Cefas contract report C2934

Refinement and in-house validation of the AOAC HPLC method (2005.06): the determination of paralytic shellfish poisoning toxins in cockles, Pacific oysters and native oysters by liquid chromatography and fluorescence detection

FSA Contract Reference: ZB1807

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Executive Summary

The AOAC HPLC method 2005.06 was approved as an Official Method by the European Commission for the detection and quantitation of a number of paralytic shellfish poisons in certain harvested shellfish products. The method describes the analysis of the acetic acid extracts of shellfish homogenates after clean up, fractionation and pre-column oxidation of PSP toxins with periodate and peroxide oxidants. The method consists of two parts, a screening step to qualitatively analyse for the presence of PSP toxins and a fully quantitative step whereby the concentrations of individual toxins and total PSP toxicity are determined. The AOAC 2005.06 method was subjected to a single laboratory validation scheme conducted at the Cefas laboratory between August 2007 and July 2009 for cockles, Pacific oysters and native oysters. As part of the validation scheme, parallel testing was performed whereby a number of shellfish acquired, both from the official control (OC) monitoring programmes and by feeding of shellfish with mass-cultured toxic algal species, were analysed by both HPLC screen/MBA and AOAC HPLC methodologies. Validation was conducted for the PSP toxins specified in the AOAC 2005.06 method and which are currently available as certified reference standards (National Research Council, Canada (NRCC)). The following toxins were included; the N-hydroxylated toxins (neosaxitoxin (NEO) and gonyautoxins (GTX) 1 and 4 together (GTX1,4), and the non N-hydroxylated toxins saxitoxin (STX), gonyautoxins (GTX) 2 and 3 together (GTX2,3), and 5 (GTX5), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl toxins C1 and C2 together (C1,2)). Additionally, the validation was extended to the non N-hydroxylated decarbamoylgonyautoxin-2 and 3 (dcGTX2,3) and the N-hydroxylated decarbamoylneosaxitoxin (dcNEO) not covered by the AOAC method but recently made available as certified reference standards. The work followed on from an earlier validation exercise conducted at Cefas of the AOAC 2005.06 method for the analysis of PSP toxins in mussels.

Validation results generally showed that the analysis of PSP toxins in the acetic acid extracts of oysters and cockles was selective enough to detect the presence of each toxin peak. Minor issues were encountered with the early eluting toxin dcGTX2,3 where naturally fluorescing co-extracted components were present at the same chromatographic retention time, thus potentially contributing to false positive toxin assignment. The presence of these interferences in cockles and oysters helped confirm the need for running unoxidised extracts alongside oxidised extracts for each sample for full quantitation of PSP toxicity, as currently carried out for mussels.

The linear relationship between the instrumental response and toxin concentration in oysters and cockles extract as well as solvent based solutions was shown to be acceptable, both visually and statistically, over the working range of 0 to 1.2 Action Limit (AL) for each toxin (0 to 0.12 AL for GTX5). Some differences were observed between the calibration slopes of matrix-matched and solvent spiked standards for some toxins in some species (e.g. non-N-hydroxylated toxins in Pacific oysters), but a lack of consistency in these deviations confirmed that the use of matrix-matched calibration standards would not be appropriate.

In relation to the European regulatory limit (0.8 µg STX eq./g shellfish tissue) and a target concentration level of 0.2 AL (0.16 µg STX eq./g) per toxin, a good level of instrumental sensitivity was determined in cockles and oysters for both the periodate screen (approximately 0.03 to 0.22 µg STX eq./g) and the full quantitation method (~0.01 to 0.16 µg STX eq./g detection limits). The method has therefore been shown to detect most toxins in all three matrices at or below the 0.2 AL target level, showing a similar level of sensitivity to that described in the AOAC method and previously for mussels. Limits of quantitation ranged from ~0.01 to 0.16 µg STX eq./g for all toxins except GTX1,4 and NEO in all three matrices (0.35 to 0.40 µg STX eq./g) and GTX2,3 in cockles and native oysters (0.24 and 0.34 µg STX eq./g respectively). Thus, with the exception of these toxins, the method can perform with an acceptable level of sensitivity at toxin concentrations less than or equal to 0.2 AL for each of these toxins. However, the acceptability of medium term precision data for GTX1,4, NEO and GTX2,3 at 0.2 AL (RSD < 20% (n=6), HorRat < 2.0) argues that quantitation of these toxins may still be carried out at levels below the LOQ with a good degree of confidence and fitness for purpose. Due to the limited availability of dcNEO, the LOD and LOQ were confirmed at 0.16 and 0.32 µg STX eq./g respectively for each shellfish matrix, although predicted LODs and LOQs were well below these concentrations.

Method recoveries at 0.2 AL and 0.5 AL showed recoveries to be similar to the levels described previously for mussels and in the AOAC 2005.06 method, with results ranging from approximately 50% to 120% for all toxins with the majority ranging from 60% to 100%. Differences were noted between recoveries for each toxin and at different concentration levels.

The precision of the method for oysters and cockles was shown to be acceptable. The instrumental precision showed low variability between toxin peak retention times (RSD = 0.4% to 1.6% in all three matrices), with the precision of toxin peak area responses also acceptable (mean RSD = 3% to 4% for the three matrices). The short term method repeatability at both 0.5 and 0.2 AL for each toxin was good with a RSD < 10% for all toxins except C1,2 (12%) in cockles, dcSTX (13%) in Pacific oysters and GTX1,4 (13%) and NEO (15%) in Pacific oysters. Medium term repeatability was also acceptable at both concentration levels. The mean percentage RSD for all PSP toxins was 7% in cockles (0.5 AL) and 12% for Pacific oysters and native oysters (0.5 AL). Mean RSDs at the lower concentration (0.2 AL) were 12%, 11% and 14% for cockles, Pacific oysters and native oysters respectively. Long term precision, or within-laboratory reproducibility was assessed with the repeated analysis (> 6 months, multiple analysts, instruments and consumables) of naturally contaminated shellfish homogenates. Data was acceptable with all long term precision values equivalent to or improved compared with the data generated in mussels. Further statistical analysis of short, medium and long term repeatability using HorRat values (HorRat < 2.0 for all toxins) provided further evidence of the acceptability of the repeatability of the method.

Ruggedness experiments were undertaken to examine the effects of method parameters on the stability of the method. Results from the analysis of PSP toxins in naturally contaminated Pacific oyster extracts and from spiked native oysters and cockles showed that the method was robust for all parameters investigated. Validation results obtained throughout the study were used to calculate preliminary standardised and expanded uncertainties for the analysis of PSP toxins in cockles and oysters. The contributions to uncertainty were assessed to ensure all factors were incorporated whilst eliminating measurement uncertainty duplication. Results showed combined

standardised uncertainties of measurement ranging from 0.16 to 0.27 (cockles), 0.14 to 0.34 (Pacific oysters) and 0.11 to 0.33 (native oysters) with expanded uncertainties (k=2) consequently ranging from 0.32 to 0.54 (cockles), 0.28 to 0.68 (Pacific oysters) and 0.23 to 0.66 (native oysters), depending on the toxin. The values are generally lower and more consistent than the range of uncertainties reported previously for mussels.

The method was trialled in comparison with the MBA, with the analysis of cockle, Pacific oyster and native oyster samples obtained from the GB biotoxin monitoring programme. The samples were analysed in parallel using both the AOAC 2005.06 method and the current HCl HPLC screen and MBA methods. Acetic acid extracts of samples were subjected to both the screening and quantitation elements of the AOAC 2005.06 method and comparisons were carried out between results obtained from the two HPLC screening methods. No false HPLC positives (in comparison with the MBA) were observed and the correlation between the methods was found to be good, with minor differences between the HCl and acetic acid screening results observed due to levels of PSP close to the LOD. Quantitation of all negative HPLC samples resulted in negative HPLC quantitation results. Four HPLC positive samples were found to contain only minor levels of STX, GTX2,3 and C1,2 toxins following full quantitation, with total toxicities lower than the detection limit of the MBA.

Results from the parallel analysis by HPLC and MBA for cockles, showed some positive bias for some samples in the HPLC as compared to MBA, but this could be explained by the use of the highest toxicity equivalence factor (TEF) as part of the toxin quantitation procedure. Results for both Pacific oysters and native oysters showed a much higher positive bias in the HPLC toxicity results as compared to the MBA (196% and 263% respectively). Parallel analysis of the same oyster samples using a post-column oxidation LC-FLD method provided evidence that the bias was not solely related to the use of the highest TEF and that other matrix effects were affecting either the HPLC or MBA analyses. Application of measurement uncertainty to the HPLC results showed the level of uncertainty varying depending on the species and toxin profile, but with the range of uncertainties comparing similarly to those described for the MBA.

Overall, the validation results presented in this report are comparable or improved compared with those presented previously for mussels, with precision results showing an improvement in oysters and cockles. As such the method uncertainties calculated show a narrower range of uncertainty for oysters and cockles compared with those calculated for mussels. In terms of method implementation, the logistics of the fully-quantitative HPLC method have been detailed in a previous report. Whilst the method is complex and time-consuming, it is not thought that extension of the method to cover cockles, native oysters and Pacific oysters will reveal any additional issues. Therefore, when applied to the routine monitoring of cockles, Pacific oysters and native oysters, the method has the potential to provide further significant reduction in the overall bioassay usage for PSP toxin determination in compliance with the 3R (Replacement, Refinement, Reduction) principles in both European Union (EU) and national legislation. However, whilst the results indicate that it may be safe to implement the HPLC method for cockles, the large disparity between the HPLC and MBA results for both oyster species indicates a strong need to further investigate the comparative performance of the HPLC and MBA methods for these two species. Future work will continue with the validation of the AOAC 2005.06 for other shellfish species (clams and scallops) of importance to the UK marine biotoxin monitoring programmes.

Cockles

Toxins	Linearity (r^2)	LOD ug/g STX equiv	LOQ ug/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged-ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL			
GTX 1,4	0.989	0.16	0.34	125%	131%	4%	3%	9%	4%	nd	20%	0.21
NEO	0.99	0.16	0.35	66%	62%	14%	4%	10%	10%	nd	23%	0.27
dcNEO	nd	<0.16	<0.32	71%	58%	3%	5%	nd	nd	nd	nd	nd
dcSTX	0.957	0.009	0.02	93%	85%	4%	7%	12%	7%	Yes	nd	0.21
GTX 2,3	0.916	0.12	0.24	80%	84%	8%	4%	15%	7%	Yes	19%	0.23
GTX 5	0.993	0.004	0.008	92%	90%	5%	7%	12%	7%	Yes	11%	0.16
STX	0.985	0.025	0.05	136%	126%	5%	1%	13%	3%	Yes	18%	0.21
dcGTX 2,3	0.966	0.047	0.09	48%	49%	6%	6%	8%	10%	Yes	nd	0.21
C 1,2	0.986	0.017	0.03	92%	76%	8%	12%	14%	11%	Yes	19%	0.25
Mean	0.973	0.068	0.14	92%	88%	7%	6%	12%	7%	na	Total 10%	na

na = not applicable. Nd = not determined. Total = long term precision of total toxicity (%RSD)

Pacific Oysters

Toxins	Linearity (r^2)	LOD ug/g STX equiv	LOQ ug/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged-ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL			
GTX 1,4	0.96	0.16	0.40	88%	97%	18%	8%	17%	16%	Yes	26%	0.34
NEO	0.991	0.16	0.40	51%	61%	24%	7%	17%	7%	Yes	23%	0.32
dcNEO	nd	<0.16	<0.32	58%	54%	4%	3%	nd	nd	nd	nd	nd
dcSTX	0.994	0.004	0.01	82%	77%	6%	13%	16%	21%	Yes	nd	0.27
GTX 2,3	0.991	0.05	0.16	90%	94%	7%	4%	17%	12%	Yes	17%	0.23
GTX 5	0.995	0.002	0.008	85%	85%	4%	2%	14%	10%	Yes	15%	0.20
STX	0.993	0.011	0.05	123%	121%	4%	1%	15%	10%	Yes	5%	0.14
dcGTX 2,3	0.991	0.029	0.09	79%	70%	5%	2%	16%	14%	Yes	nd	0.23
C 1,2	0.994	0.01	0.03	97%	99%	3%	2%	3%	6%	Yes	14%	0.15
Mean	0.989	0.053	0.14	87%	88%	9%	5%	na	na	na	Total 14%	na

na = not applicable. Nd = not determined. Total = long term precision of total toxicity (%RSD)

Native Oysters

Toxins	Linearity (r^2)	LOD ug/g STX equiv	LOQ ug/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged-ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL			
GTX 1,4	0.964	0.16	0.40	68%	94%	13%	13%	16%	16%	nd	26%	0.33
NEO	0.949	0.15	0.40	59%	82%	8%	15%	16%	16%	nd	19%	0.28
dcNEO	nd	<0.16	<0.32	60%	71%	3%	3%	nd	nd	nd	nd	nd
dcSTX	0.989	0.008	0.03	78%	81%	4%	3%	14%	3%	Yes	nd	0.19
GTX 2,3	0.977	0.1	0.34	73%	78%	6%	3%	11%	12%	Yes	15%	0.19
GTX 5	0.987	0.003	0.01	83%	79%	3%	3%	4%	16%	Yes	18%	0.22
STX	0.983	0.02	0.07	111%	108%	3%	2%	7%	8%	Yes	8%	0.11
dcGTX 2,3	0.988	0.037	0.12	70%	56%	7%	2%	8%	12%	Yes	nd	0.20
C 1,2	0.988	0.017	0.06	82%	68%	8%	4%	13%	10%	Yes	12%	0.18
Mean	0.978	0.062	0.18	78%	81%	7%	6%	11%	12%	na	Total 11%	na

na = not applicable. Nd = not determined. Total = long term precision of total toxicity (%RSD)

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Glossary

AL	Action Limit
AOAC	AOAC International
GTX5 (B-1)	Gonyautoxin 5
GTX6 (B-2)	Gonyautoxin 6
Cefas	The Centre for Environment, Fisheries and Aquaculture Sciences
CFIA	Canadian Food Inspection Agency
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment UK
NRCC	Canadian National Research Council
CRL	Community Reference Laboratory for Marine Biotoxins
C1	N-sulfocarbamoyl toxin C1 (N-Sulfocarbamoyl-gonyautoxin-2)
C2	N-sulfocarbamoyl toxin C2 (N-Sulfocarbamoyl-gonyautoxin-3)
C3	N-sulfocarbamoyl toxin C3
C4	N-sulfocarbamoyl toxin C4
dcGTX2,3	decarnamoylgonyautoxin-2 and 3
dcNEO	decarbamoylneosaxitoxin
dcSTX	decarbamoylsaxitoxin
EX	Excitation wavelength (FLD)
EM	Emission wavelength (FLD)
EC	European Commission
EU	European Union
FLD	Fluorescence detection
GTX	Gonyautoxin
GTX2,3	Gonyautoxins 2 and 3 together
GTX1,4	Gonyautoxins 1 and 4 together
HorRat	Horwitz ratio
HPLC	High Performance Liquid Chromatography
IQC	Internal Quality Control
IUPAC	International Union of Pure and Applied Chemistry
LRM	Laboratory Reference Material
LOD	Limit of Detection
LOQ	Limit of Quantitation
MBA	Bioassay
NA	Not analysed
ND	Not detected
NEO	Neosaxitoxin
NG (-ve)	Negative
OC	Official Control
PS (+ve)	Positive
PCOX	Post-column oxidation
PSP	Paralytic Shellfish Poisoning
Rt	Retention time
SPE	Solid Phase Extraction
SOP(s)	Standard Operating Procedure(s)
STX	Saxitoxin
UKNRL	UK National Reference Laboratory for Marine Biotoxins
µg STX eq./g	Micrograms of STX equivalence per gram of edible shellfish tissue

1. Introduction

Filter feeding bivalves, such as cockles and oysters, may accumulate marine biotoxins which have been derived from marine phycoplankton. Contaminated shellfish may subsequently impact on the health of the human consumer. Eight major classes of marine phycotoxins have been identified to date and are distinguished by their chemical structure and physico-chemical behaviour, resulting in five major human seafood poisoning syndromes [1]. One of the groups known to induce human illness is the Saxitoxin derived compounds, known as paralytic shellfish poisoning (PSP) toxins. To ensure consumer protection, monitoring of PSP toxins in shellfish is a statutory requirement. The current European Union's (EU) reference method for the detection of PSP toxins is the mouse bioassay (MBA) [2,3].

Cefas and the UK competent authority are committed to moving away from animal assays in the statutory monitoring programme and have pursued the development and implementation of sensitive and validated alternative methods to ensure the continued safety of shellfish products. These methods must be able to deal with complex matrices as well as differentiate toxins from non-toxic compounds and from other toxins [4,5]. The increased commercial availability of analytical standards over recent years for PSP toxins has facilitated the development of quantitative HPLC methods.

An HPLC method, commonly referred to as the "Lawrence method", has been developed and gone through single and inter-laboratory validation [6,7,8,9,10,11]. In 2005, this method was adopted by the AOAC as an official, first action method (method AOAC 2005.06) [12] and was approved by the EU as an alternative to the MBA for those toxins and shellfish species detailed in the published validation reports (Regulation EC/2006/1664) [2]. In 2007 Cefas undertook the single-laboratory validation of this method for the analysis of PSP toxins in mussels [13]. The validation followed the requirement of EC regulation 882/2004 that official control methods should be validated and quality assured prior to adoption into EU monitoring programmes [14]. Annex III of this regulation sets out the following requirements: "the method of analysis should be characterised by the following criteria: a) accuracy, b) applicability (matrix and concentration range), c) limit of detection, d) limit of determination, e) precision, f) repeatability, g) reproducibility, h) recovery, I) selectivity, j) sensitivity, k) linearity, l) measurement of uncertainty, m) ruggedness n) other criteria that may be selected, as required". The validation included a period of parallel testing whereby HPLC results were compared with those obtained from MBA analysis of mussels obtained from the routine GB biotoxins monitoring programmes. The method was deemed fit for purpose following a period of review and consultation with stakeholders and the method implemented at Cefas for the measurement of PSP toxins in mussels from May 2008 [15].

The AOAC 2005.06 method exists at several levels of complexity depending on the PSP toxins identified in a sample [13]. The method involves the extraction of PSP toxins from shellfish tissue using 1% acetic acid solution, followed by extract clean up using a C18 Solid Phase Extraction (SPE) cartridge. Toxins can only be identified by HPLC following periodate oxidation derivatisation to form fluorescent products. This oxidation and analysis step enables samples to be "screened" for the presence of the whole suite of PSP toxins. Samples where toxins are detected are passed onto a full quantitation analysis. Non-N-hydroxylated PSP toxins can be quantified by subjecting the C18-SPE-cleaned extracts to peroxide oxidation, followed by LC-FLD analysis. However, if N-

hydroxylated toxins are suspected to be present, the extract is fractionated using ion-exchange SPE cartridges, followed by periodate oxidation of the individual fractions.

The objective of the work presented here was to establish the performance characteristics of the method when applied to cockle (Cerastoderma edule), Pacific oyster (Crassostrea gigas) and native oyster (Ostrea edulis) matrices and validate the AOAC 2005.06 official method (OM) for use in these matrices at the Cefas Laboratory. The validation was to be applied to those PSP toxins described in the OM, and was extended to include those toxins commercially available as certified reference standards. Two phases were undertaken. Phase 1, an in-house validation of the method was performed following as closely as possible the guidelines laid down by the International Union of Pure and Applied Chemistry (IUPAC) [4] to obtain methodological information regarding selectivity, linearity, limits of detection, limits of determination, accuracy, recovery, instrumental precision, repeatability and reproducibility. For Phase 2, an assessment was made of the method through testing of cockles and oysters obtained through the GB biotoxin monitoring programmes and through shellfish feeding experiments.

