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Surface Plasmon Resonance Biosensor Screening Method for Paralytic Shellfish Poisoning Toxins: A Pilot Interlaboratory Study

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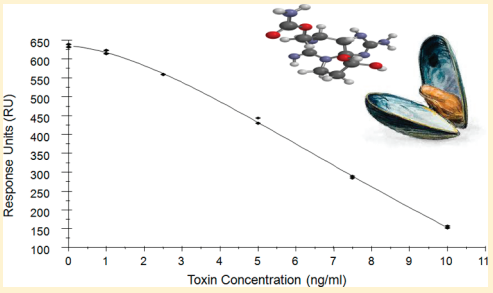
ABSTRACT: A surface plasmon resonance (SPR) optical biosensor method was developed for the detection of paralytic shellfish poisoning (PSP) toxins in shellfish. This application was transferred in the form of a prototype kit to seven laboratories using Biacore Q SPR optical biosensor instrumentation for interlaboratory evaluation. Each laboratory received 20 shellfish samples across a range of species including blind duplicates for analysis. The samples consisted of 4 noncontaminated samples spiked in duplicate with a low level of PSP toxins (240 μg STXdiHCl equivalents/kg), a high level of saxitoxin (825 μg STXdiHCl/kg), 2 noncontaminated, and 14 naturally contaminated samples. All 7 participating laboratories completed the study, and HorRat values obtained were <1 demonstrating that the method performance was acceptable. Mean recoveries expressed as STXdiHCl equivalents/kg were 94.6 ± 16.8% for the low level PSP toxin mix and 98.6 ± 5.6% for the high level of saxitoxin. Relative standard deviations for within-laboratory variations (RSD_r: repeatability) and between-laboratory variations (RSD_R = reproducibility) ranged from 1.8 to 9.6% and 2.9 to 18.3% respectively. This first ever reported SPR biosensor interlaboratory study demonstrated this PSP application to be an empowering tool in the drive toward the reduction and replacement of the mouse bioassay within Europe.



Paralytic shellfish poisoning (PSP) has been reported recurrently in various regions of the world,[1] and because of its severity, it is a life-threatening public health concern.[2] Incidences arise following the consumption of shellfish that have fed on toxic dinoflagellates of the *Alexandrium, Gymnodinium*, and *Pyrodinium* genera, which produce PSP toxins.[3−7] The parent toxin has been designated saxitoxin (STX) from which there have been reported greater than thirty derivatives.[8,9] The routine worldwide reference method of analysis to test shellfish for PSP toxicity is the mouse bioassay (MBA),[10] which is recognized as an official AOAC method.[11] However, test conditions, such as animal strain and sex, salt concentration, and sample treatment, considerably affect the test results.[12−15]

The European Food Safety Authority (EFSA) recently published a scientific opinion in relation to the PSP (saxitoxin-group) toxins,[16] which included the following statement: "In order for a 60 kg adult to avoid exceeding the acute reference dose, a 400 g portion of shellfish should not contain more than 30 μg of STX equivalents, that is, 75 μg of STX equivalents/kg shellfish meat". The limit of detection (LOD) for the MBA is 370 μg STX dihydrochloride (STXdiHCl) equivalents/kg shellfish tissue,[16] a level approximately just 2-fold less than the current European Union (EU) regulatory level of 800 μg STXdiHCl equivalents/kg shellfish tissue, at which point the harvesting and placing on the market of shellfish is prohibited.[17] The CODEX Alimentarius Commission which develop food standards and guidelines worldwide also implement this current EU regulatory level.[5,18] The increasing scientific interest on chronic long-term exposure of toxins as opposed to symptomatic acute toxic episodes may have added connotations. For example, if the EFSA opinion for PSP toxins were to lead to a significant reduction of the EU regulatory limit, then the MBA would be unable to detect the level required for monitoring and would be invalid. Additionally, differences in regulatory levels between two regulatory authorities would have further implications but are outside the scope of this article. Consequently, the technical difficulties (variability, nonspecificity and detection capability) and ethical concerns in using this MBA motivated a new wave of research for alternative methods over the last two decades.

The AOAC 2005.06 HPLC method[19] has been adopted as an alternative method by the EU but this method, as a screening tool, has a laborious sample preparation with two oxidation steps and requires highly trained personnel for the interpretation of results following analysis and conversion of the data using toxicity equivalency factors (TEFs) to correlate to the toxicity of the MBA. Lack of standard material, lengthy purification procedures, and insufficient detection capability has inhibited the progress in the development of other techniques such as mass

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spectrometry for routine monitoring. However, considering the high cost and the need for skilled scientists, physicochemical methods are more suitable for confirmatory analysis than screening methods. Nevertheless, during this time other methods of analysis have been developed but the most challenging requirement, stipulated by the EU regulations, to these alternative methods has been that intralaboratory and interlaboratory validation must be performed to an internationally agreed protocol prior to being recommended as an official method of analysis.

