 μg/kg; • NEO >40 μg/kg; • GTX 1/4 together >50 μg/kg; • C1/2 together >93 μg/kg; • C3/4 together >725 μg/kg	SPR-based biosensor method would be able to detect STXdiH equivalents at <10 μg/kg (This is a variable limit depending on the last dilution of the extraction method)	ELISA method would be able to detect STXdiH equivalents at <10 ng/kg (This is a variable limit depending on the last dilution of the extraction method)

Table 6 (*Continued*)

Main characteristics	Mouse bioassay (AOAC official method 959.08)	HPLC method (AOAC official method 2005.06)	SPR–BC67 antibody	ELISA–BC67 antibody
Specificity	Detection of all PSP toxins but there is interference from other toxic substances too. High false positive rate	Suitable for the analysis of • dcGTX2/3 (together), • C1/2 (together), • dcSTX, • GTX2/3 (together), • GTX5, • STX, • dcNEO, • NEO, • GTX1/4 (together), • GTX6 (through hydrolysis to NEO), • identification of C3/4 possible In the periodate oxidation step some peaks may co-elute from the column. When peaks are overlapping extra clean-up steps are required and long calculations are necessary to quantify these toxins individually	Good detection of all PSP toxins except some R1 hydroxylated analogues that have a lower cross-reactivity (neosaxitoxin, GTX 1/4 and decarbamoyl neosaxitoxin)	Good detection of all PSP toxins except some R1 hydroxylated analogues that have a lower cross-reactivity (neosaxitoxin, GTX 1/4 and decarbamoyl neosaxitoxin)
Sample preparation in terms of speed	Estimated time of sample preparation: 2 h	Sample preparation involves a double extraction with acetic acid and a SPE–C18 clean-up For a set of 10 samples the extraction step can last around 2 h For a set of 10 samples SPE–C18 clean-up is a long step SPE–COOH would be required for certain samples in which the Cs toxins, GTXs (GTXs and dc-can last 3 h (pH adjustment GTX2,3) toxins and STXs (STX, dc-STX, dc-NEO, NEO) toxins must be separated into 3 fractions. For a set of 10 samples SPE–COOH clean-up can last 2 h if the concentration step is not applied afterwards. Some laboratories can have automated equipments for SPE–C18 clean-up and hence save time	Sample preparation: 1 h	Sample preparation: 1 h
Speed of analysis	Total speed of analysis for a set of 10 samples when only one person is employed can last >12 h	Total speed of analysis (including extraction, clean-up, HPLC analysis and results evaluation) for a set of 10 samples, when only one person is employed on the analysis is approximately 2 days if the samples are negative or if quantified following periodate and peroxide oxidation for all toxins is 4–5 days if SPE–COOH clean-up and hydrolysis steps are required	Total speed of analysis (extraction, analysis and results evaluation for a set of 10 samples when only one person works on it can last approximately 5h	Total speed of analysis (extraction, analysis and results evaluation for a set of 10 samples when only one person works on it can last approximately 24h due to overnight incubation step

respectively. When compared to the MBA the SPR method had three false negatives by extraction method 1 with only one false positive by extraction method 2. In general, the sodium acetate extraction provides a greater recovery of PSP toxins and appears to reduce matrix effects in samples compared to the ethanol extraction.

For some samples the specificity profile and the toxicity factors of the toxins compensates for the low recovery of toxin using the ethanol extraction in comparison to the HPLC analysis in particular if GTX5 is present in the sample. Similarly, the improved recovery of the extraction procedure 2 could lead to an overestimation of toxin in comparison to HPLC when this toxin is present. For the ELISA and SPR method the underestimation of the toxin quantity with the ethanol extraction in comparison to HPLC was due to the samples containing predominately GTX1/4. This was improved with the increased recovery of the sodium acetate extraction especially for SPR.

4. Discussion

The main characteristics of all four procedures described in the present study are compared in Table 6. Each method utilized could potentially have a role to play depending on the level of testing required, whether it is for screening or confirmatory regulatory monitoring, end-product testing or as an early warning tool.

The main concerns from EFSA in 2009 and other regulatory authorities are that although antibodies are very sensitive, to date the cross-reactivity profile of antibodies for PSP toxins has not matched the toxicity factors of the toxins. STXdiH has a toxicity factor of 1.00 equating to an action limit of 80 μg/100 g. However, the 20+ different analogues diversely ranging in toxicity factor from 1.09 for neosaxitoxin to 0.04 for C3/C4 toxins equate to action limits of 73.4–2000 μg/100 g respectively. For quantitative correlation between the immunological method against either the MBA and/or HPLC method this can pose a problem of over and underestimation depending on the antibody specificity. The binder in this study has limited cross-reactivity to the hydroxylated toxins in particular GTX1/4. It should also be noted that the ELISA detected in the pg/mL range for STXdiH and that even a cross-reactivity of 0.2% provided a detection limit of approximately 4 ng/mL. Hence, at the first dilution of the extract for each extraction the ELISA could distinguish between samples containing and not containing PSP toxins at levels of 1 μg STXdiH/100 g. In countries where PSP toxin contamination is not ordinarily detected this method could effectively screen samples from further confirmatory analysis. However, if PSP toxin contamination is recurrent at low levels the number of false positive in relation to the action limit at this dilution could be severe but the further dilution of 1 in 200 would provide semi-quantification.

From the profiles observed in samples, individual PSP toxins do not appear to occur in isolation. Although PSP toxin profiles with only *N*-hydroxylated PSP toxin analogues in samples are unusual they may occur and, therefore, the method must be capable of detecting these samples. For either ELISA or SPR the use of threshold limits with the assay using either extraction procedure may compensate and ensure that no false negatives occur but would potentially cause a higher number of false positive results. This does not detract from the use of antibody-based methods as screening tests. Either screening method with the sodium acetate extraction procedure could be extremely effective in substantially reducing the number of samples requiring confirmatory analysis. This is especially true in countries such as Canada where samples are transported extensive distances at a high expense for regulatory testing.

Currently, HPLC and LC–MS methods for detecting PSP toxins require prolonged sample preparation in addition to highly trained personnel [49]. The AOAC HPLC method requires boiling of the sample, solid-phase extraction and oxidation of the sample extract prior to analysis compared to a simple buffer extraction followed by dilution used in this SPR method. Similarly, to achieve low limits of detection a 4 h freezing step is required for LC–MS sample preparation [50]. The faster throughput, real time monitoring SPR method which requires limited analytical expertise to perform the PSP analysis would also appear a faster option than the 24 h ELISA kit method. SPR-based testing does have greater start up costs and requires more antibody reagent than ELISA whereas SPR does not require toxin for an enzyme label and the chip surfaces are stable for greater than 2000 analysis based on on-going validation studies. The potential for SPR to be linked to mass spectrometry [51,52] has been demonstrated and in the future this could be utilized as the confirmatory tool. The differences in level of analysis required, sample turn around time, cost, manual labor time and availability of reagents could decide which immunoassay technique could be employed most effectively as the first action screening tool for PSP toxins.

5. Conclusion

A direct comparison of the immunoassay techniques demonstrated that the corner stone for each test was the quality of the antibody. The extraction in sodium acetate buffer was quicker and more efficient than using the 90% ethanol. The results for each immunological format generally resulted in data that correlated with the MBA and HPLC analysis. Using either immunological technique with designated threshold limits the use of animals in toxin testing world wide could be significantly reduced. In combination with the AOAC HPLC method or coupled with confirmatory MS techniques for identification and quantification for those toxins with existing standards immunological methods have the potential to eliminate the use of the MBA for PSP toxin analysis.

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