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2. Materials and methods

2.1 Overview of the AOAC 2005.06 Method

Cockle and oyster samples are shucked and homogenised and the shellfish homogenates extracted with acetic acid, before being cleaned up on a C18 Solid Phase Extraction (SPE) cartridge. After pH adjustment, aliquots of the extract are oxidised by periodate reagent in the presence of a matrix modifier, prior to high-performance liquid chromatography with fluorescence detection (HPLC-FLD) alongside periodate-oxidised standards of certified toxin standards. This provides a qualitative screen for the presence of the toxins GTX1,4, NEO, dcNEO, dcSTX, GTX2,3, dcGTX2,3, C1,2, GTX5 and STX. Samples are assigned positive if PSP toxin peaks are present, and positive samples are progressed to full quantitation. This involves peroxide oxidation of the C18-cleaned extracts in order to calculate the amounts of the non-N-hydroxylated PSP toxins (STX, dcSTX, GTX2,3, dcGTX2,3, C1,2 and GTX5) and ion-exchange fractionation and subsequent periodate oxidation of fractions for the quantitative determination of the N-hydroxylated toxins (GTX1,4, NEO and dcNEO). Each toxin is quantified by direct comparison of peak area responses to external, certified analytical standards prepared at known concentration levels for each individual toxin or toxin epimeric pair (e.g. GTX1,4 together).

Both periodate and peroxide oxidation reactions are used during the course of this method as neither oxidant will alone successfully oxidise every toxin to give a suitable level of analytical sensitivity. Peroxide oxidation is utilised for the oxidation of all non-N-hydroxylated toxins (STX, dcSTX, GTX2,3, GTX5, dcGTX2,3 and C1,2) and is deemed to be a sensitive and reliable oxidation technique. N-hydroxylated toxins (GTX1,4, NEO and dcNEO), however, do not respond to peroxide oxidation, and must be oxidised using the periodate reagent. This oxidation method is inherently less reliable, due in part to the significant effect of small pH variations [17] and as such, the AOAC 2005.06 method describes the use of a matrix modifier to be used in all periodate oxidations for both standards and samples, which is believed to result in a more repeatable oxidation, particularly for the oxidation of NEO (Lawrence, personal communication). This modifier is the C18 SPE cleaned up, acetic acid extract of a Pacific oyster sample, which has been shown to be free from chromatographic peaks at the same retention times as any of the PSP toxin standards.

With the inherent ambiguity in the AOAC 2005.06 regarding the use of pre or post fractionation oxidation, a quantitation approach was taken to reduce the overall number of analyses per sample to 4 (Table 1; Figure 1). This is the same approach as used for the validation and implementation of the HPLC method for the analysis of mussels as described previously [13, 15].

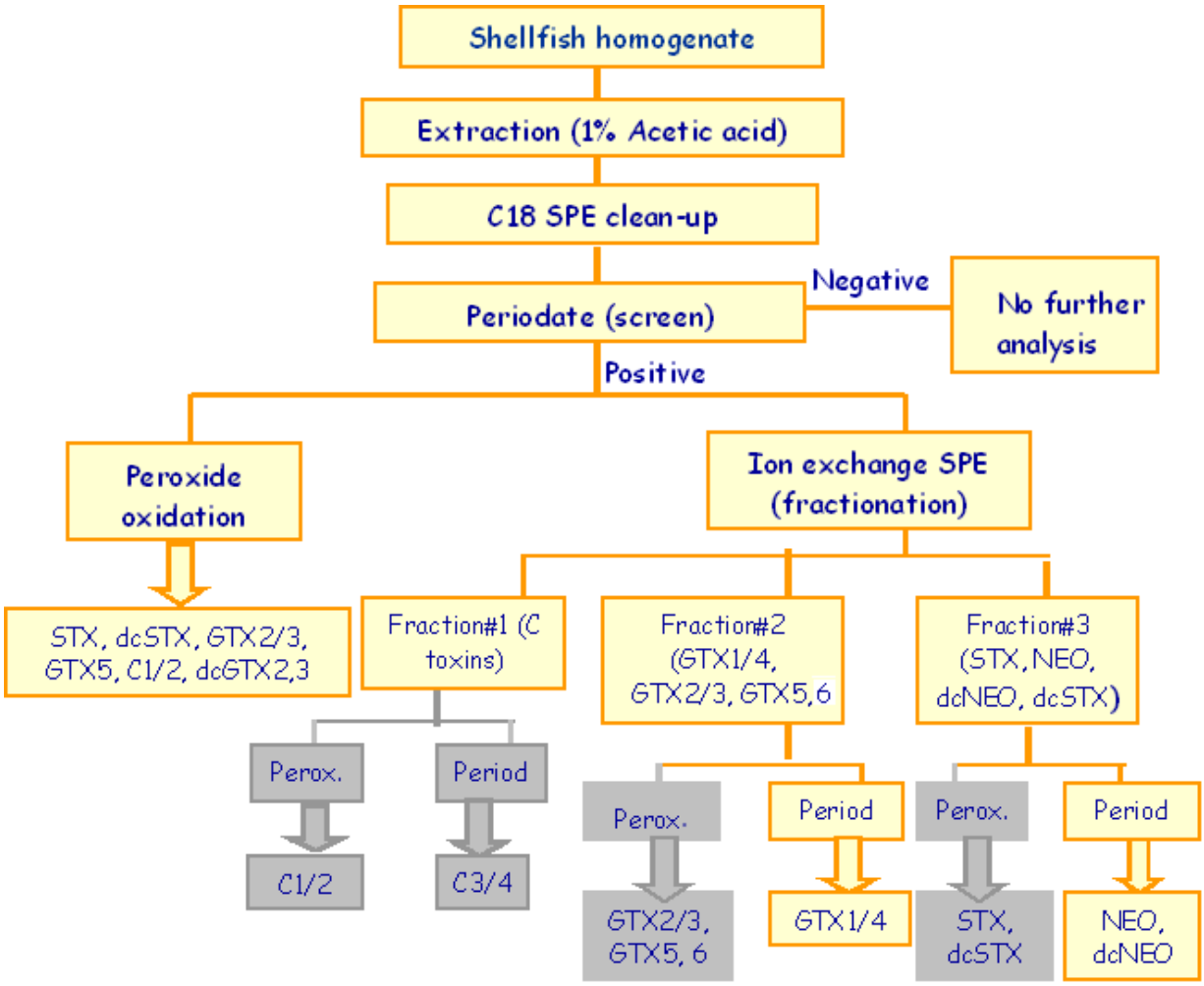

Figure 1. Scheme utilised for screening and quantitation of PSP toxins in oyster and cockle samples. Parts of method in grey not carried out.

Table 1. Oxidation methods for screening and quantitation of PSP toxins

Toxin	Screening method	Quantitation method
GTX1,4	Periodate C18 extract	Periodate fraction F2
NEO	Periodate C18 extract	Periodate fraction F3
dcNEO	Periodate C18 extract	Periodate fraction F3
dcSTX	Periodate C18 extract	Peroxide C18 extract
GTX2,3	Periodate C18 extract	Peroxide C18 extract
GTX5	Periodate C18 extract	Peroxide C18 extract
STX	Periodate C18 extract	Peroxide C18 extract
C1,2	Periodate C18 extract	Peroxide C18 extract
dcGTX2,3	Periodate C18 extract	Peroxide C18 extract

2.2 Laboratory equipment

The following general laboratory equipment was used throughout the validation scheme: hot water bath capable of holding boiling water, calibrated pH meters, 50 and 15mL polypropylene centrifuge tubes, 5mL plastic graduated “DEC” tubes (with caps), vortex homogenisers, centrifuge, calibrated analytical balance (4 decimals), calibrated (10 to 1000µL) pipettes, precision volumetric flasks (series A; 10, 100, 250 and 500mL),

nylon syringe filters (0.45µm), 2mL autosampler vials with screw caps, 3mL vials, C18 SPE cartridges (Phenomenex, 500mg/3mL cartridge volume), SPE-COOH ion exchange cartridges (Strata X-CW, Phenomenex, 200mg/3mL), ice bath, 250mL beakers, 500mL solvent vessels, calibrated timer, Gilson automated SPE systems, glass Pasteur pipettes.

2.3 Chemicals

Certified reference toxins were obtained from National Research Council Canada (NRCC, Halifax, Nova Scotia, Canada). Toxins are supplied at the certified concentrations listed in Table 2 and prepared in acetic acid and/or hydrochloric acid.

Table 2. Concentrations of certified PSP calibration solutions.

Toxin	Mol weight as supplied	Concentration as supplied (µg/mL)	Diluent
GTX1	411.4	43.6	0.01M acetic acid
GTX4	411.4	14.4	0.01M acetic acid
NEO	388.2	25.2	0.003M HCl
dcNEO	345.2	10.4	0.003M HCl
dcSTX	329.2	20.4	0.003M HCl
GTX2	395.4	46.7	0.003M HCl + 0.01M acetic acid
GTX3	395.4	15.4	0.003M HCl + 0.01M acetic acid
GTX5	379.4	24.7	17µM (pH5) acetic acid
STX-di HCl	372.2	24.2	0.003M HCl
C1	475.4	54.2	17µM (pH5) acetic acid
C2	475.4	16.6	17µM (pH5) acetic acid
dcGTX2	352.3	40.2	0.003M HCl
dcGTX3	352.3	11.3	0.003M HCl

Acetonitrile was of HPLC-grade (Rathburn Chemicals Ltd., Scotland) and water either HPLC-grade (Rathburn) or de-ionised water produced in-house. Analytical reagent grade acetic acid (99.9 % pure), ammonium formate (99 % pure), formic acid (>98 % pure), ammonium acetate (99 % pure), hydrogen peroxide, sodium hydrogen phosphate (99 % pure), periodic acid (99 % pure) were from Sigma-Aldrich (Poole, Dorset, UK), sodium chloride (99 % pure) and sodium hydroxide (99 % pure) were from BDH. Toxin standards were diluted in ~4.5g water to give concentrated stock standard solutions. These were subsequently diluted in appropriate volumes of 0.1 mM acetic acid to produce working analytical standards for instrument calibration purposes. The toxicity equivalence factors (TEF) quoted [21] for each toxin were incorporated into the calculations for preparation of calibration solutions for each toxin mix, so that the calibration range for each toxin equated to 0.2 to 1.0 AL in terms of STX equivalence. The exception was GTX5 where calibration solutions were prepared at 10% of the concentration of other toxins (0 to 0.12 AL) due to the very low relative toxicity of GTX5 to STX (Appendix 6). In the case of isomeric pairs (GTX1,4, GTX2,3, C1,2 and dcGTX2,3), the highest toxicity equivalence factor was used for each pair (Appendix 6). Individual toxin results obtained are therefore quoted in terms of µg STX eq./g of flesh and the total PSP toxicity was calculated by summing the individual concentration

contributions from all quantified toxins and is quoted in terms of µg STX eq./100 g of flesh.

2.4 Samples

Bulk shellfish samples for use in homogenate and extract spiking studies were obtained from M&J Seafood of Poole, Dorset. Approximately 0.5kg each of live Pacific oysters (*Crassostrea gigas*), native oysters (*Ostrea edulis*) and cockles (*Cerastoderma edule*) were shucked and homogenised on arrival and stored at –20 °C until use. Shellfish was acquired during winter months (late November) when Alexandrium and other PSP-producing algae are not present in the marine water column. Randomly selected triplicate 5.0 (± 0.1g) sub-samples were transferred to 50 mL polypropylene centrifuge tubes and extracted and analysed according to the AOAC 2005.06 method and results compared against PSP toxin standards to confirm that samples were free from all PSP toxins. In addition, blank (non-spiked) 5.0g aliquots were analysed alongside spiked homogenate samples at each stage of the validation process to give further confirmation that the shellfish homogenates acquired were free from PSP toxins. For practical reasons, all validation work involving the spiking of oysters and cockles with toxins was carried out on homogenate aliquots taken from the same bulk sample. The potential variability between the matrix components present in shellfish grown and harvested at different times of the season and in different locations is however noted, as highlighted in previous validation studies within our laboratory [16].
Shellfish samples analysed during the phase 2 comparison were acquired from the GB Official Control monitoring programmes and the majority analysed fresh without any low temperature storage. A low number of sample homogenates (4) were previously frozen (-20°C) and analysed once thawed and extracted. In addition, due to the low prevalence of naturally contaminated oysters and cockles, shellfish feeding experiments were undertaken to provide further materials for comparative testing (section 2.9).

2.5 Analysis of PSP toxins by High Performance Liquid chromatography-fluorescence detection (HPLC-FLD)

Liquid chromatographic elution of injected samples was performed on a Gemini C18 HPLC column (150mm x 4.6mm, 5µm) (Phenomenex, Manchester, UK) with a Gemini C18 guard column, and using a gradient solvent system (Table 3). Mobile phase (A): 0.1M ammonium formate, adjusted to pH6 +/- 0.1 with 0.1M acetic acid, (B): 0.1M ammonium formate with 5% acetonitrile, also adjusted to pH6 +/- 0.1 with 0.1M acetic acid. The mobile phase (2mL/min) was delivered by an Agilent 1200 series LC gradient pump equipped with a mobile phase vacuum degassing module, a 100-vial capacity thermostatically controlled autosampler and a column oven (set at 35 °C).

Table 3. HPLC mobile phase gradient for the separation of PSP toxins.

Time (min)	A (%)	B (%)
0	100	0
5	95	5
9	30	70
10	30	70
12	100	0

An Agilent fluorescence detector (1200 model FLD) was used for the detection of the oxidation products of all PSP toxins. Fluorescence excitation was set to 340nm and

emission to 395nm. The peak width was set to >0.2min and the detector gain (PMT) set to 11.

2.6 Optimisation of HPLC and FLD parameters

A previous report has described the initial work carried out to optimise the parameters associated with the AOAC 2005.06 method [13]. Specifically, toxins not included in the AOAC validation (dcNEO, dcGTX2,3) and the additional toxin C1,2 not currently employed in our routine HPLC screening method [15] were optimised in terms of oxidation method and chromatographic retention time. Results were presented for shellfish for the entire suite of available certified toxins (STX, NEO, dcSTX, GTX1,4, GTX2,3, GTX5, dcNEO, C1,2 and dcGTX2,3). However, current availability issues with dcNEO prevented the full use of dcNEO in the present study, so this toxin was utilised only for sensitivity (LOD and LOQ confirmation at 0.2 and 0.4 AL) and recovery (at 0.2 and 0.4 AL) tasks. The method employed during this study matches as closely as possible that of the original Lawrence method [11, 12]. Small deviations from the original procedure were highlighted in the previous report, specifically the use of an improved ion exchange fractionation step, the use of cooled autosampler and automated solid phase extraction technologies [13,15]. The scheme in 2.1 (Figure 1) details the steps involved in the method. As defined by the FSA, the "target" detection limit in terms of concentration for all PSP toxins was set at 0.2 times the regulatory action limit (AL) per toxin and thus it was one of the purposes of this work to demonstrate the performance of the method, in terms of sensitivity, at this concentration level (0.16 μg STX eq./g).

2.7 Toxin extraction, clean-up and oxidation prior to HPLC-FLD analysis

The scheme in 2.1 (Figure 1) details the steps involved in the method. Pacific oyster, native oyster and cockle samples were shucked and homogenised and the shellfish homogenates extracted by heating with a 1% acetic acid solution and the supernatants collected post-centrifugation. A second extraction of the homogenate was performed with a further aliquot of 1% acetic acid at room temperature and the subsequent supernatant added to the first. Extracts were diluted to a known volume (10.0mL) and cleaned-up using a solid phase extraction (SPE) SPE cartridge. After conditioning the cartridge with methanol and water, the extract was added to the top of the cartridge and the effluent collected into a suitable graduated collection tube. The cleaned-up extract was then pH-adjusted to pH 6.5 (± 0.5) before diluting the extract with water. Aliquots of this extract were then used for oxidation and subsequent LC-FLD analysis. SPE-COOH ion-exchange clean-up was used for all samples thought to contain N-hydroxylated PSP toxins (GTX1,4 and NEO). A sample of cleaned-up extract was passed through an ion-exchange cartridge pre-conditioned with 0.01M ammonium acetate and the effluent collected into a graduated tube labelled fraction 1 (F1). A further volume of water was added to the cartridge and the effluent collected also in F1. Further volumes of sodium chloride (NaCl) were passed through the cartridge; first 0.3M NaCl solution, then 2M NaCl solution, each enabling further fractions (F2 and F3) to be collected. The exact conditions used for this fractionation were developed and optimised in-house during this work and were described previously [13]. F1 contains the N-sulfocarbamoyl C-toxins (C1,2 and C3,4), F2 contains the Gonyautoxins (GTX) group of toxins (GTX1,4, GTX2,3, GTX5 and dcGTX2,3) leaving the carbamates (STX, dcSTX and NEO) to elute in F3. Sample extracts were analysed by first oxidising the relevant extracts and/or fractions to form fluorescent oxidation products. Oxidation methods used throughout the validation work were exactly those detailed in the AOAC 2005.06 method [12].

2.8 Validation of the AOAC 2005.06 method (extraction, clean-up and LC-FLD analysis) for PSP toxins.

In this study, validation was applied to the refined AOAC 2005.06 LC-FLD method, for the quantitation of PSP toxins and total saxitoxin equivalents in oyster and cockle homogenates. The primary aim was to check that the method performs adequately for the purpose over an appropriate range of PSP toxin concentrations in each shellfish species. Validation experimental design should ideally describe sources and sizes of error in analysis which includes random measurement error, run effect, laboratory effect, method bias and matrix variation, as well as the effects of analyte concentration on method performance. Availability of an appropriate certified reference material, traceable to international standards with a known level of uncertainty, will allow a laboratory to assess method bias and laboratory bias in a single step with the replicate analysis of materials in different batches over a specified time period [4]. Ideally, such materials should be naturally contaminated with the analytes of interest, in order for the materials to best mimic the behaviour of the analytes in any naturally contaminated samples. With such materials the accuracy of the method within the laboratory can be determined. Without the availability of certified materials, non-certified but well characterised reference materials may be used [4], although the assigned values for toxin concentrations will be subject to less defined levels of measurement uncertainty. Without any certified or characterised materials, a third option is to use analyte spiking and recovery information in order to estimate the method bias. It is noted however, that there may be additional levels of uncertainty associated with this approach, as recovery studies will only assess bias due to method performance effecting spiked analyte, with such effects not necessarily affecting the native, naturally occurring analytes. Specifically, for the determination of PSP toxins in shellfish homogenates, there is the potential for heterogeneous distribution of toxins throughout spiked samples and for differences in extraction efficiency and subsequent toxin recovery between spiked and naturally contaminated samples. Method validation work previously conducted for mussels [13] involved the repeat analysis of a well characterised mussel reference material, naturally contaminated in part with PSP toxins. With no such materials available for oysters and cockles, estimation of method bias was conducted through the repeat analysis of spiked homogenates. It is therefore noted that good recovery of analytes from spiked samples is not a guarantee of method accuracy. However, it is also noted that previous validation work conducted on mussels did not show much difference between using spiked samples and the candidate mussel reference material [13].

2.8.1. Method selectivity

Oyster and cockle tissue was extracted according to the AOAC method and as described above. A sub-sample of each extract was cleaned-up using C18 SPE cartridges prior to pH adjustment and aliquots then analysed using the optimised HPLC-FLD method with both periodate and peroxide oxidants. Periodate oxidation was carried out in the presence of matrix modifier as specified in the method [12]. Aliquots were analysed along-side un-oxidised sample extracts and standards containing PSP toxins in order to determine qualitatively whether oyster and cockle extracts contained any fluorescing compounds which may interfere with the presence of any PSP toxins. Results are presented in section 3.2.

2.8.2 Linearity and linear range of the HPLC-FLD method

In order to determine the range of selected toxin concentrations over which the quantitation method can be applied, PSP toxins were spiked into oyster and cockle extracts and also shellfish extract-free solvent (0.1mM acetic acid) to give a range of toxin concentrations, before subsequent oxidation and HPLC-FLD analysis. To prepare each calibration level, specific volumes of each PSP toxin mix were spiked into known volumes of C18-cleaned shellfish extract or solvent to produce the following concentrations (expressed in terms of fraction of the action level for STX i.e. 80 μg STX eq./100g): 0.0, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0 and 2.5 AL. The exception was GTX5, which was spiked at 10% of the concentrations described above. Toxin mixes were the same as utilised previously [13; Table 4]. The linearity of the calibrations was assessed over the standard working range (0 to 1.2 AL; GTX5 = 0 to 0.12 AL) and over an extended range (0 to 2.5 AL; GTX5 = 0 to 0.25 AL). Three aliquots of each calibrant solution were oxidised and analysed. Linear regression equations were generated and no weighting was placed on the calibration plot. The linearity of the analytical method was evaluated graphically, with visual inspection of calibration plots generated for individual toxins. Linear calibration graphs are presented (section 3.2) along with the associated correlation coefficients, gradients and intercepts. Correlation coefficients were generated using all triplicate data points rather than the mean value at each concentration level and calibration graphs are also plotted as such. Results are displayed together with calibrations generated by the analysis of toxins spiked into a shellfish extract-free solvent (0.1 mM acetic acid). This enables a visual comparison of gradients between solvent and shellfish-spiked standards and indicates whether there may be issues with fluorescence enhancement or suppression in any of the shellfish matrices.