One technique that has risen to this challenge of in-house validation and transferability for interlaboratory validation has been optical surface plasmon resonance (SPR) analysis. SPR analysis has proven itself in both the pharmaceutical industry and food analysis for the determination of veterinary drug residues, antibiotics and toxins. Within the EU Sixth Framework Programme project "BioCop"[20] SPR analytical methodology for PSP toxins was developed[21] and evaluated using different shellfish matrices.[22] Further advances in the technology have demonstrated that the SPR method can be coupled to LC-MS for confirmatory analysis of toxins present.[23] Since 2007 the method has been modified with changes in binding protein to improve specificity to the whole PSP toxin family and the extraction procedure simplified to obtain an improved recovery of the toxins. Variations in the method during development have been compared to the AOAC HPLC method[24] and an ELISA developed using the same binder as in the SPR method.[25] The variations included a change of antibody and extraction solution which improved the specificity to more toxins within the PSP toxin family and improved the efficiency and recovery of the assay respectively. The intralaboratory validation, considering European Commission Decision 2002/657/EC[26] but also considering IUPAC and AOAC guidelines[27,28] for a finalized method[20] has recently been evaluated and published.[29] This evaluated SPR method can be considered a more refined technique with enhanced specificity, precision, and improved detection capability. Although the MBA has been reliable as an indicator of human oral toxicity, it cannot serve as a method for early warning because of its high limit of detection of approx 370 μg STXdiHCl equivalents/kg shellfish tissue. In contrast to the MBA the SPR limit of detection is currently fit for purpose at 120 μg STXdiHCl equivalents/kg shellfish tissue, and this method is therefore more capable of detecting the early stages of toxic algal blooms which is potentially useful in a monitoring and regulatory application. In addition, with minor modifications to the assay, the limit of detection can be lowered should the regulatory limit be lowered as a consequence of the EFSA-opinion.[16]

Because of the successful performance of the intralaboratory study, an interlaboratory assessment of the method was conducted. Seven international laboratories involved in either food analysis or marine biotoxin research participated in this first ever reported SPR biosensor interlaboratory study. The study involved the determination by SPR of the total PSP toxin content present in shellfish samples expressed as STXdiHCL equivalents. Participants received 20 individual samples (including blind duplicates) of shellfish homogenate, a prototype kit, study instructions and reporting sheets. The aim of this study was to obtain data about the transferability, accuracy, repeatability and between-laboratory reproducibility of this SPR biosensor method. A full description of the study and the outcomes of this assessment are described herein.

MATERIALS AND METHODS

Reagents for Calibration Curve and Spiked Samples. Saxitoxin dihydrochloride (STXdiHCl, 65 μM), neosaxitoxin (NEOSTXdiHCl, 65.6 μM), decarbamoyl gonyautoxin 2/3 (dcGTX2/3, 142 μM), and C1/C2 (149 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/crmp/).

Naturally Contaminated Samples. Shellfish samples, as frozen homogenates, were provided for the study to the University of Santiago de Compostela by the EU Community Reference Laboratory for Marine Biotoxins (Vigo, Spain), ANFACO (Vigo, Spain) and Queen's University (Belfast, U.K.). The determination of PSP toxin content was previously performed using the AOAC HPLC and mouse bioassay methods of analysis. The homogenates were thawed, mixed thoroughly and separated into aliquots of approximately 1.5 g and frozen for distribution.

The study samples included shellfish tissue from different European sites and contained a variety of PSP toxin patterns. The rationale for selecting different shellfish species and different locations was to assess the method with different matrices and different mixtures of toxins predominant in these regions.

Spiked Samples. The spiked samples were prepared from cockle and mussel homogenates that tested negative using the AOAC 2005.06 HPLC protocol[19] for PSP toxins against the current commercially available standards.