A further examination of the extended data set involved the examination of the residuals after linear regression, graphs for which are also displayed (Appendix 1). Additionally an F-test “Goodness of fit” check, specifically the square of the standard deviation of the residuals divided by the standard deviation of the response factors, was compared against the F-critical (F_{crit}) value at 95% confidence associated with the appropriate degrees of freedom. An F-test value less than f-critical provides further evidence that linearity is indicated whilst a significant result ($F > F_{crit}$) test indicates that the residuals are more widely scattered than expected, providing evidence of non-linearity. The combination of visual, coefficient and residual analysis gives a good overall insight into the linearity of PSP toxins spiked into mussel extracts.

Table 4. Toxin mix nomenclature utilised during this study.

Toxin Mix	Toxins
I	GTX1,4, NEO
II	STX, dcSTX, GTX2,3, GTX5
III	C1,2, dcGTX2,3
dcNEO	dcNEO run separately due to limited availability of certified reference material

2.8.3 Determination of limits of detection

In this study, the limit of detection (LOD) is taken as the lowest injected amount of toxin that results in a chromatographic peak height at least three times as high as the baseline noise level surrounding the peak. LODs were determined for both the screening step and the full quantitation method. Values for screening method LODs were first predicted from the HPLC-FLD analysis of matrix-matched calibration solutions using the following calculation:

Predicted LOD = 3C/S

Where S = signal to noise (s/n) ratio of the toxin peak of the sample spiked and C = concentration of the spiked sample (μg STX eq./g).

Once these predicted LOD values were established, matrix extract spiking was performed at the predicted LOD concentration and triplicate oxidations and injections of the spiked solutions were carried out to confirm the s/n and establish the actual LOD and the variability of the screening step. Results are presented in section 3.3.1.
Full quantitation method LODs were estimated using s/n ratios measured in repeat analyses of oyster and cockle tissue homogenates spiked at 0.2 AL (the target concentration) for each toxin. Predicted method LODs were calculated using the same calculation as above and further triplicate homogenate spiking was performed at the predicted LOD concentration in order to confirm the measured s/n ratios and LOD values for each toxin. Triplicate oxidations for each triplicate spike were used to assess variability of the amount. Results are presented in section 3.3.2.

2.8.4 Determination of limits of quantitation of the method

Limits of quantitation (LOQ) are defined in this study as the concentration of analyte which gives rise to an analytical peak with a signal to noise ratio of 10:1. Shellfish tissue homogenates were spiked in triplicate at 0.2 AL for each toxin and the samples extracted, cleaned, fractionated and oxidised according to the method. Using the same approach as above, signal-to-noise ratios for each LC-FLD peak were measured to calculate the predicted concentration which would result in a signal to noise ratio of 10:1. This predicted concentration of each toxin was subsequently spiked in triplicate into cockle and oyster homogenates, extracted and analysed to experimentally confirm the predicted LOQs. Results are presented in section 3.3.3.

2.8.5 Determination of the accuracy of the extraction and analytical method for the quantitation of PSP toxins in cockles and oysters

The accuracy is defined here as the extent of the agreement between the analytical data generated and the 'true' value. In order to undertake such accuracy studies, a certified reference material should be extracted and analysed. As currently no such material is available in certified form for cockles and oysters, no accuracy studies can be undertaken using reference materials for these particular shellfish matrices and method bias was estimated through spiking and recovery studies.

2.8.6 Determination of the method recovery

Assessment of the recovery of PSP toxins from cockle and oyster tissues involved the spiking of homogenates with known amounts (addition by volume) of each toxin. Each 5 g sample of shellfish tissue homogenate was spiked with a PSP toxin mix to provide expected concentrations relating to 0.2 and 0.5 AL for each toxin (with the exception of GTX5 which was spiked at 0.02 and 0.05 AL), assuming 100 % method recovery. For each concentration, three separate 5g aliquots of homogenates were spiked, the sample tube was capped and vortex mixed for 1 min. Tissues were extracted and analysed according to the method described above, with oxidation and analysis carried out in triplicate. Analysis was carried out as soon as possible post-clean-up and oxidation, but in some instances extracts had to be stored overnight (4^{o} C) prior to oxidation. This was due to the high number of analyses required. Quantitation involved the comparison of toxin peak area responses obtained from oxidised spiked

samples with those obtained from oxidised toxin mix calibration solutions as described by AOAC 2005.06. Due to issues discussed previously [13], toxins were spiked as part of a toxin mix, as defined in Table 4. Recovery results are presented in section 3.4. Additional work was undertaken to determine the extraction efficiency of the method for Pacific oysters. Both spiked and naturally contaminated Pacific oyster homogenates were extracted and analysed for PSP toxins using the refined AOAC 2005.06 method as described. The remainder of the tissue material was extracted three more times, each with a further 3mL of 1% acetic acid, before the combination of all three extract supernatants were diluted to 10mL, cleaned-up and analysed. The relative concentrations of PSP toxins quantified following the initial extraction and the subsequent exhaustive extraction enabled the extraction efficiency of the method to be determined. Results are also presented in section 3.4.

2.8.7 Determination of method precision

Instrumental precision was assessed with the repeated analysis (n=10) over one analytical sequence of shellfish extract containing PSP toxins. Naturally contaminated material was used where possible (Pacific Oysters) to assess the precision of toxin peak areas, otherwise toxin-free extracts were spiked with toxin standards (cockles and native oysters). Limited availability of some toxin standards at the time of study restricted the levels at which some toxins could be spiked.

Method repeatability was assessed with the repeated extraction, clean-up and analysis of fortified cockle and oyster tissue. As noted in the previous study, limitations on batch length and the high numbers of analyses required for each experiment result in the need to place limitations on the experimental design. As such, the experiment was designed as follows:

- short term (intra-batch) repeatability was assessed on 3 repeat sample analyses in the same run at both 0.2 AL and 0.5 AL;
- medium term (inter-batch) repeatability was assessed on 6 replicates (0.2 AL and 0.5 AL) analysed in 2 batches of 3 samples, more than two weeks apart;
-long term precision was assessed with the repeated extraction, clean-up and analysis of a naturally contaminated laboratory reference material (LRM) over a period longer than three months. Due to the non-availability of naturally contaminated oysters and cockles through the official control monitoring programmes, live shellfish were fed mass-cultured toxic Alexandrium strains through in-house feeding experiments. Algal strains were chosen to represent most closely those observed in UK waters and which also contained a relatively high number of PSP toxins. As a result of these feeding experiments, two LRMs were created for native oysters (at different toxin concentrations) and one each for Pacific oysters and cockles. Shellfish contaminated with PSP toxins were shucked and homogenised according to laboratory protocol and 6-7g aliquots measured into plastic vials before long-term storage at –20^{o}C. Over a period of > 6months, aliquots were removed from storage, thawed and the required amount of homogenate weighed into a 50mL centrifuge tube and extracted according to AOAC 2005.06. Extracts were C18 cleaned, fractionated and quantified by HPLC-FLD.

The acceptability of the precision characteristics of the method was further assessed with the calculation of HorRat values. RSD values calculated for each toxin were compared against the Horwitz value derived from the Horwitz equation:

RSD_R (predicted) = $2^{(1-0.5\log C)}$ where C = concentration of analyte and RSD_R (predicted) is the Horwitz-predicted RSD for medium term repeatability.

The HorRat value for medium term repeatability ($HorRat_R$) was then calculated using the equation:

$HorRat_R = RSD_R$ (observed) / RSD_R (predicted)

The predicted RSDs for short term repeatability (RSD_r (predicted)) were calculated assuming predicted $RSD_r = 0.66$ predicted RSD_R. HorRat values were then calculated using the equation:

$HorRat_r = RSD_r$ (observed) / RSD_r (predicted)

HorRat values were calculated with values ≤ 2.0 inferring satisfactory levels of precision. Precision results are presented in section 3.5.

2.8.8 Method ruggedness

Ruggedness was assessed with the deliberate introduction of small variable changes into the method and the subsequent statistical analysis of any variations in analytical data emerging from these changes. A Plackett-Burman experimental design was used to analyse the effects of 7 key method parameters on the stability of the method, comparing the variability of these effects against method precision data. Due to the complexity of the method and the need to run each ruggedness experiment in a single analytical batch, two ruggedness experiments were performed for Pacific oysters where N-hydroxylated toxins were present in the naturally contaminated matrix. Due to the recent low availability of certified reference standards, only the non-N-hydroxylated standards were spiked into homogenates of cockles and native oysters. Experimental parameters chosen for study were those thought most likely to be matrix-dependent. These included extract pH, concentrations and volumes of oxidation reagents, oxidation parameters and matrix modifier pH. One 5g homogenate of each shellfish species was extracted according to the method. Each extract was cleaned eight times by C18 SPE and each cleaned up sample progressed through the ruggedness experiment as described in Tables 5 and 6.

Table 5. Experimental design for ruggedness testing of non N-hydroxylated toxins in Pacific oysters, native oysters and cockles

	Experiment number							
Parameter	1	2	3	4	5	6	7	8
C18 extract pH	pH6	pH6	pH6	pH6	pH7	pH7	pH7	pH7
Peroxide concentration	9.9%	9.9%	10.1%	10.1%	9.9%	9.9%	10.1%	10.1%
Vortex mixing time	3 sec	6 sec	3 sec	6 sec	3 sec	6 sec	3 sec	6 sec
Ambient temp during oxidation	22 °C	22 °C	25 °C	25 °C	25 °C	25 °C	22 °C	22 °C
Oxidation time	115 sec	125 sec	115 sec	125 sec	125 sec	115 sec	125 sec	115 sec
Volume glacial acetic acid	19 µl	21 µl	21 µl	19 µl	19 µl	21 µl	21 µl	19 µl
Sodium hydroxide concentration	0.9 M	1.1 M	1.1 M	0.9 M	1.1 M	0.9 M	0.9 M	1.1 M

Table 6. Experimental design for ruggedness testing of N-hydroxylated toxins in Pacific oysters

	Experiment number							
Parameter	1	2	3	4	5	6	7	8
C18 extract pH	pH6	pH6	pH6	pH6	pH7	pH7	pH7	pH7
Periodate pH	pH 8.15	pH 8.15	pH 8.25	pH 8.25	pH 8.15	pH 8.15	pH 8.25	pH 8.25
Vortex mixing time	3 sec	6 sec	3 sec	6 sec	3 sec	6 sec	3 sec	6 sec
Ambient temp during oxidation	22 °C	22 °C	25 °C	25 °C	25 °C	25 °C	22 °C	22 °C
Oxidation time	55 sec	65 sec	55 sec	65 sec	55 sec	65 sec	55 sec	65 sec
Volume glacial acetic acid	4 μl	6 μl	6 μl	4 μl	4 μl	6 μl	6 μl	4 μl
Matrix modifier pH	pH6	pH7	pH7	pH6	pH7	pH6	pH6	pH7

N-hydroxylated toxins (GTX1,4 and NEO) in Pacific oyster were analysed following fractionation of each of the eight C18 extracts and oxidation of fractions F2 and F3 respectively. Alongside the ruggedness analysis, eight C18 cleaned extracts and related fractions for each species were analysed following the normal method. This enabled the comparison of results against method precision using a significance test (t-test). Results are presented in section 3.6.

2.8.9 Method uncertainty

Results were used from the phase 1 validation studies to calculate an overall value of uncertainty for the measurement of PSP toxins in cockles, Pacific oysters and native oysters. In order to assess the overall method uncertainty, it was first necessary to identify each of the method parameters that contribute to measurement uncertainty. Once sources were described, the individual component uncertainties were calculated and propagated to calculate an overall measurement uncertainty. Expanded uncertainties were calculated using an appropriate coverage factor (k), in order to provide “an interval expected to encompass a large fraction of the distribution of values that may be attributable to the measurand” [17]. The contribution and effects of different sources of matrix modifier and sampling uncertainty are not discussed within this study. Measurement uncertainty results are presented in section 3.7.

2.9 Phase II. Analysis of samples using PSP-contaminated shellfish samples.

Phase II of the validation study involved the analysis of Pacific oysters, native oysters and cockles obtained through the PSP Official Control monitoring programme. In Phase II, the AOAC 2005.06 method (screen and quantitation elements) was tested in parallel with the mouse bioassay method (MBA). The aims of this exercise were to demonstrate that:

(1) the full method performs adequately as an analytical procedure for the quantitative analysis of PSP toxins in cockles and oysters,

(2) the screen successfully discriminates between contaminated and non-contaminated samples and

(3) the screen successfully identifies samples which contain PSP toxins at or above half of the regulatory action limit (0.5 AL).

However, non-availability of PSP-contaminated oysters and cockles initially prevented the full assessment of all three criteria (section 4.1). Due to the absence of such material in the official control monitoring programmes, experiments were conducted at

three different laboratories: Cefas, Scottish Association of Marine Science, Dunnstaffnage Marine Laboratory, Oban (SAMS) and Integrin Advanced Biosystems, Argyll, to feed oyster and cockle shellfish with mass cultured toxic *Alexandrium* strains. Different strains were utilised at the three laboratories and different culturing and feeding regimes conducted according to individual laboratory protocols. Once bulk batches of shellfish were fed algae for an appropriate length of time, the shellfish were separated into groups of sub-samples, each being shucked and homogenised. 5g aliquots of each sample or sub-sample were extracted and analysed according to the AOAC 200.06 method, whilst a 30g aliquot was extracted in HCl prior to MBA. MBA was only conducted if the samples were found to contain appropriate concentrations of PSP toxins by HPLC analysis. In the case of the cockle samples sourced from Cefas, three feeding experiment were conducted to produce high volumes of PSP-contaminated cockle homogenate, two of which were subsequently diluted using PSP-free cockle homogenate from the same source of cockles (cockles which were not fed *Alexandrium*). Appropriate sample dilutions were performed prior to thorough homogenisation to allow multiple HPLC/MBA comparisons to be performed using the three sources of cockles.

The criterion on which a shellfish sample was assigned positive was the following:
If PSP toxins exhibiting single oxidation product peaks were present with a diagnostic peak height showing a signal-to noise ratio of equal to or greater than 3:1 following periodate oxidation, then the sample was assigned positive.
If peaks were present corresponding to the diagnostic peak and the most significant secondary peak of those toxins exhibiting multiple oxidation products, then the sample was assigned positive.

Ordinarily, only positive samples would be progressed to full quantitation using the full AOAC 2005.06 method for further evaluation. However, for the purposes of this study, all samples were fully quantified to ensure none of the samples screened negative exhibited any positive toxin concentrations in subsequent quantitation. PSP concentration data derived from LC-FLD analysis were compared with MBA results and the correlation between the two data sets analysed statistically to determine whether there was any significant difference between the two methods. Results from the phase II testing are given in section 4.1.

Internal quality controls (IQC):

SOPs have been implemented in our laboratory for the analysis of mussels by the AOAC 2005.06 method, which detail the application of IQC to the routine monitoring of shellfish. The same procedures were utilised during the analysis of oysters and cockles.
The full quantitation sequence begins with a number of instrumental blanks and subsequently consists of initial calibrations of the three toxin standard mixes at five different concentration levels, evenly spaced over the 0 to 1.0 AL working range. Correlation coefficients calculated from these calibrations are checked to ensure toxin oxidation of standards is successful and the calibration curve fit for purpose for toxin quantitation. The sensitivity of analysis is checked by ensuring the signal to noise ratios of all quantitation peaks are ≥ 3.0. Retention times of initial calibration toxin standards are used to assign retention time windows enabling the integration and quantitation of toxins in samples. The retention time drift of toxin peaks within an analytical batch must be $\leq \pm 2.5\%$. A system suitability test is performed on each toxin mix to assess the chromatographic efficiency of the instrumental system, with values generated for peak

resolution, peak tailing and column efficiency (plates per metre). Acceptable tolerances have been defined and will be applied to the method. Within each batch, a procedural blank is run alongside the samples throughout the entire procedure (using 5mL 1% acetic acid instead of shellfish homogenate) and oxidised with periodate and peroxide oxidants prior to LC analysis. Additionally, an un-oxidised aliquot of C18 cleaned extract of each sample is analysed alongside the oxidised samples, so as to determine whether any of the peaks were due to the presence of naturally-fluorescent co-extractive interferences. Any components identified in either of these controls, are subtracted from the peak responses of any PSP toxins with the same retention times. A laboratory reference material (currently mussel homogenate) is extracted, cleaned, fractionated and analysed alongside each batch of samples. The toxin concentrations calculated for the peaks of interest are recorded on Shewhart quality control charts and must fall within ± 3 standard deviations (SD) for each toxin analysed. The continuing calibration check (0.6 AL) standard for each toxin mix is run through the sequence after every 20 injections. The results from this check are used to ensure that the initial calibration utilised for quantitation remains valid throughout the analytical batch. The analytical batch may continue for up to 24 hours.

Toxin quantitation

Each toxin was quantified by direct comparison of peak areas to external, certified analytical standards prepared at 0.2, 0.4, 0.6, 0.8 and 1.0 AL in 0.1mM acetic acid for each toxin (GTX5 prepared at 10% concentration of other toxins). Calibration solutions were prepared and oxidised in suitable mixes of toxins as advised by the AOAC 2005.06 method. As discussed in a previous report [13] toxicity equivalence factors (TEF) used are those published by Oshima [21].

3. Results and discussion

3.1. Selectivity of the method

In order to assess whether components of the shellfish matrices may have an effect on the quantitation of PSP toxins, the cockle, Pacific oyster and native oyster tissue extracts were analysed un-oxidised and after both periodate and peroxide oxidations. The results indicate an example of the interferences observed in chromatograms, but it is noted that variability of co-extractive interferences is expected to vary from sample to sample, as highlighted by Cefas in previous work [16].

Un-oxidised extracts

A profile of co-extractive matrix components was observed eluting up to 2 minutes in the chromatograms for cockles, Pacific oysters and native oysters (Figures 2 to 4). No additional chromatographic peaks were observed likely to interfere with diagnostic or secondary peaks of any toxin oxidation products. As such, the only peak affected is the tertiary oxidation peak of GTX1,4 (1.8min), which elutes close to the third significant matrix peak (1.9min) present in the cockles matrix.

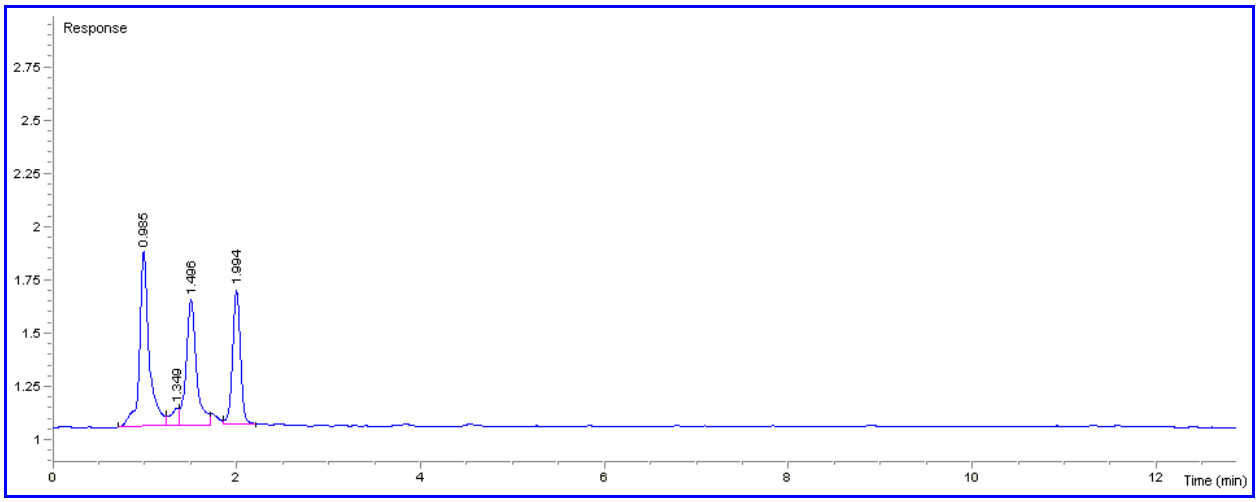

Figure 2. LC-FLD chromatogram of un-oxidised C18 cleaned cockle extract.

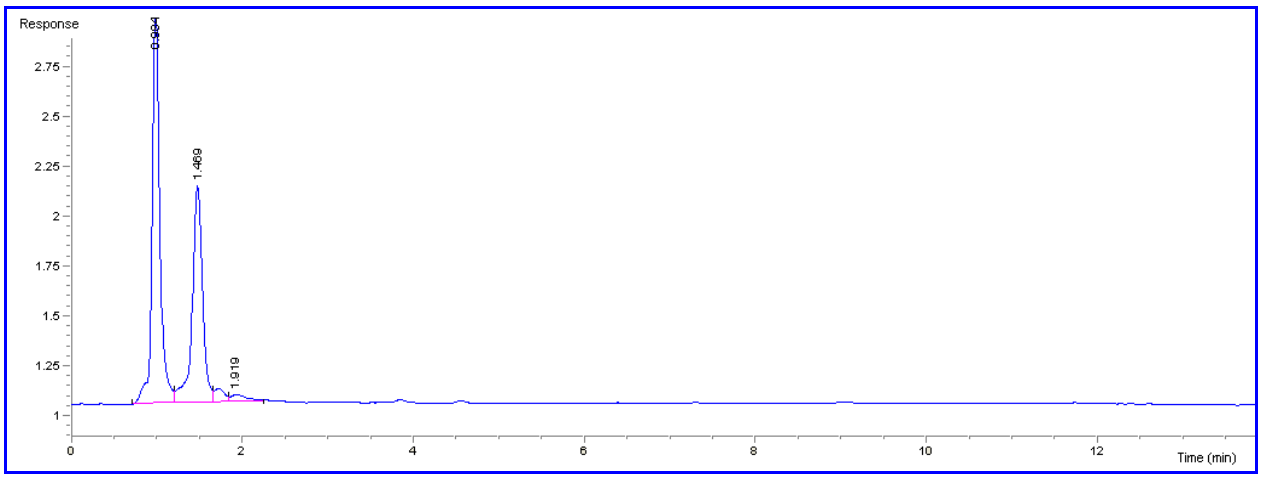

Figure 3. LC-FLD chromatogram of un-oxidised C18 cleaned Pacific oyster extract.

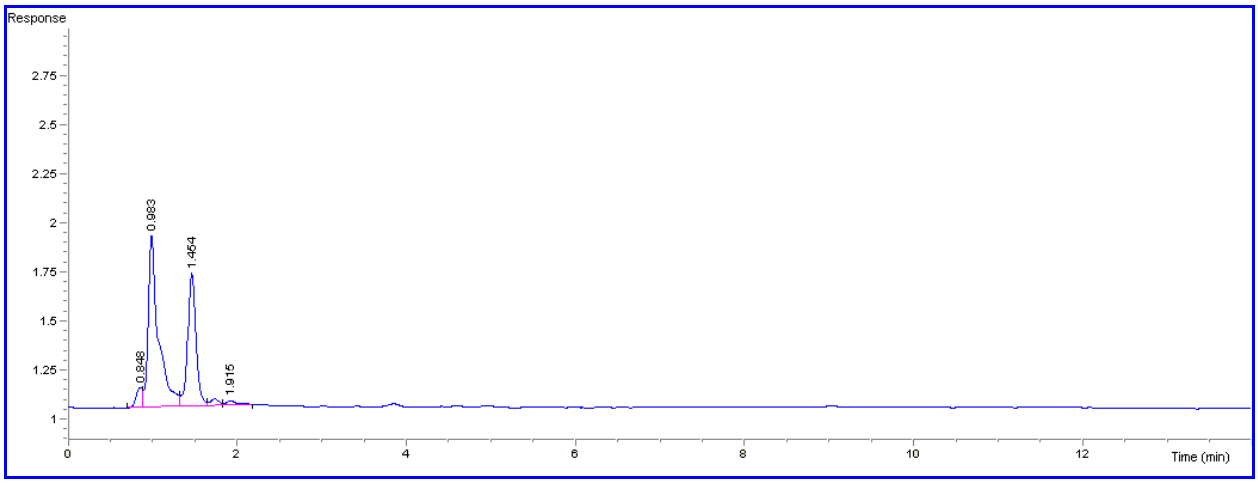

Figure 4. LC-FLD chromatogram of un-oxidised C18 cleaned native oyster extract.

Periodate oxidation

A profile of co-extractive matrix components was observed eluting up to 2 minutes in the chromatograms for the periodate oxidation of cockles, Pacific oysters and native oysters (Figures 5 to 7). No additional chromatographic peaks were observed in either of the oyster samples likely to interfere with diagnostic or secondary peaks of any toxin oxidation products. Two additional matrix components were observed in the cockles chromatogram at 3.8 and 5.1 mins (Figure 5). Whilst these are low in intensity and do not elute within 2.5% of the expected retention times of any of the PSP toxin oxidation products, they are close to the retention times of the tertiary NEO/dcSTX peak (3.4min) and the C1,2 (4.1min) and dcSTX (4.3min) diagnostic peaks. As such, any variability in such components or chromatographic shifts of matrix components as observed in the laboratory previously could potentially result in the presence of chromatographic interferences.

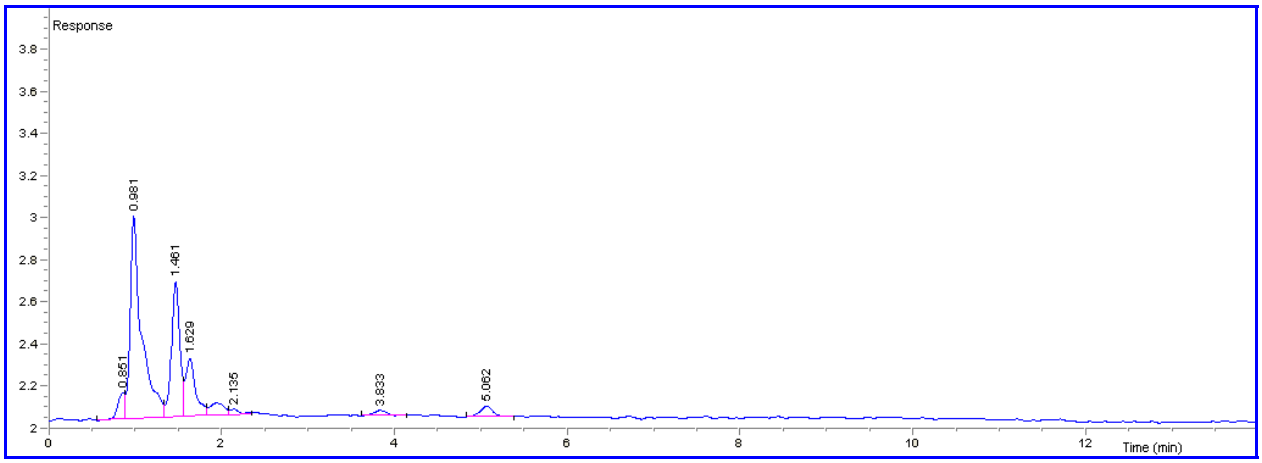

Figure 5. LC-FLD chromatogram of periodate-oxidised C18 cleaned cockle extract.

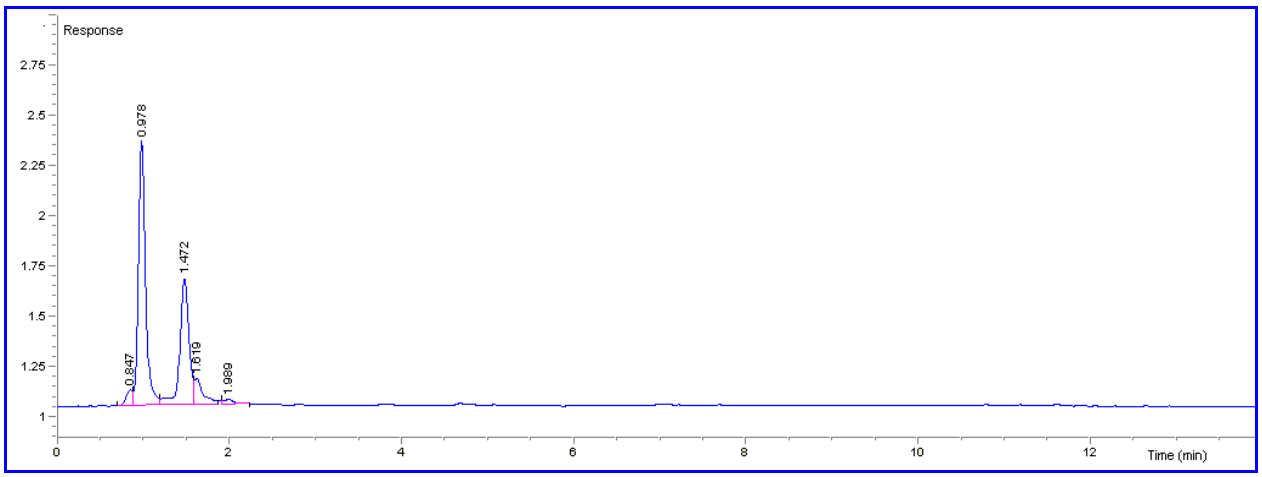

Figure 6. LC-FLD chromatogram of periodate-oxidised C18 cleaned Pacific oyster extract.

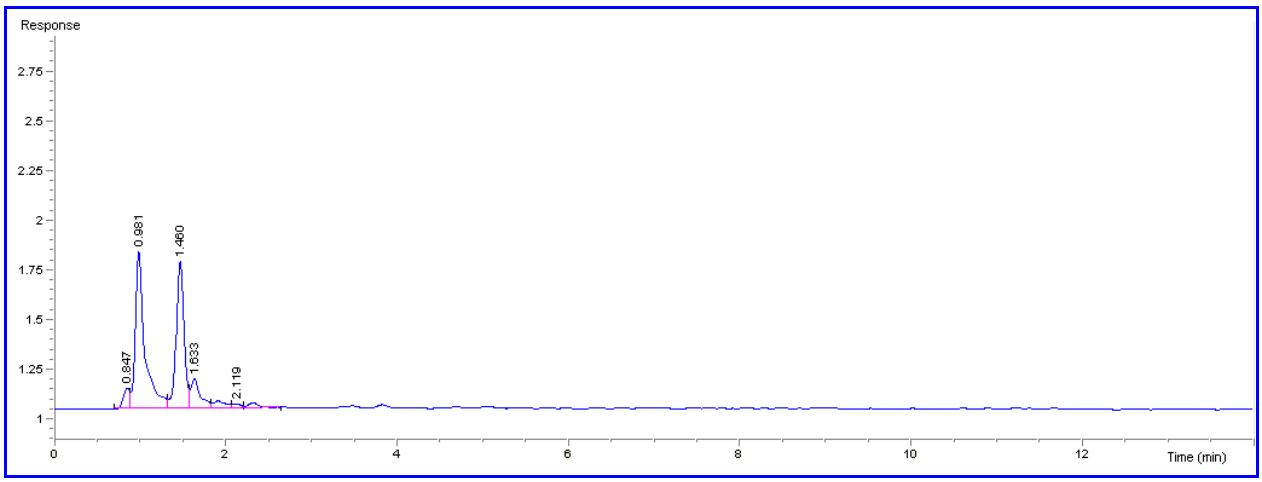

Figure 7. LC-FLD chromatogram of periodate-oxidised C18 cleaned native oyster extract.

Peroxide oxidation

31.

Analysis of the cockle and oyster extracts following peroxide oxidation showed similar chromatograms to those obtained for periodate and the unoxidised matrices (Figures 8 to 10). Two major matrix peaks are observed eluting before 1.5 minutes, which would not interfere with any of the toxin oxidation products. Furthermore, the chromatograms all show the absence of any further matrix components after 2.5 minutes. Cockles and native oysters both show a matrix component eluting at 2.4 minutes, therefore co-eluting with the diagnostic peak for dcGTX2,3, as also observed in mussels previously [13,15].

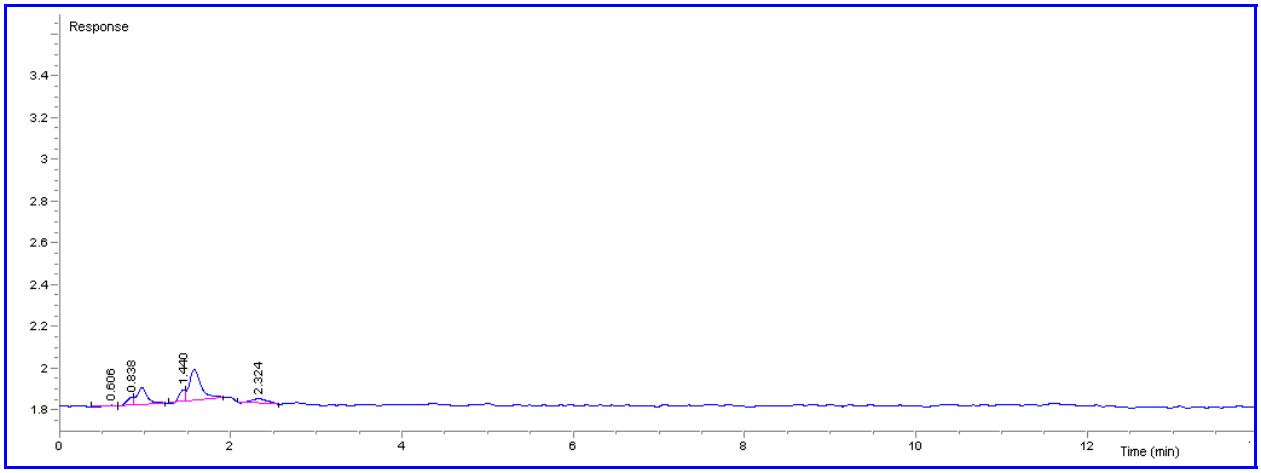

Figure 8. LC-FLD chromatogram of peroxide-oxidised C18 cleaned cockle extract.

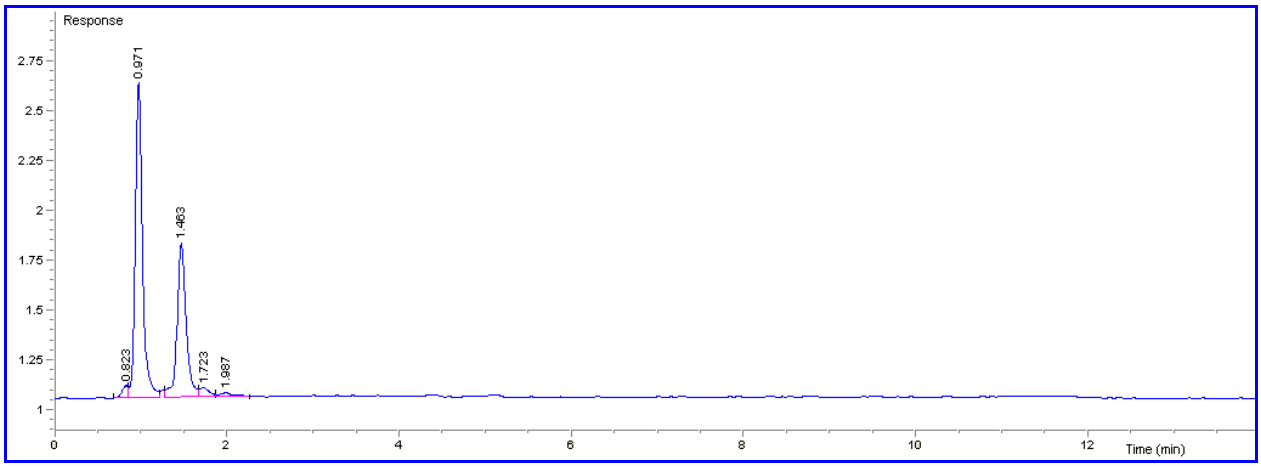

Figure 9. LC-FLD chromatogram of peroxide-oxidised C18 cleaned Pacific oyster extract.

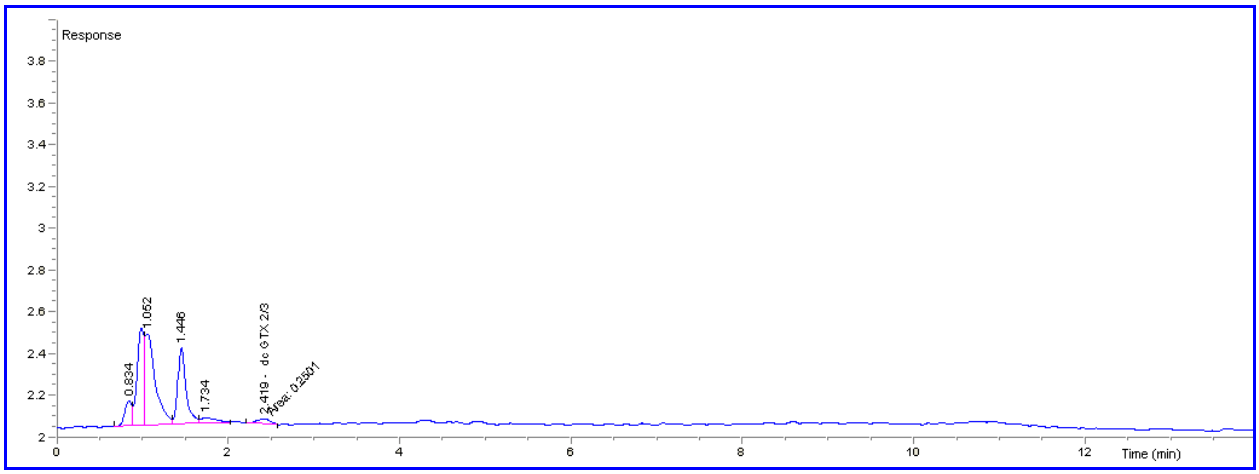

Figure 10. LC-FLD chromatogram of peroxide-oxidised C18 cleaned native oyster extract.

In summary, the chromatograms from the unoxidised, periodate and peroxide oxidised extracts of cockles, Pacific oysters and native oysters show relatively uncomplicated patterns of matrix co-extractive components. These appear less complicated than those observed previously for mussels [13,15]. Only a small number of peaks were shown to elute at the same retention times as toxin oxidation products, with the 2.4 min diagnostic peak of dcGTX2,3 the only primary toxin peak affected. As such, it is still recommended that unoxidised extracts are run alongside periodate and peroxide oxidised extracts during quantitation of cockle and oyster samples for PSP toxins to ensure any naturally fluorescence matrix co-extractives are accounted for.

3.2. Linearity of the analytical method

The LC-fluorescence detector was calibrated with individual PSP toxins prepared in either acetic acid or oyster/cockle extracts (after C18 and fractionation by COOH SPE). In all cases, results showed that a linear-fit model is the preferred model, with separate slopes for each matrix (solvent and extract/fraction). A summary of all the results is shown in Table 7 and the graphical results for each toxin are presented individually as follows.

3.2.1. Linearity for GTX1,4

Quantitation of GTX1,4 is performed by analysing the presence of this toxin in fraction F2, generated post ion exchange fractionation. Linearity observed in the fraction calibration graphs (Figure 11) appears acceptable for all 3 matrices (Table 7) and the random errors measured in the response factors range from 11 to 15%. The values calculated for the linearity of GTX1,4 in fraction matrix result in calibration gradients in good agreement between solvent and all 3 matrices (Figure 11, Table 7). The F-test goodness of fit results and F-critical value for the data set and visual examination of the residual plots (Appendix 1) for the fraction plot indicates a linear relationship exists between toxin concentration and analytical response.