The negative cockle sample, denoted as J, was spiked with a mixture of four key toxins (STXdiHCl, dcGTX2/3, NEOSTXdiHCl and C1/C2) to be equivalent to half the EU regulatory level. A mixture containing 116 μL of STXdiHCl (65 μmol/L), 111 μL of NEOSTXdiHCl (65.6 μmol/L), 40 μL of C1/C2 (149 μmol/L), 55 μL of dcGTX2/3 (142 μmol/L), and 198 μL of deionized water was prepared. The cockle homogenate (26.5 g) was spiked with 500 μL of this mixture (each toxin at ~100 μg/kg and a combined total equal to 406 μg/kg). When Oshima's[30] toxic equivalency factors were applied the total mass fraction represents 240 μg STXdiHCl equivalents/kg.

The negative homogenized mussel sample (26 g), denoted as K, was spiked with 890 μL of STXdiHCl (65 μM) to be close to the action level of 800 μg STXdiHCl equivalents/kg. The exact total mass fraction represents 825 μg STXdiHCl equivalents/kg.

The homogenates for each spiked sample were mixed thoroughly and separated into aliquots of approximately 1.5 g and frozen for distribution. In addition, six 2 g aliquots of the spiked samples were saved for HPLC quantification of STX to confirm homogeneity. The determination was performed following the AOAC 2005.06 HPLC protocol[19] (repeatability c.v. = 9.2%) but scaled down for a reduced amount of shellfish homogenate. Homogeneity tests were performed according to ISO 13528:2005 on the prepared materials and materials showed to be sufficiently homogeneous (RSD_R (%) of 6 subsamples < 0.3 times predicted RSD_R (%)).

SPR Instrumentation. Biacore Q SPR optical biosensors with computer control software version 3.0.4 and BIA evaluation computer software version 4.1 obtained from Biacore AB (GE Healthcare, Uppsala, Sweden) were used in the study.

PSP Toxin Prototype Kit. The PSP toxin prototype kit contained a carboxymethyl dextran (CM5) sensor chip with saxitoxin immobilized, saxitoxin binding protein (SBP), HBS-EP buffer pH7.4, Greiner round bottomed 96 well microtiter plates, vials

and instructions for performing the assay as previously described elsewhere.[29] The instructions included the preparation of STXdiHCl calibration standards, the extraction procedure in shellfish and the operating and setup instructions for PSP toxin assay on the Biacore Q instrument.

In brief, 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.

Shellfish samples (1 g of homogenate) were weighed into centrifuge tubes and 0.2 M 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 3600*g* 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).

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 μL/min for 120s was employed. Report points were taken before (10 s) and after (30 s) each injection 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 μL/min. Calibrants and samples were analyzed in duplicate.

INTERLABORATORY STUDY

The design and planning of the interlaboratory study was divided over three institutes: Queen's University prepared and distributed the PSP toxin prototype kits; the University of Santiago de Compostela prepared and distributed the samples; RIKILT acted as study director (design of study, collection and processing of results, statistical evaluation). All materials were distributed in the same week with a four week turn around requested for the reporting of the results.

Seven laboratories in seven different countries (within the EU and North America) with a Biacore Q SPR instrument previously installed commenced the study. Each participant purchased saxitoxin dihydrochloride (STXdiHCl-65 μM) from the NRC in Canada for the preparation of calibrants for the assay. The PSP toxin prototype kits were distributed by courier service at room temperature, with participants being requested to store the kit at +4−8 °C on receipt. In addition, the computer operating software (commonly referred .blw file) specific for the Biacore Q PSP assay was distributed via electronic mail to all participants. Shellfish samples (20 × 1.5 g) were labeled 1−20 and distributed to each participant frozen by courier service. Participants were requested to store the samples at −20 °C immediately upon receipt until defrosting just before the analysis whereby the samples should be thawed and thoroughly mixed prior to weighing.

The participants were instructed to weigh 1 g amounts of each of the 20 samples for analysis and follow the instructions provided in the prototype kit for analysis of PSP toxins by SPR. The condition and weight of the sample were recorded. Following analysis the result for each sample, the midpoint (IC_{50}) and dynamic range (IC_{20} to IC_{80}) of the calibration curve were reported using the reporting sheets. These parameters were investigated to determine the reproducibility of the calibration curve between laboratories.

STATISTICAL EVALUATION

Results were initially reviewed and only valid data were included in the statistical evaluation. Statistical evaluation of the results was carried out following the approach described in Collaborative Study Guidelines of AOAC INTERNATIONAL.[31] The use of blind duplicates facilitated the use of the Cochran test to identify laboratories showing significantly greater variability among replicates (within day) when compared to the other participants (1-tail test at a probability value of 2.5%). The Grubbs test that identifies laboratories with extreme averages, the single value test (2-tail, P = 2.5%) followed by a paired value test (P = 2.5%) were performed.

To facilitate comparison of the variability for different test materials included in the study, the relative standard deviation (RSD) under repeatability (RSD_r) and reproducibility (RSD_R) conditions were calculated. All laboratories performed duplicate analyses. In addition the HorRat value was calculated as the ratio of the RSD_R (%) to the predicted RSD_R (%).[32]

RESULTS AND DISCUSSION

Spiked and Naturally Contaminated Samples. The results of the homogeneity evaluation by HPLC analysis show that for both spiked materials toxin distribution was homogeneous. For the six 2 g aliquots of cockle homogenate, spiked with STXdiHCl, Neo-STXdiHCl, dcGTX2/3 and C1/C2, the average amount of STXdiHCl (only determined) was 62.7 ± 3.3 μg/kg with a coefficient of variation of 5.2%. For the six 2 g aliquots of mussel homogenate, spiked with STXdiHCl, the average amount of STXdiHCl determined was 537.3 ± 27.4 μg/kg with a coefficient of variation of 5.1%.

Interlaboratory Study. In relation to the study design, the prototype kit and all samples were received in good condition within a week after dispatch. All seven participants completed the interlaboratory study. However, out of the 7 participants, 6 returned the results on time using the reporting sheet provided. One participant reported that there were problems with the analysis and thus had to submit results after these problems were resolved.

The results reported were initially reviewed to remove possible nonvalid data. The determination of the mean value for all samples was carried out by the consensus from participants (mean of means) after checking for nonvalid data. No nonvalid data were submitted by participants. The values participants obtained for each sample (including the blind duplicates indicated alphabetically) are presented in Table 1. The dynamic ranges IC_{20}−IC_{80} reported are presented, in addition to values recalculated at Queen's University from the raw data files for standardization because participants originally used different methods for calculating these IC_{20}−IC_{80} values. This reported range varied from 258−864 μg STXdiHCl/kg to 478−1896 μg STXdiHCl/kg. The standardization of the calculation for the dynamic range resulted in dynamic ranges that were more consistent between participants. Based on this it was recommended by participants that the kit instructions should include details on how to calculate these parameters from calibration curves. The standardized average IC_{20}, IC_{50}, and IC_{80} values from all 7 laboratories were 327 ± 38, 621 ± 62, and

Table 1. Values of Toxin Contents (μg STXdiHCl Equivalents/kg Shellfish) Found in Individual Samples as Used for Calculations

		concentration of PSP toxin detected at each laboratory (μg STXdiHCl equivalents/kg shellfish)							
sample coding for participants	sample identification for paired samples and reporting	Lab 01	Lab 02	Lab 03	Lab 04	Lab 05	Lab 06	Lab 07	mean of means
4	A	472.6	484.5	475.5	427.0	539.5	432.0	511.5	**481.6**
10	A	425.7	478.5	460.0	426.5	535.0	400.5	673.5	
6	B	1142.5	1165.0	1095.0	1200.0	1200.0	1107.0	1200.0	**1158.5**
9	C	low	41.5	<1.2	105.7	98.0	<1.2	<1.2	**< 117**
17	C	low	57.0	<1.2	89.9	135.5	<1.2	<1.2	
3	D	1186.2	>1200	1100.0	>1200	>1200	1120.2	>1200	**> 1100**
12	E	1231.5	>1190	1200.0	>1200	>1200	>1200	>1200	**> 1190**
18	E	1240.0	>1190	>1200	>1200	>1200	>1200	>1200	
15	F	1165.2	1170.0	1130.0	1200.0	1200.0	1172.4	1200.0	**1174.3**
19	F	1088.2	1180.0	1150.0	1200.0	1200.0	1185.0	1200.0	
7	G	962.8	995.0	966.5	1090.0	1170.0	943.5	1065.0	**1028.1**
16	G	1014.6	994.5	943.0	1055.0	1170.0	943.5	1080.0	
2	H	576.1	582.5	568.5	565.5	687.5	530.5	646.5	**600.0**
20	H	600.3	608.0	576.5	584.5	703.5	528.0	642.0	
11	I	1080.6	1095.0	1080.0	1200.0	1200.0	1042.2	1170.0	**1117.4**
14	I	1005.9	1100.0	1080.0	1200.0	1200.0	1039.8	1150.0	
1	J (spike)	263.5	222.5	243.0	208.0	266.5	165.5	211.0	**227.1**
13	J (spike)	225.5	217.0	233.5	207.0	305.5	151.0	260.0	
5	K (spike)	786.4	791.5	833.0	826.5	847.5	744.5	842.0	**813.6**
8	K (spike)	804.6	813.0	810.5	817.0	846.5	719.0	908.0	
dynamic range (IC_{20}–IC_{80})		292–1040	456–936	258–864	720–1080	1308–486	1896–478	433–1262	
recalculated at QUB		303–895	326–933	289–878	282–875	385–1016	345–923	359–984	
IC_{50}		588	621	563	553	722	624	680	