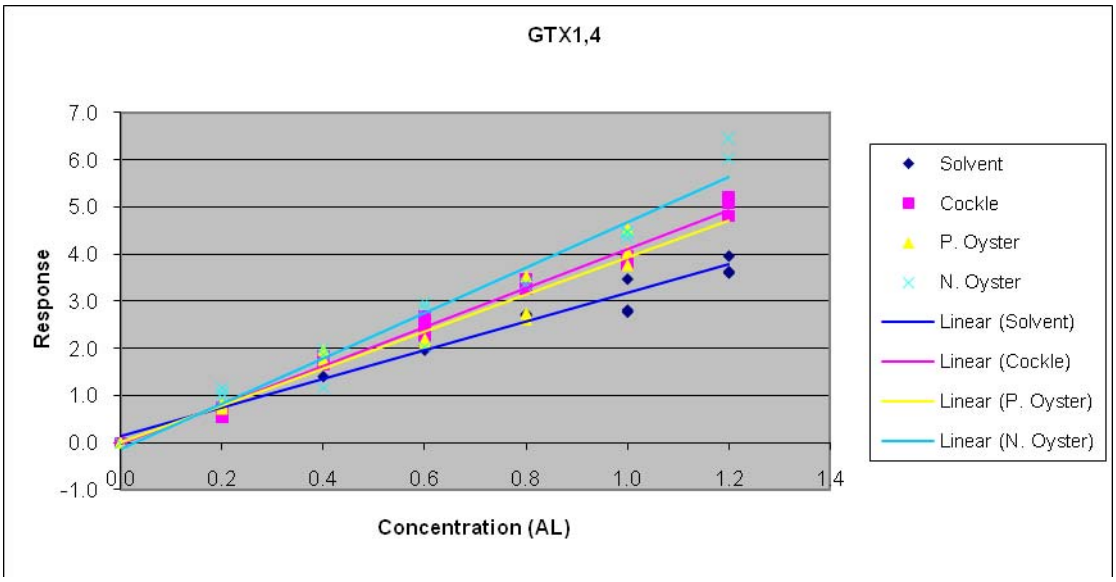

Figure 11. Calibration plots of GTX1,4 concentration against detector response for standard prepared in fraction extract and solvent over the working calibration range of 0 to 1.2 AL.

3.2.2. Linearity for NEO

Figure 12 displays the calibration graphs in fraction (F3) for cockles and oysters plus the solvent matrix for the periodate oxidation of NEO toxin. Visual inspection indicates good linearity for fractions and solvent, with the exception of native oysters where a correlation coefficient of 0.95 is returned. F-test goodness of fit checks for all three matrices indicate a linear relationship exists for all matrix calibrations, with values all significantly lower than F-critical. Visual examination of residual plots (Appendix 1) confirms that there is no apparent systematic deviation of the residuals from zero. The calibration slopes appear almost identical for solvent and cockles, with Pacific and native oysters both similarly exhibiting higher gradients (Table 7).

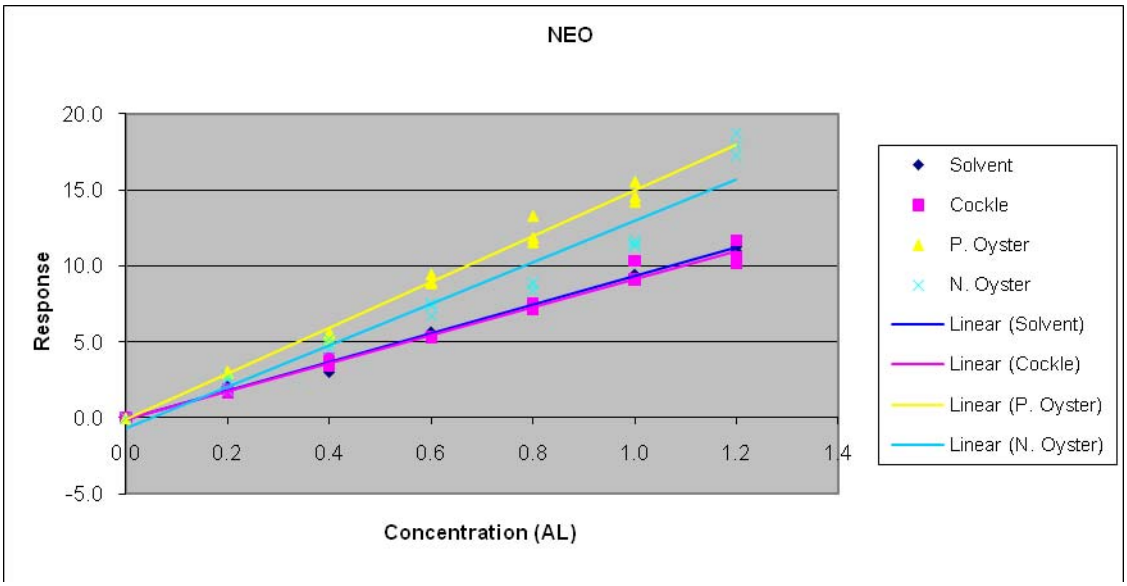

Figure 12. Calibration plots of NEO concentration against detector response for standard prepared in cleaned-up tissue extract, fraction extract and solvent over the working calibration range of 0 to 1.2 AL.

3.2.3. Linearity for dcSTX

Peroxide oxidation of solvent and matrix dcSTX-fortified solutions reveals three calibrations very close in gradient (solvent, native oysters and cockles; Figure 13), with Pacific oysters exhibiting a significantly higher calibration slope. The random errors associated with the calibrations appear small with an RSD of 5% for the response factors of the dcSTX calibration in Pacific oyster extract. The F-test check furthermore indicates a linear relationship (Table 7) for all matrices, and insignificant intercept values confirms the qualitative observation that there are no significant interferences associated with the quantitation of this toxin. Hence it appears as though the relative increase in gradient slope associated with the Pacific oysters is truly indicative of matrix-triggered peak enhancement and the results obtained from recovery experiments in this matrix should be analysed carefully for any further evidence of this behaviour.

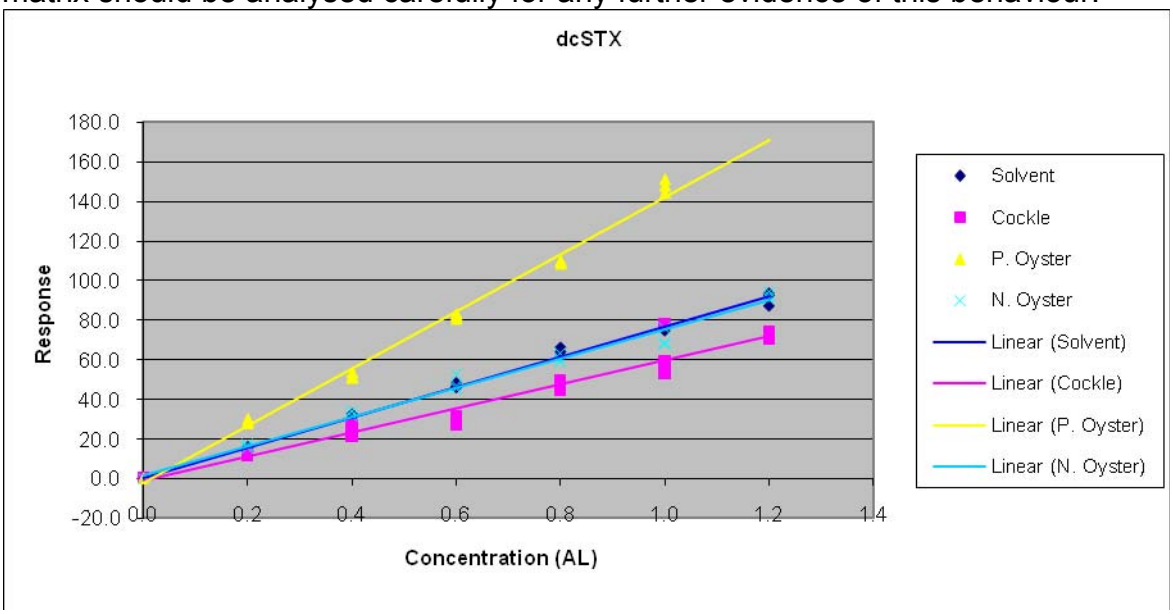

Figure 13. Calibration plots of dcSTX concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.2.4. Linearity for GTX2,3

Visual inspection indicates a good linear fit for both all matrices (Figure 14). F-test goodness of fit checks again indicate a linear relationship between concentration and response and the closeness of agreement between cockle and native oyster extracts and solvent standards shows there are no matrix effects present in these shellfish extract for this particular toxin. However, as with dcSTX, a significant enhancement in Pacific oysters is evidence by an increased calibration gradient.

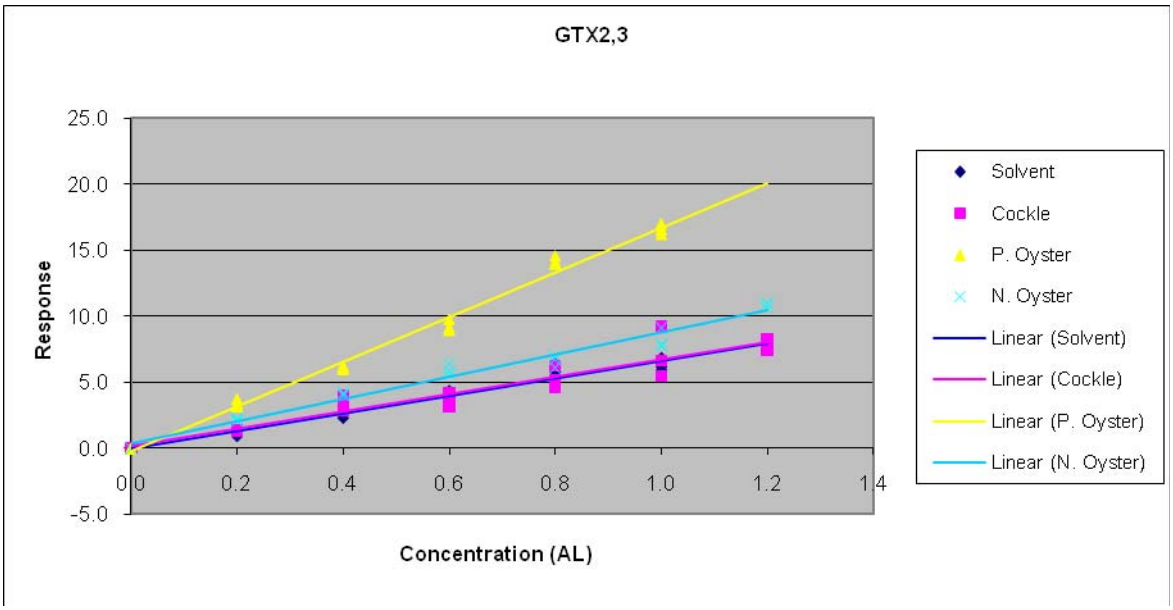

Figure 14. Calibration plots of GTX2,3 concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.2.5. Linearity for GTX5

Figure 15 shows that the gradients of the cockle and native oyster extracts and solvent calibration plots are very similar and appear linear over the working range (0 to 0.12 AL). The F-test results again indicate a linear relationship between concentration and detector response in both all extracts and solvent standards and this is further evidence by the random nature of the residual scatter plot (Appendix 1). Again, enhancement is evident in the Pacific oyster matrix, which could result in a positive analytical bias seen in this matrix.

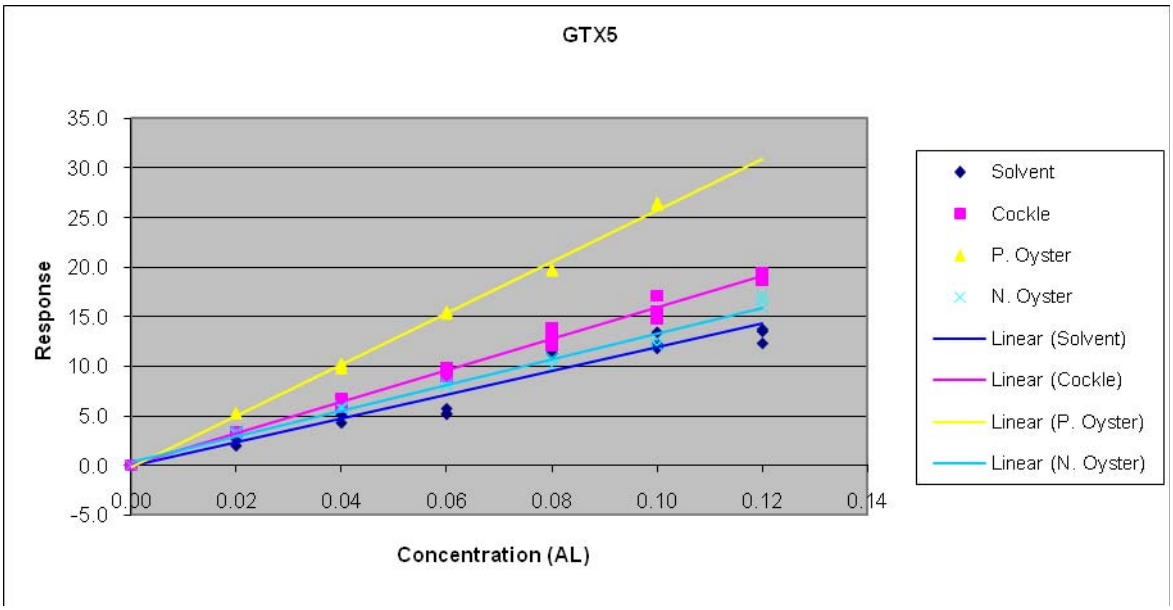

Figure 15. Calibration plots of GTX5 concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 0.12 AL.

3.2.6. Linearity for STX

Examination of the calibration graphs and gradients for STX in native oyster and cockle extracts and solvent (Figure 16; Table 7), indicates close similarities between the calibrations. However, again the responses of toxin in Pacific oyster extracts appear significantly enhanced, in this instance generating a calibration slope double the mean of the other three matrices (including solvent). The correlation coefficients of all three shellfish matrix calibrations demonstrate excellent linearity and the variation in response factors over the entire calibration range is low. F-test goodness of fit checks for all extract calibrations, together with a visual examination of the residual plots (Appendix 1) indicates a linear relationship between detector response and STX concentration.

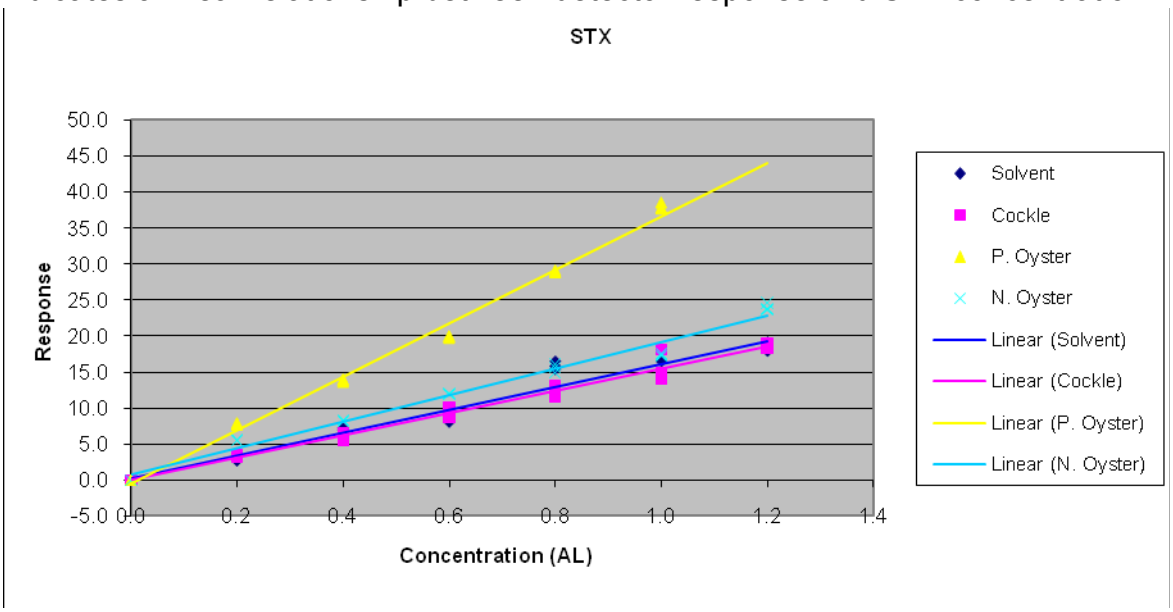

Figure 16. Calibration plots of STX concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.2.7. Linearity for C1,2

Visual inspection of calibration graphs for all matrices and solvent (Figure 17) and residual plot (Appendix 1) indicates a good degree of linearity, with F-test linearity test values all significantly below F-critical, inferring linearity is still associated with the relationship. RSDs of the response factors are low for all matrices indicating the low degree of random scatter associated with the calibrations (Table 7). As opposed to previous non-N-hydroxylated toxins, the response of C1,2 is not enhanced in the Pacific oyster matrix, with calibrations in all matrices showing fairly similar values, although the response in native oysters does show a slightly elevated gradient.

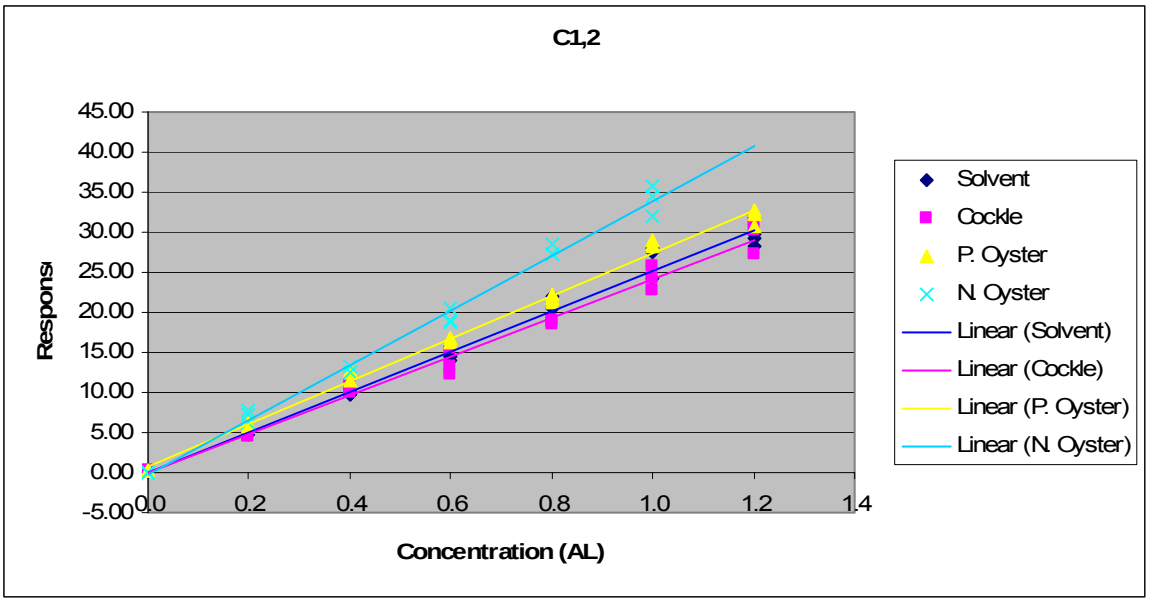

Figure 17. Calibration plots of C1,2 concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.2.8. Linearity for dcGTX2,3

The calibration graphs of the three shellfish extracts (Figure 18), do not show any issues relating to the possible matrix interferences discussed previously (section 3.1), with intercept values appearing low in all cases. The linearity of the calibrations appear visually acceptable although the correlation coefficients are < 0.98 for cockles and in solvent. Residual plots (Appendix 1) show low residual values for dcGTX2,3 in all extracts resulting in acceptable F-test goodness of fit. The spread of calibration gradients is noticeable, with all matrices exhibiting different slopes. As with C1,2 toxin, native oyster extract results in the highest gradient, with Pacific and cockles seemingly exhibiting a degree of toxin peak suppression (Figure 9)

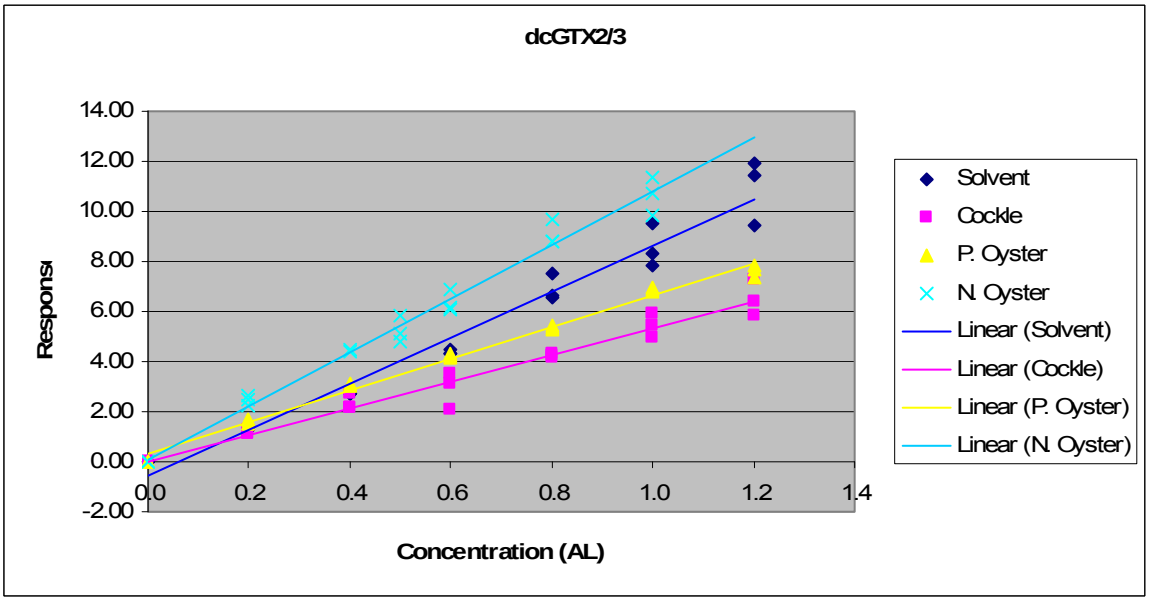

Figure 18. Calibration plots of dcGTX2,3 concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

Table 7. Linear regression gradients, intercepts and coefficients, plus relative standard deviations of response factors and F-test goodness of fit (F critical = 2.0) calculated for each PSP toxin in extracts of cockles, native oysters, Pacific oysters and solvent over working calibration range (0 to 1.2 AL).

Toxin	Matrix	Calibration gradient	Intercept	r^2	RSD% of response factors	F-test
GTX1,4	Solvent	3.05	0.138	0.970	13%	0.26
	Cockles	4.12	-0.025	0.989	11%	0.17
	P. Oysters	3.51	0.008	0.960	12%	0.46
	N. Oysters	4.81	-0.127	0.964	15%	0.31
NEO	Solvent	9.39	-0.056	0.996	8%	0.11
	Cockles	9.21	-0.064	0.990	5%	0.63
	P. Oysters	13.74	-0.084	0.991	6%	0.55
	N. Oysters	13.58	-0.652	0.949	13%	0.65
dcSTX	Solvent	76.66	0.469	0.996	4%	0.34
	Cockles	61.32	-1.154	0.957	13%	0.55
	P. Oysters	128.80	-2.115	0.994	5%	0.86
	N. Oysters	74.15	1.575	0.989	7%	0.37
GTX2,3	Solvent	6.63	0.026	0.971	13%	0.30
	Cockles	6.54	0.196	0.916	18%	0.42
	P. Oysters	15.53	-0.168	0.991	7%	0.42
	N. Oysters	8.43	0.355	0.977	12%	0.24
GTX5	Solvent	119.3	0.034	0.930	17%	0.04
	Cockles	158.4	0.056	0.993	5%	0.04
	P. Oysters	258.0	-0.212	0.995	3%	0.04
	N. Oysters	129.6	0.333	0.987	10%	0.02
STX	Solvent	15.91	0.252	0.956	13%	0.45
	Cockles	15.51	0.027	0.985	7%	0.50
	P. Oysters	37.06	-0.458	0.993	6%	0.26
	N. Oysters	18.45	0.748	0.983	16%	0.10
C1,2	Solvent	25.10	0.113	0.988	6%	0.63
	Cockles	24.16	0.051	0.986	7%	0.48
	P. Oysters	26.68	0.781	0.994	6%	0.23
	N. Oysters	34.16	-0.415	0.988	7%	0.21
dcGTX2,3	Solvent	9.20	-0.536	0.971	14%	0.35
	Cockles	5.34	0.026	0.966	13%	0.36
	P. Oysters	6.36	0.302	0.991	10%	0.12
	N. Oysters	10.73	0.150	0.988	8%	0.23

3.2.9 Summary of conclusions from linearity results

Statistical analysis of calibrations using both correlation coefficients and F-test goodness of fit of the residuals, indicates there are no significant systematic deviations from linearity within any of the matrices examined for the working range of 0 to 1.2 AL for any of the PSP toxins studied (Table 7). RSDs of response factors are generally low (3 to 18%, mean 10%) indicating an acceptable level of random scatter within the calibration plots. The statistical results are enhanced by visual examination of the regression and residual plots, which do not indicate the presence of any systematic deviation of the residuals from zero.

Differences in slopes of the calibrations between solvent and the three shellfish extracts were noticeable and variable between toxins. The non N-hydroxylated toxins dcSTX, GTX5, GTX2,3 and STX all exhibited a significant level of toxin enhancement in Pacific oyster matrix, but not in cockles or native oysters. No such enhancement was evident for the N-hydroxylated toxins and for C1,2, and conversely a significant level of dcGTX2,3 suppression was evident in Pacific oyster and cockle extracts.
Whilst the linearity of the method has been demonstrated for all toxins in Pacific oysters, native oysters and cockles, the presence of possible enhancement and suppression may result in a positive bias for the analysis of some toxins in Pacific oysters and the negative bias of dcGTX2,3 analysis in cockles and native oysters. As such, these signal enhancements could result in subsequent enhanced or reduced apparent recoveries (section 3.4).

Extension of the calibration study to incorporate higher concentration values indicated that for most toxins the linearity of the working range was extended successfully to higher concentrations (2.5 to 3.0 AL). The exception is GTX1,4 in Pacific oysters where a quadratic component to the regression is noticeable and the deviation from linearity above 1.0 AL is clear (Appendix 2). Replacement of the linear regression coefficient with a polynomial regression, results in a correlation coefficient (r^2) of 1.000, illustrating the degree of curvature associated with this calibration graph. This infers that the quantitation of GTX1,4 in Pacific oysters will be compromised at concentration levels above 1.0 AL.

It is noted that C1,2 toxin, found to exhibit a non-linear behavior in mussels, exhibits perfectly linear calibration statistics up to 3.0 AL in all matrices studied here. A summary of linear regression gradients, intercepts and coefficients, plus relative standard deviations of response factors calculated for each PSP toxin in each matrix over the extended calibration range is tabulated in Appendix 3.

3.2.9.1 Repeatability of matrix effects

In order to assess whether the apparent matrix effects described above are repeatable in other Pacific oyster samples, a different source of oysters was acquired and the linearity experiment repeated over the working calibration range (0 to 1.2 AL) for Pacific oysters. Results are tabulated in Table 8 and show that with exception of GTX1,4 and NEO (which illustrate a degree of matrix suppression in the oyster calibrants) a close correlation between the calibration gradients prepared in solvent and oyster matrix. It is therefore evident that the matrix effects illustrated in table 7 for Pacific oysters are not repeatable when using a different source of oysters obtained from a different geographical location at a different time of year. Without a full investigation into the variable matrix effects observed with different sources of Pacific oysters, these results indicate the likelihood for variable degrees of matrix effects between samples from different temporal and spatial origins. It should also be noted that the use of Pacific oysters from different sources as a matrix modifier as specified by the AOAC 2005.06 method may potentially result in variability in chromatographic response. Whilst such effects would be expected to effect the calibration standards and the samples to the same extent, this has not yet been studied within the laboratory.

Table 8. Linear regression gradients and coefficients calculated for PSP toxins in extracts of Pacific oysters and solvent over the working calibration range (0 to 1.2 AL).

Toxin	Matrix	Calibration gradient	r2	Percentage difference in PO gradient compared to solvent calibration
GTX1,4	Solvent	0.973	1.000	
	P. Oysters	0.865	0.993	89%
NEO	Solvent	3.316	0.995	
	P. Oysters	2.509	0.996	76%
dcSTX	Solvent	93.138	1.000	
	P. Oysters	91.516	0.998	98%
GTX2,3	Solvent	18.250	0.999	
	P. Oysters	18.516	0.998	101%
GTX5	Solvent	12.884	0.999	
	P. Oysters	12.679	0.999	98%
STX	Solvent	22.943	0.999	
	P. Oysters	22.304	1.000	97%
C1,2	Solvent	55.000	0.999	
	P. Oysters	55.444	0.999	101%
dcGTX2,3	Solvent	15.561	0.999	
	P. Oysters	15.057	0.998	97%

3.2.9.2 Options for overcoming problems with fluorescence enhancement and suppression

Results (Table 7) have indicated the potential for fluorescence enhancement and suppression for individual PSP toxins in each of the three shellfish matrices studied here. Additional evidence (Table 8) has shown that such matrix effects are not repeatable within same species animals, when samples are obtained from different locations and at different times of the year. Overcoming such effects is typically performed through use of matrix-matched calibration standards, use of standard addition or with use of a suitable internal standard. In the first instance, toxin analytes are spiked into matrix extract instead of pure solvent, as was conducted in the linearity experiments above. However, given the variability in signal enhancement observed here with two different sources of Pacific oysters (Tables 7 and 8), use of a single oyster extract for matrix-matched standard preparation will not be an appropriate measure to take to resolve any potential issues with toxin recovery in oysters. It can also be assumed that such variability would also be expected in other species. Secondly, use of standard addition for quantitation of PSP toxins in shellfish would require spiking known concentrations of PSP toxins into each shellfish sample received for PSP testing. Given the required high throughput within the laboratory (up to 40 samples per day) and the need to spike each sample with multiple toxin mixes each at a minimum of four calibration levels, this is clearly not a practical approach to quantifying PSP toxins on a routine basis. The third approach involves spiking samples with a known concentration of a suitable internal standard. Such compounds need to be analytically selective, which without mass spectral detection would require the compound to be chromatographically separated from all other PSP toxins. The expected peak size of the internal standard would then be used to apply a recovery factor to the PSP toxins being quantified in order to eliminate or at best reduce the effects of method bias, which include signal enhancement and suppression effects. However, currently no such approach has been tested for this LC-FLD method, and even if available, the assumption that each toxin will behave similarly to any internal standard would be flawed, given the variability in fluorescence enhancement and suppression observed between different PSP toxins

here (Tables 7, 8). As such, the only practical way to quantify PSP toxins potentially exhibiting enhancement or suppression is to quantify against solvent standards as is currently performed for mussels and accept that a degree of variability in signal response will contribute to the overall measurement uncertainty for the method.

3.3. Limits of detection and quantitation for PSP toxins in oysters and cockles

3.3.1. Limits of detection for screening method

Instrumental LODs were calculated for the periodate oxidation of all toxins in cleaned-up oyster and cockle extracts in order to describe the limits of detection for the screening part of the method (2.8.3). This represents the ability of the method to detect peaks which may easily be identified and quantified by peroxide but which may possibly be missed by any inherent lower sensitivity of the periodate-oxidised initial screening analysis. All LOD values are tabulated (Table 9) in terms of mean (± one standard deviation (sd, n=3) where the standard deviation is calculated from the signal to noise values measured for each of the triplicate results for each individual toxin. Values were calculated for the primary (diagnostic) toxin peak. In addition, approximate signal to noise ratios are tabulated for the secondary toxin peaks at the LOD concentrations spiked.

For the range of toxins investigated here, LODs of ~0.02 to 0.18 µg STX eq./g (0.03 to 0.22 AL) were determined for the periodate screening method of C18-cleaned extracts of shellfish tissue. Whilst these values were not derived from homogenate spiking experiments, they give a good indication of the sensitivity of the screening method. The presence of secondary toxin peaks at such low concentrations ensures the screening step is sufficiently sensitive to enable toxins < 0.2 AL to be progressed to full quantitation, with only GTX1,4 in Pacific oysters and STX in native oysters showing an LOD close to this limit. These values for oysters and cockles appear similar to those calculated for mussels previously [13,15], with C1,2 toxins being detected at a lower level than in mussels and only GTX1,4 in Pacific oysters and STX in native oysters showing a lower level of sensitivity than in mussels. The approximate signal to noise ratios for secondary peaks tabulated in Table 9 indicate that toxins present around the LOD will exhibit a secondary peak thus resulting in a positive toxin identification at this level. For dcNEO, the mean signal to noise ratios measured in the 0.2 AL spiked sample were 18, 24 and 20 for Pacific oyster, native oyster and cockles respectively, thus demonstrating that the analytical sensitivity of the periodate screen for dcNEO is sufficient to detect this toxin at levels significantly lower than 0.16 µg STX eq./g. Predicted LODs (extrapolated from observed signal to noise ratios) are also tabulated in Table 9.

Table 9. Limits of detection (LOD; µg STX eq./g ± 1 sd) of the LC-FLD screening method for the primary toxin peaks of PSP toxins following periodate oxidation of C18-cleaned oyster and cockle extracts. Approximate signal to noise ratios of secondary toxin peaks and predicted LOD for dcNEO also shown.

		LOD (µg STX eq./g) ± sd			Approximate signal to noise ratios of secondary toxin peaks		
Toxin	Rt (min)	Cockles	Pacific Oysters	Native Oysters	Cockles	Pacific Oysters	Native Oysters
GTX 1,4	2.8	0.09 ± 0.018	0.18 ± 0.015	0.08 ± 0.015	>3	>3	>3
NEO	5.3	0.04 ± 0.010	0.06 ± 0.008	0.05 ± 0.010	>3	>3	1 – 3
dcNEO*	4.5	< 0.16 (0.024*)	< 0.16 (0.027)	< 0.16 (0.020)	Na	Na	Na
dcSTX	5.3	0.09 ± 0.016	0.04 ± 0.008	0.07 ± 0.020	<1	1 – 3	1 – 3
GTX 2,3	6.8	0.06 ± 0.014	0.05 ± 0.009	0.08 ± 0.017	Na	Na	Na
GTX 5	8.8	0.06 ± 0.008	0.03 ± 0.003	0.06 ± 0.013	Na	Na	Na
STX	9.5	0.05 ± 0.011	0.09 ± 0.007	0.18 ± 0.040	Na	Na	Na
dcGTX 2,3	2.8	0.03 ± 0.007	0.03 ± 0.004	0.02 ± 0.005	1 – 3	1 – 3	1 – 3
C 1,2	4.2	0.06 ± 0.017	0.07 ± 0.015	0.05 ± 0.016	Na	Na	na

Na: not applicable (only one oxidation product expected)
* Predicted dcNEO screening LOD

3.3.2. Limits of detection for quantitation method

Table 10 tabulates method LODs for the quantitation of PSP toxins in oysters and cockles. GTX1,4 and NEO exhibit the highest LODs (0.16 µg STX eq./g; 0.2 AL) in all three matrices, whereas all others show significantly higher analytical sensitivities (0.002 to 0.12 µg STX eq./g; ~0.003 to 0.15 AL). It is therefore clear that whilst the method is capable of detecting most toxins at concentrations significantly below the target of 0.2 AL per toxin, the sensitivity of GTX1,4 and NEO analysis is at this limit. Comparison of these results with those obtained previously in mussels [13,15] shows a good degree of similarity between the species. The method is noticeably less sensitive for NEO in oysters and cockles as compared to mussels. Table 10 also compares the LODs against the values quoted in the AOAC 2005.06 method. Similar results are illustrated with the exception of GTX1,4 and NEO. It should be noted however that the AOAC 2005.06 method validation does not give any information as to how the LODs were obtained or which species they are thought to apply to. Furthermore it is not clear whether the AOAC values relate to a method LOD or to an instrumental sensitivity. The LOD for dcGTX2,3 indicates good levels of sensitivity for this toxin in all three matrices but the toxin was not studied within the AOAC method and no direct comparison is possible here. For dcNEO, the mean signal to noise ratios measured in the fractions of the 0.2 AL spiked sample were 7, 11 and 10 for Pacific oyster, native oyster and cockles respectively, thus demonstrating that the analytical sensitivity for dcNEO quantitation is sufficient to detect this toxin at levels significantly lower than 0.16 µg STX eq./g. The predicted LODs for dcNEO are also shown in Table 10.

Table 10. Limits of detection (LOD; µg STX eq./g ± 1 sd) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions and peroxide oxidation of C18-cleaned oyster and cockle extracts.

		LOD (µg STX eq./g) ± sd			
Toxin	Quantitation method	Cockles	Pacific Oysters	Native Oysters	AOAC 2005.06 LOD (µg STX eq./g)
GTX 1,4	Periodate (F2)	0.16 ± 0.04	0.16 ± 0.05	0.16 ± 0.04	0.05
NEO	Periodate (F3)	0.16 ± 0.04	0.16 ± 0.03	0.15 ± 0.02	0.04
dcNEO*	Periodate (F3)	< 0.16 (0.05*)	< 0.16 (0.07*)	< 0.16 (0.04*)	Na
dcSTX	Peroxide extract	0.009 ± 0.004	0.004 ± 0.001	0.008 ± 0.002	0.004
GTX 2,3	Peroxide extract	0.12 ± 0.05	0.05 ± 0.01	0.10 ± 0.04	0.08
GTX 5	Peroxide extract	0.004 ± 0.001	0.0017 ± 0.0004	0.0029 ± 0.0008	0.002
STX	Peroxide extract	0.025 ± 0.010	0.011 ± 0.003	0.020 ± 0.006	0.022
dcGTX 2,3	Peroxide extract	0.047 ±0.010	0.029 ± 0.007	0.037 ± 0.010	Na
C 1,2	Peroxide extract	0.017 ± 0.004	0.010 ± 0.003	0.017 ± 0.004	0.01

Na = no data available
* Predicted LOD for dcNEO shown in brackets

3.3.3. Determination of the limit of quantitation of the method

The results from the experimental confirmation of calculated LOQs are summarised in Table 11. The results show that the experimentally confirmed LOQs range from 0.006 to 0.34 µg STX eq./g for the non-N-hydroxylated toxins. The LOQs for all these toxins, with the exception of GTX2,3, are at or lower than the target concentration level of 0.2 AL. GTX2,3 exhibits higher LOQs with values ranging from 0.16 to 0.34 µg STX eq./g for the three matrices. Results obtained for the N-hydroxylated toxins GTX1,4 and NEO show higher levels of LOQ ranging from 0.34 to 0.40 µg STX eq./g. As such, the method exhibits very similar sensitivities to those reported previously for mussels [13,15]. With GTX1,4, GTX2,3 and NEO, the degree of confidence in the method at 0.2 AL may therefore be questionable. However, results from the medium-term precision analysis of these three toxins at 0.2AL show a good level of precision (RSDs 9% to 17%) and HorRat values all <2, ranging from 0.42 to 0.81 (Tables 15 to 17). From these results it is argued that the level of precision is acceptable at 0.2 AL and that quantitation of GTX1,4 can therefore be carried out with a good degree of precision well below the level of quantitation. This argument is supported by the IUPAC guidelines for single-laboratory validation of methods of analysis, which state that measurements below the limit of quantitation are not devoid of information content and may well be fit for purpose [4]. For dcNEO, the mean signal to noise ratios measured in the fractions of the 0.4 AL spiked sample were 17, 27 and 20 for Pacific oyster, native oyster and cockles respectively, thus demonstrating that the analytical sensitivity for dcNEO is sufficient to quantify this toxin at levels significantly lower than 0.32 µg STX eq./g. The predicted LOQs for dcNEO are also shown in Table 11.

Table 11. Limits of quantitation (LOQ; µg STX eq./g ± 1 sd.) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions and peroxide oxidation of C18-cleaned shellfish extracts.