Table 2. Recovery for Individual Spiked Samples (%)

	% recovery at each laboratory						
sample identification	Lab 01	Lab 02	Lab 03	Lab 04	Lab 05	Lab 06	Lab 07
J (spike)	109.8	92.7	101.3	86.7	111.0	69.0	87.9
J (spike)	93.9	90.4	97.3	86.3	127.3	62.9	108.3
K (spike)	95.3	95.9	101.0	100.2	102.7	90.2	102.1
K (spike)	97.5	98.5	98.2	99.0	102.6	87.2	110.1

929 ± 54 μg STXdiHCl/kg respectively, demonstrating that the reproducibility of the calibration curves between laboratories is satisfactory.

Recovery for Spiked Samples. Recovery values for the samples J and K were calculated for both spiked samples (Table 2). Sample J contained the different toxins STX, NeoSTX, dcGTX2/3 and C1/C2 (representing the 4 main structural and toxic variations in PSP toxins). Sample K only contained STX. The recoveries obtained for sample K indicate that the method performs well when only STX is quantified against the calibration solution (STX). The recoveries obtained for sample J indicate that with these different toxin groups and the variations in % cross reactivity for these different toxins,[29] the total mass fraction expressed as STXdiHCl equivalents/kg is close to the theoretical mass fraction.

STATISTICAL EVALUATION OF RESULTS

In relation to the statistical calculations performed on the results: only the levels for which 4 or more out of the 7 participants reported an actual value (not "higher than" or "lower than") were included for statistical evaluation. This means that for samples C, D and E no outlier tests were done and no HorRats were calculated. In the case of samples F and I (where some participants provided a result >1200 μg STXdiHCl equivalents/kg), the value of 1200 μg STXdiHCl equivalents/kg was used for indicative calculations of method performance. It should be stated that analysts were not requested to further dilute the samples greater than >1200 μg STXdiHCl equivalents/kg and reanalyse to obtain a figure. The assay was designed as a single analysis screening test and samples with levels greater than the top standard of the curve would be deemed positive for PSP toxins in relation to the cross-reactivity profile of the antibody.

Outlier Tests. The Cochran (one-tail test at $P = 2.5\%$) showed that lab 07 was detected to be an outlier for sample A, but this sample was not excluded from calculations because no technical explanation could be identified. For the blind duplicate samples (A, F, G, H, I, J, K) the Grubbs test ($P = 2.5\%$) showed that no outliers were detected.

Observations. Initially one of the blind duplicate values obtained for the spiked cockle sample J for lab no 06 was reported to be <1.2 μg STXdiHCl equivalents/kg. The reading

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Table 3. Predicted RSD_R, Overall Reproducibility (RSD_R), and Repeatability (RSD_r) in % and HorRat Values for Each Sample Level

sample identification	predicted RSD_R %	RSD_R (%)	RSD_r (%)	HorRat
A	17.9	14.8	9.6	0.83
B [a]	15.6	*3.9*		*0.25*
C				
D				
E				
F[a]	15.6	*2.9*	*1.8*	*0.19*
G	15.9	8	1.8	0.50
H	17.3	9.1	2	0.53
I[a]	15.7	*6.3*	*1.9*	*0.40*
J (spike)	20.0	18.3	8.9	0.92
K (spike)	16.5	5.8	2.6	0.35

[a] RSD_R and RSD_r partially included values of "1200" instead of the reported >1200 value

for this individual result equates to the lowest end of the calibration curve and could not be calculated. The PSP content in the blind duplicate sample could be quantified but was also low. The reason why the recovery for this sample in this laboratory was low was not clear at that time but further tests had been undertaken by the participant. Lab 06 repeated the analysis for this sample and obtained better results and these new values were included in the tables and used for calculation of the HorRat values.[32]

The predicted relative standard deviations for reproducibility between laboratories (RSD_R) for samples A, B, F, G, H, and I (Table 3) were calculated based on the average value for each sample obtained by participants (Table 1). The predicted RSD_R for the spiked samples was based on the theoretical mass fraction in μg STXdiHCl equivalents/kg.