		LOQ (µg STX eq./g) ± sd		
Toxin	Quantitation method	Cockles	Pacific Oysters	Native Oysters
GTX 1,4	Periodate (F2)	0.34 ± 0.046	0.40 ± 0.086	0.40 ± 0.10
NEO	Periodate (F3)	0.35 ± 0.055	0.40 ± 0.076	0.40± 0.04
dcNEO*	Periodate (F3)	< 0.32 (0.20*)	< 0.32 (0.28*)	< 0.32 (0.18*)
dcSTX	Peroxide extract	0.02 ± 0.004	0.01 ± 0.002	0.03 ± 0.004
GTX 2,3	Peroxide extract	0.24 ± 0.096	0.16 ± 0.024	0.34 ± 0.049
GTX 5	Peroxide extract	0.008 ± 0.003	0.006 ± 0.001	0.010 ± 0.002
STX	Peroxide extract	0.05 ± 0.010	0.04 ± 0.005	0.07 ±0.011
dcGTX 2,3	Peroxide extract	0.09 ± 0.017	0.10 ± 0.018	0.12 ± 0.026
C 1,2	Peroxide extract	0.03 ± 0.008	0.03 ± 0.006	0.06 ± 0.012

* Predicted LOQ for dcNEO shown in brackets

3.4. Determination of the recovery of PSP toxins from spiked shellfish tissues

3.4.1. Overall recoveries

Recoveries were calculated in terms of expected mean recovery for each toxin in each of the three spiked, extracted, cleaned and derivatised samples. Table 12 presents the mean recovery percentages of PSP toxins from oysters and cockles spiked at 0.5 AL and 0.2 AL, with RSDs calculated from the mean recovery of each triplicate oxidation and analysis. DcNEO was spiked at 0.2 and 0.4 AL, in order to lower the volumes of certified reference material required for spiking.

Table 12. Mean percentage recoveries (and RSDs of triplicate spikes) of PSP toxins from homogenates spiked at expected concentrations of 0.5 AL and 0.2 AL (GTX5 0.02 and 0.05 AL; dcNEO at 0.2 and 0.4 AL), plus comparison with range of mean inter-laboratory recoveries reported by AOAC 2005.06 (at variable concentration levels).

	Cockles		Pacific Oyster		Native Oyster		
	0.5 AL**	0.2 AL	0.5 AL**	0.2 AL	0.5 AL**	0.2 AL	AOAC*
	Mean	Mean	Mean	Mean	Mean	Mean	
GTX 1,4	131% (3%)	125% (4%)	97% (8%)	88% (18%)	94% (13%)	68% (13%)	67% - 79%
NEO	62% (4%)	66% (14%)	61% (7%)	51% (24%)	82% (15%)	59% (8%)	53% - 62%
dcNEO	58% (5%)	71% (3%)	54% (3%)	58% (4%)	71% (3%)	60% (3%)	na
dcSTX	85% (7%)	93% (4%)	77% (13%)	82% (6%)	81% (3%)	78% (4%)	64% - 84%
GTX 2,3	84% (4%)	80% (8%)	94% (4%)	90% (7%)	78% (3%)	73% (6%)	76% - 88%
GTX 5	90% (7%)	92% (5%)	85% (2%)	85% (4%)	79% (3%)	83% (3%)	76% - 86%
STX	126% (1%)	136% (5%)	121% (1%)	123% (4%)	108% (2%)	111% (3%)	74% - 93%
dcGTX 2,3	49% (6%)	48% (6%)	70% (2%)	79% (5%)	56% (2%)	70% (7%)	na
C 1,2	76% (12%)	92% (8%)	99% (2%)	97% (3%)	68% (4%)	82% (8%)	74% - 78%

* Spiked recoveries only calculated for mussels – no oyster/cockle AOAC data available
Na = not analysed **For dcNEO higher concentration, 0.4 AL spike used

Table 12 shows the mean recoveries of toxins spiked at 0.5 AL of C18-cleaned shellfish extracts falling in the range of 49% to 131% for cockles, 61% to 121% for Pacific oysters and 56% to 108% for native oysters. For all three matrices, the majority of toxins exhibit recoveries between 60% and 95%, with STX and GTX1,4 typically exhibiting

higher recoveries and dcGTX2,3 returning lowered recoveries in cockles and native oysters. RSDs associated with these recoveries indicate a fair degree of repeatability associated with such measurements. It therefore appears that at the 0.5 AL, the recoveries for most toxins calculated for non-N-hydroxylated PSP toxins are acceptable for such a complex method, where extraction, clean up, fractionation and oxidation steps are all expected to contribute to recovery losses. Previous work in mussels showed the majority of toxins giving recoveries between 50% and 110% at 0.5 AL so these results appear similar [13,15]. Recoveries are likely to be influenced by the effects of solvent calibrations, as both fluorescence enhancement and suppression was identified for some of these toxins (Figures 15 – 20), although these effects may be variable as noted earlier. In this specific oyster matrix used for this validation study, GTX1,4 (Figure 2) shows a degree of enhancement in all matrices which may be responsible for the enhanced recoveries observed. Similarly, a significant level of toxin enhancement observed in the calibration graphs prepared in Pacific oyster extract for STX, could explain in part the higher recoveries illustrated above. However, other toxins exhibiting toxin enhancement in Pacific oyster extracts do not appear to result in similarly enhanced recoveries (e.g. dcSTX). Similarly, the reduced recoveries observed for dcGTX2,3 in cockles could be explained in part by the apparent signal suppression observed in this matrix. However, lower recoveries in native oysters at 0.5 AL can not be explained in the same way, with no signal suppression noted in this matrix (section 3.2.8). The very low levels of matrix interferences eluting at the same retention times as the quantitation peak of dcGTX2,3 are not expected to have a significant effect on toxin recoveries, due to the relative sizes of toxin and interference peaks.

Recoveries of PSP toxins spiked into cockle and oyster matrices at the lower concentration (0.2 AL per toxin) show mean toxin recoveries very similar to those obtained at the higher concentration (0.5 AL). The exceptions are the N-hydroxylated toxins GTX1,4 and NEO in native oysters, where the mean recovery at 0.2 AL is lower. It is noted that these toxins were spiked at levels close to the method LODs, so the accuracy and precision at this level would be expected to be worse than at higher concentration levels. Recoveries of dcNEO at 0.2 and 0.4 AL appear similar to those values obtained for NEO at 0.2 and 0.5 AL in all three matrices.

Overall, with the exceptions of the lower recoveries of dcGTX2,3 in cockles and native oysters and the heightened recoveries of STX and GTX1,4 in Pacific oysters, the values obtained are similar to those quoted in the AOAC 2005.06 method in a mussel matrix. No spiked oyster or cockle homogenate recovery data was published in the AOAC 2005.06 method [11, 12], although an earlier oyster recovery experiment did return recoveries between 74% and 111% [8]. However, as these values relate to spiked extracts, they do not include the contribution of recovery losses associated with the extraction step.

3.4.2. Investigation of recovery efficiency in Pacific oysters by exhaustive extraction

Results from the exhaustive extraction of two laboratory contaminated samples and two spiked samples are shown in Table 13 in terms of the percentage of the original AOAC-extracted and quantified toxin concentrations.

Table 13. Apparent extraction recovery efficiencies as expressed by percentage of PSP toxins quantified in re-extracted samples (spiked and laboratory contaminated) as compared with levels in original AOAC-method extracted Pacific oyster tissue.

	RM1	RM2	Spike 1	Spike 2
GTX 1,4	na	79% - 85%	90%	91%
NEO	na	na	na	89%
C 1,2	85% - 87%	85% - 86%	na	88%
dcSTX	na	na	81%	na
GTX 2,3	na	87%	91%	91%
GTX 5	85% - 88%	85% - 88%	90%	90%
STX	na	85% - 87%	91%	91%

RM = Reference material

The results show that the mean extraction efficiencies varied between 79% and 91% for all samples and spiked tissues. This infers that up to 21% of the recovery losses measured previously may be attributable to the extraction process, with the toxin material being held either in the tissue matrix or in the acetic acid solution contained within the pores of the sample. This can be compared to the results obtained in mussel matrix, where a similar range of extraction efficiencies (75% to 97%) were determined [13,15].

3.5 Determination of the precision of the method

3.5.1. Estimation of instrumental precision

Instrumental precision of toxin peak retention times following the repeat analysis (n=10) of naturally contaminated Pacific oyster and spiked native oyster and cockles is presented in Table 14. The table shows that the level of precision of chromatographic retention times is high (RSD = 0.4% to 1.6% in all three matrices), hence a high degree of confidence can be placed upon the toxin peaks consistently eluting at repeatable retention times. The table also shows no significant differences between the results obtained from the three matrices (mean RSD% = 1.1% for each matrix). Table 14 also displays the results obtained from the same analysis for the precision (n=10) of toxin peak area responses. Toxin quantitation peaks were measured and recorded and the relative standard deviations of the replicate analyses calculated to fall between 1% and 6% for all toxins, other than dcGTX2,3 in native oysters (RSD=9%; present at concentrations = 0.01 μg STX eq./g). With mean RSD values between 3% and 4% for each matrix, these values are acceptable given the complexities of the chromatograms and the variable concentration levels of toxins present and furthermore illustrate no significant differences between the precision in the three matrices.

Table 14. Instrumental precision, showing variability (RSD%) of toxin retention times and peak area responses.

	Mean toxin concentration (ug STX eq./g)			Toxin peak area precision (RSD %; n=10)			Retention time precision (RSD %; n=10)		
Toxins	PO	NO	Co	PO	NO	Co	PO	NO	Co
GTX 1,4	0.77	0.51	0.56	4%	6%	6%	1.1%	1.0%	0.9%
NEO	0.38	0.14	0.17	6%	5%	4%	1.2%	1.2%	1.0%
dcSTX	0.00	0.21	0.16	nd	1%	1%	nd	1.4%	1.9%
GTX 2,3	0.39	0.47	0.43	2%	1%	1%	1.5%	1.4%	1.2%
GTX 5	0.02	0.01	0.01	4%	5%	4%	0.6%	0.6%	0.5%
STX	0.36	0.19	0.19	2%	2%	2%	0.4%	0.9%	0.6%
dcGTX 2,3	0.01	0.01	0.01	6%	9%	6%	1.3%	1.1%	1.1%
C 1,2	0.36	0.09	0.07	1%	2%	2%	1.6%	1.3%	1.1%

3.5.2. Estimation of short-term repeatability

Tables 15 to 17 present the concentrations (+/- 1 sd) calculated for triplicate cockle, Pacific oyster and native oyster homogenate spikes at 0.2 AL and 0.5 AL for each PSP toxin when extracted, cleaned-up, fractionated, oxidised and analysed within one analytical batch. Relative standard deviations calculated from the resulting concentrations illustrate an acceptable short-term method repeatability. With many of the toxins exhibiting short term precision values of <5%, the results suggest that the level of precision is excellent for these toxins. All RSD% values are less than or equal to 15% at 0.5AL with the majority < 10%. Similar results were obtained at 0.2 AL where the majority of toxins also exhibit an RSD of < 15%, with the exception of GTX1,4 and NEO at 0.2 AL in Pacific oysters. The results therefore suggest that at individual PSP toxin concentrations of 0.2 and 0.5 AL, the short term repeatability for the LC-FLD analysis of each toxin is reliable. Further evidence for statistical acceptability of precision comes from the HorRat values, all of which are <2.0 at both concentration levels (Tables 14 to 16). Lawrence and Niedwiadek [8] reported RSD% values <10% for the quadruplicate analyses of PSP toxins. However, these results related to spiked extracts rather than spiked tissue samples, so the results described here are especially encouraging, as they also include any errors associated with the tissue extraction and any possible tissue heterogeneity. Additionally, it should be noted that Lawrence does not state whether the quadruplicate analyses represent replicate injections, oxidations, clean-ups or extract spikes, so a direct comparison of precision values obtained in the two studies is not possible.

Table 15. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of triplicate spiked cockle homogenate at 0.2 AL and 0.5 AL per toxin (dcNEO at 0.4 AL; GTX5 1/10 conc), showing estimations of short-term method repeatability in terms of percentage relative standard deviation (n=3; same batch).

	Cockles					
	0.5 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.52 ± 0.014	3%	0.22	0.20 ± 0.008	4%	0.28
NEO	0.25 ± 0.009	4%	0.34	0.11 ± 0.014	14%	1.08
dcNEO*	0.19 ± 0.009	5%	0.44	0.11 ± 0.003	3%	0.23
dcSTX	0.34 ± 0.025	7%	0.66	0.15 ± 0.006	4%	0.33
GTX 2,3	0.33 ± 0.013	4%	0.35	0.13 ± 0.010	8%	0.58
GTX 5	0.04 ± 0.003	7%	0.89	0.01 ± 0.0007	5%	0.54
STX	0.50 ± 0.007	1%	0.11	0.22 ± 0.012	5%	0.39
dcGTX 2,3	0.20 ± 0.011	6%	0.53	0.08 ± 0.004	6%	0.47
C 1,2	0.30 ± 0.036	12%	1.38	0.15 ± 0.012	8%	0.86

**dcNEO spiked at 0.4 AL due to standard availability issues*

Table 16. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of triplicate spiked Pacific oyster homogenate at 0.2 AL and 0.5 AL per toxin (dcNEO at 0.4 AL; GTX5 1/10 conc), showing estimations of short-term method repeatability in terms of percentage relative standard deviation (n=3; same batch)

	Pacific oysters					
	0.5 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.39 ± 0.031	8%	0.65	0.14 ± 0.025	18%	1.29
NEO	0.25 ± 0.017	7%	0.63	0.08 ± 0.019	24%	1.89
DcNEO	0.19 ± 0.006	3%	0.26	0.09 ± 0.003	4%	0.31
dcSTX	0.31 ± 0.039	13%	1.15	0.13 ± 0.007	6%	0.44
GTX 2,3	0.38 ± 0.014	4%	0.32	0.14 ± 0.010	7%	0.55
GTX 5	0.03 ± 0.0005	2%	0.20	0.01 ± 0.0006	4%	0.47
STX	0.48 ± 0.007	1%	0.12	0.20 ± 0.008	4%	0.30
dcGTX 2,3	0.28 ± 0.005	2%	0.18	0.13 ± 0.007	5%	0.44
C 1,2	0.40 ± 0.008	2%	0.24	0.16 ± 0.005	3%	0.33

*dcNEO spiked at 0.4 AL due to standard availability issues

Table 17. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of triplicate spiked native oyster homogenate at 0.2 AL and 0.5 AL per toxin (dcNEO at 0.4 AL; GTX5 1/10 conc), showing estimations of short-term method repeatability in terms of percentage relative standard deviation (n=3; same batch)

	Native oysters					
	0.5 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.38 ± 0.048	13%	1.06	0.11 ± 0.014	13%	0.92
NEO	0.33 ± 0.049	15%	1.37	0.10 ± 0.008	8%	0.65
dcNEO	0.22 ± 0.007	3%	0.25	0.10 ± 0.003	3%	0.22
dcSTX	0.32 ± 0.010	3%	0.25	0.13 ± 0.005	4%	0.27
GTX 2,3	0.31 ± 0.009	3%	0.26	0.12 ± 0.007	6%	0.49
GTX 5	0.03 ± 0.0010	3%	0.28	0.01 ± 0.0004	3%	0.21
STX	0.43 ± 0.009	2%	0.25	0.18 ± 0.006	3%	0.34
dcGTX 2,3	0.22 ± 0.005	2%	0.25	0.11 ± 0.008	7%	0.74
C 1,2	0.27 ± 0.009	4%	0.34	0.13 ± 0.010	8%	0.65

3.5.3. Estimation of medium-term repeatability

Tables 18 to 20 show the precision for cockles, Pacific oysters and native oysters relating to the extraction, clean up, oxidation and analysis of six replicate spiked samples (both 0.2 and 0.5 AL) performed over a longer period of time (> 2 weeks) as described in section 2.8.7. For cockles, RSD percentages range from 3% to 11% at 0.5 AL and 8% to 15% at 0.2 AL for all toxins. For Pacific oysters RSD percentages range from 6% to 21% at 0.5 AL and from 3% to 17% at 0.2 AL. For native oysters, RSD percentages range from 3% to 16% and 4% to 16% at 0.2 AL. As such, the degree of precision associated with the analysis of PSP toxins in cockles, Pacific oysters and native oysters is acceptable for the majority of toxins at both concentration levels. Good precision is further evidenced from the HorRat values, which are <2.0 for all toxins at both concentration levels (also tables 18 to 20) and with the majority < 1.0. As such, these results show a significant improvement in medium term precision as reported previously for mussels and in the AOAC 2005.06, where in both cases some toxins reported HorRat values > 2.0 [12, 13, 15]. Considering the potential high variability inherent in such a multi-step method, the level of precision illustrated by these results indicates strongly that the method is repeatable within the laboratory over the medium term. Due to toxin availability, the assessment was not extended for dcNEO.

Table 18. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of six replicate spiked cockle homogenates at 0.2 AL and 0.5 AL per toxin (GTX5 1/10 conc), showing estimations of medium-term method repeatability in terms of percentage relative standard deviation (n=6; > 2 weeks)

	Cockles					
	0.5 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.53 ± 0.023	4%	0.24	0.20 ± 0.018	9%	0.42
NEO	0.24 ± 0.023	10%	0.58	0.11 ± 0.010	10%	0.52
dcSTX	0.33 ± 0.023	7%	0.42	0.13 ± 0.016	12%	0.64
GTX 2,3	0.32 ± 0.021	7%	0.38	0.12 ± 0.017	15%	0.76
GTX 5	0.03 ± 0.002	7%	0.60	0.01 ± 0.0016	12%	0.88
STX	0.49 ± 0.014	3%	0.16	0.19 ± 0.025	13%	0.62
dcGTX 2,3	0.22 ± 0.022	10%	0.65	0.08 ± 0.007	8%	0.44
C 1,2	0.29 ± 0.031	11%	0.83	0.13 ± 0.018	14%	0.92

Table 19. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of six replicate spiked Pacific oyster homogenates at 0.2 AL and 0.5 AL per toxin (GTX5 1/10 conc), showing estimations of medium-term method repeatability in terms of percentage relative standard deviation (n=6; > 2 weeks)

	Pacific oysters					
	0.5 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.45 ± 0.073	16%	0.87	0.16 ± 0.027	17%	0.81
NEO	0.24 ± 0.017	7%	0.44	0.08 ± 0.014	17%	0.89
dcSTX	0.38 ± 0.078	21%	1.24	0.15 ± 0.024	16%	0.85
GTX 2,3	0.35 ± 0.041	12%	0.68	0.16 ± 0.028	17%	0.86
GTX 5	0.03 ± 0.003	10%	0.79	0.02 ± 0.002	14%	1.03
STX	0.47 ± 0.046	10%	0.53	0.21 ± 0.031	15%	0.69
dcGTX 2,3	0.25 ± 0.035	14%	0.88	0.11 ± 0.017	16%	0.87
C 1,2	0.38 ± 0.022	6%	0.46	0.16 ± 0.005	3%	0.22

Table 20. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of six replicate spiked native oyster homogenate at 0.2 AL and 0.5 AL per toxin (GTX5 1/10 conc), showing estimations of medium-term method repeatability in terms of percentage relative standard deviation (n=6; < 2 weeks)

	Native oysters					
	0.5 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.40 ± 0.064	16%	0.91	0.10 ± 0.017	16%	0.80
NEO	0.30 ± 0.049	16%	0.93	0.12 ± 0.019	16%	0.38
dcSTX	0.33 ± 0.010	3%	0.20	0.13 ± 0.018	14%	0.76
GTX 2,3	0.28 ± 0.032	12%	0.71	0.11 ± 0.012	11%	0.58
GTX 5	0.03 ± 0.005	16%	1.42	0.01 ± 0.0005	4%	0.30
STX	0.46 ± 0.036	8%	0.31	0.19 ± 0.014	7%	0.25
dcGTX 2,3	0.24 ± 0.029	12%	0.80	0.12 ± 0.009	8%	0.46
C 1,2	0.26 ± 0.026	10%	0.81	0.12 ± 0.015	13%	0.89

3.5.4. Estimation of long-term repeatability

Concentration data from the extraction, clean up and analysis of shellfish reference materials were generated over a longer period of time (> 6 months), by different analysts and using different batches of consumables and reagents. As such, the data represents a realistic expression of the within-laboratory reproducibility of the method, incorporating variable changes such as different working calibration solutions, instruments, analysts and other laboratory conditions experienced through the year including ambient temperature. The results obtained from the repeat analysis of these LRMs are tabulated in Tables 21 and 22.

Table 21. Mean concentration +/- s.d and %RSD data generated from long term extraction, clean-up, fractionation, oxidation and analysis of Pacific oyster and cockle LRMs. Toxins present at concentration levels < 0.2 AL are shaded.