With the exception of samples B and D, all samples were provided as blind duplicates, therefore an indication of the repeatability in % (RSD_r) for each individual participant per level was calculated (Table 4). Lab 07 obtained a high value for repeatability for sample A. The high variance could not be explained by the participant and showed to be an outlier (extreme value for the within laboratory variances) with the Cochran test. Since there is no technical explanation for this high variance for A and because the variance for the other levels from this laboratory were within a normal range, the laboratory results for A were not excluded from statistical calculations.

Overall repeatability (RSD_r, %) and reproducibility (RSD_R, %) and HorRat values were calculated for each level (Table 3). It was shown that in all cases the RSD_r is very low. The method explicitly includes the duplicate measurement in the SPR of every single extract. This duplicate measurement of each extract may have an influence on the RSD_r and RSD_R as the detection of outliers and statistical treatment were done based on the mean of these two measurements. The HorRat values obtained in this study all are <1 for the samples tested using this SPR method by the seven participating laboratories. The method performance, therefore, in terms of interlaboratory precision can be considered as acceptable.

Table 4. Repeatability in % (RSD_r) for the Blind Duplicate Samples as Obtained by Individual Participants

	% (RSD_r)						
sample identification	lab 01	lab 02	lab 03	lab 04	lab 05	lab 06	lab 07
A	7.4	0.9	2.3	0.1	0.6	5.4	19.3
B							
C							
D							
E							
F	4.8	0.6	1.2			0.8	
G	3.7	0.0	1.7	2.3	0.0	0.0	1.0
H	2.9	3.0	1.0	2.3	1.6	0.3	0.5
I	5.1	0.3	0.0			0.2	1.2
J	11.0	1.8	2.8	0.3	9.6	6.5	14.7
K	1.6	1.9	1.9	0.8	0.1	2.5	5.3

COMPARISON OF METHODS

An overview of all samples used in this study including the analysis data obtained from the HPLC AOAC 2005.06[19] together with toxin composition, MBA where available and average SPR data from all participants are presented in Table 5. For HPLC, the results are expressed as STXdiHCl equivalents. To calculate the total amount of STXdiHCl equivalents, both the TEFs provided by the NRC in the Supporting Information based on those published by Oshima[30] and the EFSA TEFs[16] were used. For the EFSA TEFs implemented in Europe[16] there are some changes in the results expressed as STXdiHCl equivalents especially when (high) amounts of dc-STX are present in the sample. The data from the three methods are compared based on the regulatory limit (Table 6). At this level for the naturally contaminated samples the result from each of the three methods matched with the exception of three samples. For sample B the difference in TEFs caused this sample to be lower when using the Oshima TEFs compared to the EFSA TEFs when analyzed by HPLC. The result based on the EFSA TEFs matched the other two methods of analysis. Following the implementation of the EFSA TEFs in Europe the standardization of TEFs worldwide may need to be readdressed. For sample G the biosensor detected PSP toxins above this level whereas both HPLC and MBA detected PSP toxins but below this level and for sample H the HPLC method detected PSP toxins above the level whereas the MBA and biosensor detected PSP toxins below the level.

Extraction Conditions. Differences between the HPLC (corrected for TEF) and MBA results (Table 5) may be caused by the method of extraction. The MBA method uses a 0.1 M HCl in water and 5 min boiling to extract the toxins. During this procedure some of the *N*-sulfocarbamoyl toxins can be transformed to their carbamate analogues. The toxicity of these carbamate analogues may be up to 10 times higher than their N-sulfocarbamoyl forms. The HPLC method uses a 0.2 M acetic acid in water and milder heating. In all the samples where the MBA result is notably higher than the results found in the HPLC method, N-sulfocarbamoyl toxins (C1/C2, C3/C4, GTX5, and GTX6) are present. It is probable that during the extraction for the MBA, some hydrolysis has taken place and therefore toxicity increased as compared to the HPLC analysis. The SPR extraction uses a single extraction with sodium acetate buffer 0.2 M at pH 5 and no heating, therefore it is

Table 5. Comparison of Analytical Results for Each Method, with Naturally Contaminated and Spiked Samples (Values above the EU Regulatory Limit Are in Bold Text)

	toxin profile in μg/kg PSP toxins (based on HPLC AOAC 2005.06 analysis)						
sample	toxins quantified (μg/kg)	<LOQ	other toxins detected	HPLC analysis (μg STXdiHCl equivalents/kg) based on Oshima TEFs	HPLC analysis (μg STXdiHCl equivalents/kg) based on EFSA TEFs	MBA analysis (μg STXdiHCl/kg)	biosensor average all participants (μg STXdiHCl/kg)
A. Scallops	**GTX2/3**: 120	STX C1/C2 GTX5		72	68	360	482
B. Mussels	**dcGTX2/3**: 72 **C1/C2**: 1808 **dcSTX**: 408 **GTX5**: 1616 **dcNEO**: 304		***C3/C4***[a] ***GTX6***[a]	672	**923**	**1730**	**1159**
C. Mussels	-			<LOD	<LOD	<LOD	<117[b]
D. Mussels	**dcGTX2/3**: 816 **C1/C2**: 23440 **dcSTX**: 2952 **GTX5**: 8720 **dcNEO**: 1952		***C3/C4***[a] ***GTX6***[a]	**5435**	**7215**	**11600**	**> 1100**[c]
E. Mussels	**GTX2/3**: 440 **STX**: 984 **GTX1/4**: 912 **NEO**: 136	**dcSTX**		**2189**	**2188**	**1340**	**>1190**[c]
F. Clams	dcGTX2/3: 1768 dcSTX: 1384 dcNEO: 944	**GTX5**	***C3/C4***[a] ***GTX6***[a]	**2029**	**2719**	Not available	**1174**
G. Cockles	**C1/C2**: 456 **GTX5**: 376	**dcGTX2/3** **dcSTX**	***C3/C4***[a] ***GTX6***[a]	58	73	700	1028
H. Mussels	**GTX2/3**: 120 **STX**: 88 **GTX1/4**: 544 **NEO**: 320	**C1/C2**		**933**	**955**	440	600
I. Mussels	**GTX2/3**: 168 **STX**: 264 **GTX1/4**: 560 **NEO**: 336	**C1/2** **dcSTX** **GTX5**		**1166**	**1188**	**810**	**1117**
J. Cockles	**dcGTX2/3**: 99 **STX**: 102 **C1/2**: 102 **NEO**: 103			240[d]	250[d]	not available	227
K. Mussels	**STX**: 825			825[d]	825[d]	not available	814

[a] Toxin is present in the sample. Quantification not possible due to lack of certified standards. [b] Four or more laboratories reported a value "lower than", therefore the highest reported value is mentioned in this table. All reported values for this sample are lower than the value mentioned here. [c] Four or more laboratories reported a value "higher than", therefore the lowest reported value is mentioned in this table. All of the reported values for this sample are higher than the value mentioned here. [d] Theoretical value based on the TEF.

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Table 6. Qualitative Comparison of Each Method in Relation to the Regulatory Limit[a]

sample	HPLC analysis (μg STXdiHCl equivalents/kg) based on Oshima TEFs	HPLC analysis (μg STXdiHCl equivalents/kg) based on EFSA TEFs	MBA analysis (μg STXdiHCl/kg)	biosensor average all participants (μg STXdiHCl/kg)
A. Scallops	+/−	+/−	+/−	+/−
B. Mussels	+/−	+	+	+
C. Mussels	−	−	−	−
D. Mussels	+	+	+	+
E. Mussels	+	+	+	+
F. Clams	+	+	not available	+
G. Cockles	+/−	+/−	+/−	+
H. Mussels	+	+	+/−	+/−
I. Mussels	+	+	+	+

[a] −, No PSP toxins detected at the limit of detection; +/−, PSP toxins detected but <800μg STXdiHCl equivalents/kg shellfish; +, PSP toxins detected but >800μg STXdiHCl equivalents/kg shellfish

expected that hydrolysis will not occur during this extraction procedure, similar to that with the HPLC method. This phenomenon of acid hydrolysis raises difficulties in the comparison of results using different extraction procedures when N-sulfocarbamoyl toxins are present in the samples.

Cross-reactivity. Differences between the SPR and both HPLC and MBA results (Table 5) may be caused by differences in cross-reactivity of the antibody. For sample G both the HPLC method and MBA provided a toxin level lower than the present EU regulatory limit whereas the SPR prototype method displayed a toxin level greater than the present regulatory limit. However, as the SPR method is a screening technique to single out negative (blank) samples, this overestimation would not provide a health risk but may result in a situation that in the laboratory more samples need to be reanalyzed. This sample G contains only C1/C2 and GTX5 as the two quantified toxins (HPLC). When the cross reactivity in mussel extract was expressed as the PSP toxin concentration in STX equivalents,[29] the toxins C1/C2 and GTX5 have a cross-reactivity relative to STXdiHCl of 217 and 1300% respectively. The high result obtained with the SPR prototype method for this sample is explained by the high cross-reactivity for these toxins and is therefore not surprising.

In addition, the cross reactivity for GTX1/4 both in HBS-EP buffer and mussel extract is very low at <1.1%. This means that in the unlikely event of only GTX1/4 being present in a sample at a mass fraction above or close to the present regulatory limit the sample will be determined to be below the LOD of <120 μg/kg and thus compliant to the regulatory limit of 800 μg STXdiHCl equivalents/kg. The high relative toxicity of GTX1 (TEF = 0.9940)[30] in combination with the low cross-reactivity is a weaker point in the method that may be overcome when antibodies with a better cross reactivity to GTX1/4 are incorporated. Further development of this method may be needed to better detect toxins like GTX1/4 with a better cross-reactivity on the antibody.

Screening Method. For the blank sample C the SPR method obtained a highest reported result of <117 μg STXdiHCl equivalents/kg. For this sample most participants reported values below the lowest point in the calibration curve (<1.2 ng/mL). Where participants reported an actual value, the average for this sample was calculated and the highest of these values was the value reported for Lab 05 in Table 1. All other values are below this highest reported average "mean of means" value.

From the single lab validation study performed,[29] a limit of detection based on the CCβ was established of 120 μg STXdiHCl equivalents/kg. The results for the blank sample (Table 1) in this study were in agreement with this established LOD. The method has shown in this study that the one sample where no PSP toxins were present was identified. If a result of this method shows a value above the LOD (>120 μg STXdiHCl equivalents/kg) the sample should be investigated with a second method to determine the actual concentration. This decision level of <120 μg STXdiHCl equivalents/kg, whereby a sample is considered negative, is low. Further improvements or gaining experience with this method may result in increasing this decision level to a higher value and reduce the amount of samples that would need to be analyzed again with a quantitative method.

After the method is fully developed for testing on a larger scale, it is recommended that a significant amount of shellfish samples originating from different sites in the EU are tested with the SPR method in parallel to the regular testing method(s). These parallel tests could be centralized in one or two laboratories experienced with the SPR method. Yearly, EU National Reference Laboratories on Marine Biotoxins (NRLs) could distribute previously investigated samples from their own lab/country to these central laboratories for testing using this biosensor method. This would allow insight into the performance of the method in practice and based on the results of such an extensive comparison, the decision level may be adapted to allow semiquantification of the samples. The feasibility of such a trial is currently under discussion in the EU NRL network, with Queen's University acting as a central repository, where samples would be investigated with the SPR method.

One perceived disadvantage to this method is where concerns have been raised over the cost of this specific SPR instrumentation from one supplier. Although there may be a high initial outlay in relation to purchasing the instrumentation, the long-term benefits outweigh this if the method can be successfully used to reduce, and with the aid of dedicated confirmatory methods replace, the mouse bioassay or animal testing for PSP toxin analysis. Although the Biacore Q was employed as a model instrument, ongoing research has shown that the prototype kit is transferable and can be used on another SPR instrument with good agreement between results.[33] It would be envisaged to investigate the transferability of this assay further using SPR and biosensor instrumentation, from different manufacturers, to illustrate its potential for wider implementation thus minimizing costs and additional expenditure where possible.

CONCLUSION

The interlaboratory study of the SPR method for the determination of PSP toxins was successfully completed by seven laboratories. The statistical analysis of the data generated in this study, particularly where the HorRat values are all <1 for the samples tested, demonstrated that the method performance, in terms of interlaboratory precision, can be considered as acceptable. This interlaboratory study indicates that this method has the potential to become a candidate screening method for PSP analysis. Samples that result in a toxin value greater than 120 μg STXdiHCl equivalents/kg should be analyzed by a second method for further quantification or toxin determination. Samples that result in a value less than 120 μg STXdiHCl equivalents/kg should be considered to be compliant, that is, below the current EU regulatory limit of 800 μg STXdiHCl equivalents/kg. It is recommended that the method is investigated in trials parallel with the existing EU methods for regulatory testing for an extensive sampling period to explore its potential as an official method of analysis for PSP toxins in shellfish. The demonstrated transferability of this SPR technology between laboratories indicates the feasibility of this technology being applicable as a routine screening tool for other toxins and chemical contaminants provide suitable binders are available.

SAFETY

Saxitoxin and its analogues are responsible for incidents of PSP. Therefore, when using PSP toxin standard solutions or contaminated shellfish 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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Notes

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