	Pacific oyster LRM (n=13)			Cockle LRM (n=18)		
Toxin	Mean (ug/g STX equiv)	%RSD	HorRat	Mean (ug/g STX equiv)	%RSD	HorRat
GTX 1,4	0.66 ± 0.17	26%	1.51	0.96 ± 0.19	20%	1.25
NEO	0.36 ± 0.09	23%	1.27	0.37 ± 0.09	23%	1.26
C 1,2	0.090 ± 0.01	14%	0.88	0.134 ± 0.03	19%	1.26
GTX 2,3	0.13 ± 0.02	17%	0.85	0.17 ± 0.03	19%	0.98
GTX5	0.001 ± 0.0002	15%	0.52	0.003 ± 0.0003	11%	0.41
STX	0.15 ± 0.007	5%	0.23	0.21 ± 0.04	18%	0.90
Total	1.38 ± 0.19	14%	0.93	1.85 ± 0.18	10%	0.68

Table 22. Mean concentration +/- s.d and %RSD data generated from long term extraction, clean-up, fractionation, oxidation and analysis of native oyster LRMs. Toxins present at concentration levels < 0.2 AL are shaded.

	Native oyster LRM1 (n=15)			Native oyster LRM2 (n=9)		
Toxin	Mean (ug/g STX equiv)	%RSD	HorRat	Mean (ug/g STX equiv)	%RSD	HorRat
GTX 1,4	0.48 ± 0.13	26%	1.45	1.73 ± 0.39	23%	1.53
NEO	0.51 ± 0.10	19%	1.10	2.24 ± 0.29	13%	0.93
C 1,2	0.066 ± 0.008	12%	0.72	0.307 ± 0.046	15%	1.11
GTX 2,3	0.11 ± 0.016	15%	0.71	0.52 ± 0.067	13%	0.78
GTX5	0.001 ± 0.0002	18%	0.61	0.005 ± 0.0004	8%	0.33
STX	0.21 ± 0.016	8%	0.38	0.92 ± 0.066	7%	0.44
Total	1.38 ± 0.15	11%	0.72	5.74 ± 0.50	9%	0.70

Results indicate an acceptable level of long-term precision for the four LRMs studied. Long-term RSDs for non-N-hydroxylated toxins (analysed following peroxide oxidation of the C18 cleaned extracts) exhibited values between 5% and 19%, whereas toxin quantitations requiring the additional fractionation step (GTX1,4 and NEO) exhibited slightly higher variability as expected (13% to 26%). All HorRat values were < 2 with the majority < 1.3. The precision did not seem to worsen at concentrations < 0.2 AL for any of the species analysed. Given the long-term analysis of these LRMs, the results overall indicate a good level of within-laboratory reproducibility and are similar to those reported previously for mussels [13,15]. The similarity in results is also noted between the two native oyster LRMs, with slight overall improvement in precision seen in LRM2, containing toxins at significantly higher concentration levels. Native oyster LRM1 is therefore more indicative of the performance of the method for samples containing toxins at concentrations within the working calibration range (0 to 1.2 AL).

3.6. Ruggedness of the method

Main effects were calculated as the difference of means for each paired set of parameter levels (parameter differences) and compared against method precision (single batch; n=8) using a t-test (two-tailed, 95% confidence). Results from the analyses (concentration and parameter difference data) are shown in Appendix 4. Tables 23 to 26 show the t-test results for each parameter in each matrix.

Table 23. T-test results (n=8, t-critical = 2.37) from ruggedness experiment of non N-hydroxylated toxins (Pacific oysters)

Toxin	Extract pH	Peroxide concentration	Vortex time	Ambient temp	Oxidation time	Acetic volume	NaOH concentration
dcGTX 2,3	-0.04	0.02	-0.01	-0.05	0.00	-0.01	0.04
C1,2	-0.92	0.20	0.00	-1.09	-0.67	-0.50	0.91
dcSTX	-0.01	0.01	0.01	0.00	-0.01	0.00	-0.01
GTX 2,3	-0.93	0.15	0.02	-0.87	-0.50	-0.48	1.44
GTX 5	0.00	0.02	0.03	0.00	0.00	-0.03	-0.01
STX	0.01	0.38	0.43	0.17	0.08	-0.56	0.37

Table 24. T-test results (n=8, t-critical = 2.37) from ruggedness experiment of N-hydroxylated toxins (Pacific oysters)

Toxin	Extract pH	Periodate pH	Vortex time	Ambient temp	Oxidation time	Acetic volume	Matrix modifier pH
GTX1,4	0.08	0.46	0.86	-1.14	0.20	-2.17	-0.69
NEO	0.39	-0.35	0.29	-0.05	0.27	-0.22	-0.37

Table 25. T-test results (n=8, t-critical = 2.37) from ruggedness experiment of non N-hydroxylated toxins (native oysters)

	Extract pH	Peroxide concentration	Vortex time	Ambient temp	Oxidation time	Acetic volume	NaOH concentration
dcGTX 2,3	-0.01	-0.01	0.01	-0.02	-0.01	-0.01	-0.01
C1,2	-0.13	0.03	-0.01	-0.16	-0.09	-0.16	0.09
dcSTX	-0.01	0.12	0.12	-0.11	-0.11	-0.33	-0.06
GTX 2,3	-0.47	0.03	0.08	-0.57	-0.32	-0.86	0.87
GTX 5	0.01	0.00	0.01	0.00	-0.01	-0.02	0.00
STX	0.07	0.02	0.14	0.11	-0.02	-0.31	0.10

Table 26. T-test results (n=8, t-critical = 2.37) from ruggedness experiment of non N-hydroxylated toxins (cockles)

	Extract pH	Peroxide concentration	Vortex time	Ambient temp	Oxidation time	Acetic volume	NaOH concentration
dcGTX 2,3	-0.02	-0.01	-0.01	-0.03	-0.01	-0.01	0.03
C1,2	-0.10	0.04	0.03	-0.17	-0.09	-0.09	0.14
dcSTX	-0.17	0.11	0.27	-0.36	-0.12	-0.27	0.04
GTX 2,3	-0.45	0.04	0.10	-0.59	-0.36	-0.34	0.81
GTX 5	0.00	0.00	0.01	0.00	0.00	-0.02	0.00
STX	-0.21	0.13	0.32	-0.05	-0.25	-0.46	0.11

Overall, Tables 23 to 26 show that all t-test values were lower than t-critical (n=8, 95% confidence). As such, none of the ruggedness parameters investigated had a statistically significant effect on the stability of the method, with the assumption that parameters investigated do not interact. Whilst not statistically significant, the most sensitive parameter investigated appears to be the volume of glacial acetic acid during the oxidation of GTX1,4 in Pacific oysters (table 24). Furthermore, results in Appendix 4 and tables 23 and 24 show for Pacific oysters that changes in concentration of sodium hydroxide, ambient temperature during oxidation and C18 extract pH all result in slightly different oxidation efficiencies for C1,2 and GTX2,3, but not dcGTX2,3, dcSTX, GTX5 or STX. In contrast, results for native oysters and cockles (Tables 25 and 26) do not show the same effects, with the exception of sodium hydroxide concentration. Whilst the method has shown to be stable with respect to the parameters investigated, this does emphasise the importance of precise and accurate preparation and dispensing of reagents for the oxidation of toxins prior to LC-FLD analysis.

3.7. Measurement of uncertainty

The uncertainty of measurement within the AOAC 2005.06 method is dependent upon a multitude of variables, which are assessed through the propagation of standard uncertainties inherent in the precision, recovery assessment and repeatability/reproducibility of the method. It is thought that the assessment of these parameters and calculation of uncertainties obtained through the validation studies

described in this report is the most effective way of summing the total method uncertainty without duplication of uncertainty components. As described previously [13], the uncertainties associated with sample sampling, toxicological correction factors and the use of different matrix modifiers is not included in the overall assessment of method measurement uncertainty.

3.7.1. Precision – Repeatability

The measurement of uncertainty inherent in the precision component is evaluated from the statistical distribution of the results of a series of measurements and can be characterised by standard deviations [23]. Uncertainties are calculated for medium term precision (both at 0.2 AL and 0.5 AL concentration levels). These RSDs are pooled to give total standardised precision uncertainties (table 27):

$$u_c(y) = \sqrt{\frac{(n_a - 1) \times a^2 + (n_b - 1) \times b^2 + \ldots}{(n_a - 1) + (n_b - 1) + \ldots}}$$

Where:
$u_c(y)$ = pooled uncertainty of precision uncertainty components
a,b = RSDs of components
n = number of replicates used in precision studies for each component

Table 27. Precision values (RSDs) and pooled uncertainties calculated for PSP toxins in cockles, Pacific oysters and native oysters.

	Cockles			Pacific Oysters			Native Oysters		
	Medium term		"Pooled"	Medium term		"Pooled"	Medium term		"Pooled"
Toxin	0.2 AL	0.5 AL	Uncertainty	0.2 AL	0.5 AL	Uncertainty	0.2 AL	0.5 AL	Uncertainty
GTX 1,4	0.09	0.04	0.07	0.17	0.16	0.17	0.16	0.16	0.16
NEO	0.10	0.10	0.10	0.17	0.07	0.13	0.16	0.16	0.16
dcSTX	0.12	0.07	0.10	0.16	0.21	0.19	0.14	0.03	0.10
GTX 2,3	0.15	0.07	0.12	0.17	0.12	0.15	0.11	0.12	0.11
GTX 5	0.12	0.07	0.10	0.14	0.10	0.12	0.04	0.16	0.12
STX	0.13	0.03	0.09	0.15	0.10	0.12	0.07	0.08	0.08
dcGTX 2,3	0.08	0.10	0.09	0.16	0.14	0.15	0.08	0.12	0.10
C 1,2	0.14	0.11	0.12	0.03	0.06	0.05	0.13	0.10	0.11

3.7.2. Reproducibility or long-term repeatability

The uncertainties associated with long term precision (Table 28) are estimated from the precision data generated by the repeated extraction, clean-up, fractionation, oxidation and analysis of LRMs (>6 months; multiple analysts, instruments, consumable and reagent batches and calibrations). For toxins not present in the current LRMs, uncertainties were taken from the mean of all other toxins present. For native oysters, reproducibility values were taken from LRM1 as this material contained toxins at concentrations within the working calibration range (0 to 1.2 AL).

Table 28. Within-lab reproducibility uncertainties calculated from repeat analysis (>6 months) of LRMs

Toxin	Cockles	P. Oyster	N. Oyster
GTX 1,4	0.20	0.26	0.26
NEO	0.23	0.23	0.19
dcSTX	0.18*	0.17*	0.16*
GTX 2,3	0.19	0.17	0.15
GTX 5	0.11	0.15	0.18
STX	0.18	0.05	0.08
dcGTX 2,3	0.18*	0.17*	0.16*
C 1,2	0.19	0.14	0.12

* Mean values from other toxins present in LRMs

3.7.3. Recovery

Recovery was calculated using the levels spiked into the tissues as the expected values. The uncertainties present in the determination of recovery were estimated by calculating the RSD for each toxin at each spiking level, thus generating information on the uncertainty in recovery determination. Values are tabulated for each toxin at 0.2 AL and 0.5 AL in Table 29 below. Pooled uncertainties are calculated for each toxin using the same formula as in section 3.7.1 (above) and are shown to be of relatively small magnitude.

Table 29. RSDs and pooled uncertainties associated with determination of recovery.

	Cockles			Pacific Oysters			Native Oysters		
	Recovery uncertainty		"Pooled"	Medium term		"Pooled"	Medium term		"Pooled"
Toxin	0.2 AL	0.5 AL	Uncertainty	0.2 AL	0.5 AL	Uncertainty	0.2 AL	0.5 AL	Uncertainty
GTX 1,4	0.038	0.027	0.033	0.18	0.08	0.14	0.13	0.13	0.13
NEO	0.135	0.037	0.099	0.24	0.07	0.18	0.08	0.15	0.12
dcSTX	0.041	0.073	0.059	0.06	0.13	0.10	0.04	0.03	0.03
GTX 2,3	0.076	0.040	0.061	0.07	0.04	0.06	0.06	0.03	0.05
GTX 5	0.049	0.071	0.061	0.04	0.02	0.03	0.03	0.03	0.03
STX	0.055	0.014	0.040	0.04	0.01	0.03	0.03	0.02	0.03
dcGTX 2,3	0.057	0.056	0.056	0.05	0.02	0.04	0.07	0.02	0.05
C 1,2	0.084	0.118	0.102	0.03	0.02	0.03	0.08	0.04	0.06

3.7.4. Calculation of combined standard uncertainty

Using method performance data reported earlier in this report and uncertainties pooled as described above, preliminary combined standardised uncertainties for each PSP toxin (Table 30) was calculated from the square root of the sum of squares:

$$u_c = \sqrt{u_1^2 + u_2^2 + u_3^2 \ldots\ldots}$$

where:

u_c = combined standardised uncertainty

$u_1 - u_n$ = individual standardised uncertainties

Table 30. Combined uncertainties calculated from validation data for cockles, Pacific oysters and native oysters showing uncertainties as (a) standardised uncertainty and (b) expanded uncertainty (k=2).

	Standardised uncertainties			Expanded uncertainties (k=2)		
Toxin	Cockles	P. Oyster	N. Oyster	Cockles	P. Oyster	N. Oyster
GTX 1,4	0.21	0.34	0.33	0.43	0.68	0.66
NEO	0.27	0.32	0.28	0.54	0.64	0.55
dcSTX	0.21	0.27	0.19	0.43	0.54	0.38
GTX 2,3	0.23	0.23	0.19	0.46	0.46	0.39
GTX 5	0.16	0.20	0.22	0.32	0.39	0.44
STX	0.21	0.14	0.11	0.41	0.28	0.23
dcGTX 2,3	0.21	0.23	0.20	0.42	0.46	0.39
C 1,2	0.25	0.15	0.18	0.50	0.30	0.35

The standardised combined uncertainties given in Table 30 are preliminary as further work on the method and its use on the monitoring programme should help build up further data on long term repeatability of the method for oysters and cockles. The current results above show a range of combined standardised uncertainties for individual toxins, ranging from 0.11 to 0.34. Expanded uncertainties, calculated using a coverage factor (k) of 2, subsequently result in a range of values from 0.23 to 0.68. The coverage factor, k was taken to be 2 in order to provide a 95% confidence in the distribution of values, assuming a normal distribution [17]. Uncertainty results show a narrower range of values for the toxin suite compared with values reported previously for mussels with overall uncertainties either equivalent to or lower for these species than for mussels [13,15]. Standardised uncertainties could not be calculated for the toxin dcNEO, due to both the limited availability of a reference standard and the absence of the toxin in the contaminated samples, resulting in the subsequent inability to perform both the medium term and long-term precision analysis.

4. Phase II. Analysis of naturally contaminated oyster and cockle samples

4.1 Results overview

The number and availability of fresh cockle and oyster samples with PSP toxicity were found to be low at the time this part of the study was conducted. As such, the experimental study was supplemented with samples of Pacific oysters, native oysters and cockles previously fed mass-cultured toxic *Alexandrium* in the laboratory. Appendix 5a tabulates the results of the LC-FLD qualitative screen and full LC-FLD quantitation of 43 oyster and cockle samples found to be PSP negative by either HPLC screen or MBA. Of these, 16 samples were native oysters, 14 were cockles and 13 Pacific oysters. Overall, the agreement between the HCl and acetic acid screening results was good, with the majority of HCl screen negative samples also found negative following the acetic acid screen. A low number of samples found to be HPLC-positive by the HCl screening method but subsequently negative by MBA. Of these seven samples, 3 were found to be positive by the acetic acid screen. Three of the four samples found to be positive by the HCl screen positive but consequently negative by the AOAC 2005.06 HPLC screen, were only identified as PSP positive in the former with the presence of 3 or more toxin peaks each with a signal to noise ratio < 3.0. The fourth sample was found to contain only a low level of GTX5 in the HCl screen and as such was suspected to represent a chromatographic interference. One additional sample was found to be positive following the acetic acid screen and negative following the HCl screen. This sample was only assigned positive with the identification of 3 potential toxin peaks each with a signal to noise ratio < 3.0. As such, the differences observed between the two screening methods are minor and are to be expected when screening samples very low in PSP toxicity and with toxin peaks close to or less than LOD. Quantitation of all negative HPLC samples resulted in negative HPLC quantitation results, with no toxins detected using peroxide oxidation. Four MBA-negative HPLC positive samples obtained through the monitoring programmes (samples BTX/2008/874, 1475, 1709 and 2504) were found to contain minor levels (close to LOD) of STX, GTX2,3 and C1,2 toxins (Appendix 5a).

Appendix 5b tabulates the quantitative HPLC (individual toxin concentrations and total toxicities) and MBA results obtained from the analysis of PSP-positive oysters and cockles. In total, 19 cockles, 14 Pacific oyster and 15 native oyster samples were analysed using the two quantitative methods. Correlations between the methods for all three species are displayed in Figures 19 to 21. Visual inspection of the results indicates that there is a degree of correlation between the two data sets, but with a noticeable positive bias in the HPLC results for each species as compared to the MBA and with noticeable differences in correlation between the three species. A summary of these results is shown in Table 31.

Table 31. Summary of results from HPLC and MBA analysis of PSP-contaminated oysters and cockles (total toxicity in μg STX eq./100g flesh)

	Cockles	Pacific Oysters	Native Oysters
Number of samples	19	14	15
Mean HPLC concentration	75	120	112
Mean MBA concentration	53	63	43
Mean HPLC/MBA	143%	196%	263%
RSD of HPLC/MBA ratios	39%	24%	20%
Pearson correlation coefficient (r)	0.76	0.82	0.84
Linear regression slope equation	y=0.69x+4.8	y=0.54x-2.0	y=0.30x+9.6

4.2 Cockles

The mean HPLC/MBA ratio for the 19 cockle samples is 143% showing there is a degree of positive bias in the cockle HPLC results. 5 out of the 19 cockle samples exhibit HPLC toxicities above the action limit (80μg STX eq./100g) with the MBA below AL (Table 32), although 3 of these return HPLC results very close to the regulatory limit (81, 82 and 83 μg STX eq./100g; Appendix 5b). Sample results in Appendix 5b indicate that differences are observed between the groups of samples sourced from the three different laboratories (Cefas, SAMS and Integrin). Removing the Cefas samples from the dataset (also those frozen prior to MBA at an external laboratory), the mean HPLC/MBA bias becomes 109% (RSD = 19), Cefas-contaminated cockles alone giving a mean HPLC bias of 181%. This includes one noticeable outlier (sample Co LRM5) which when removed from the complete dataset gives an overall HPLC bias of 134% (RSD 27%) in all remaining cockle samples analysed. Figure 19 shows the visual comparison of toxicity results with the outlier removed, highlighting the linear regression between the two methods and showing its relation to equality. Confidence bands shown are the 95% confidence for the predicted mean at each x value and give further evidence for a slight positive bias in the HPLC method as compared with the MBA. It is noted however that the differences observed between the two methods fall within the size of the measurement uncertainty determined earlier for the HPLC method (section 3.7.4).

This bias in the Cefas-sourced cockles, and to a lesser extent the SAMS samples, can be explained primarily (with the exception of sample Co LRM5) by the use of the highest toxicity equivalence factor (TEF) for each epimeric pair. The strains of *Alexandrium* used to contaminate cockles at Cefas and SAMS contained high levels of GTX1,4, quantitation of which requires the assumption that the highest toxicity epimer (GTX1; Appendix 6) is exclusively present. Results re-calculated using the lowest TEF for all epimeric pairs result in a mean HPLC bias of 103% for all cockles analysed. This is further evidenced with the analysis of the same cockle samples at the Canadian Food Inspection Agency (CFIA) using a post-column oxidation method (LC-PCOX-FLD). Comparative results between the two HPLC methods show a degree of positive bias for the Cefas-sourced cockles in the Cefas LC-FLD method as compared with the CFIA LC-PCOX-FLD method (Appendix 5c). However, a good agreement was shown between the two methods for the remainder of the cockle samples, found to contain lower proportions of epimeric pairs (GTX1,4 in particular). One notable exception (Co LRM5) showed a high HPLC bias in both pre-column (318%) and post-column (236%) results from the two laboratories, thus indicating that in this one cockle sample there is either significant enhancement in the fluorescence signals in both LC methods or that there is potential suppression in the MBA. It is noted that differences in matrix effects

are possible in this particular instance, given that sample Co LRM5 was contaminated in a separate feeding experiment to the other Cefas cockles and involved cockles harvested at a different time from those incorporated into the other cockle samples.

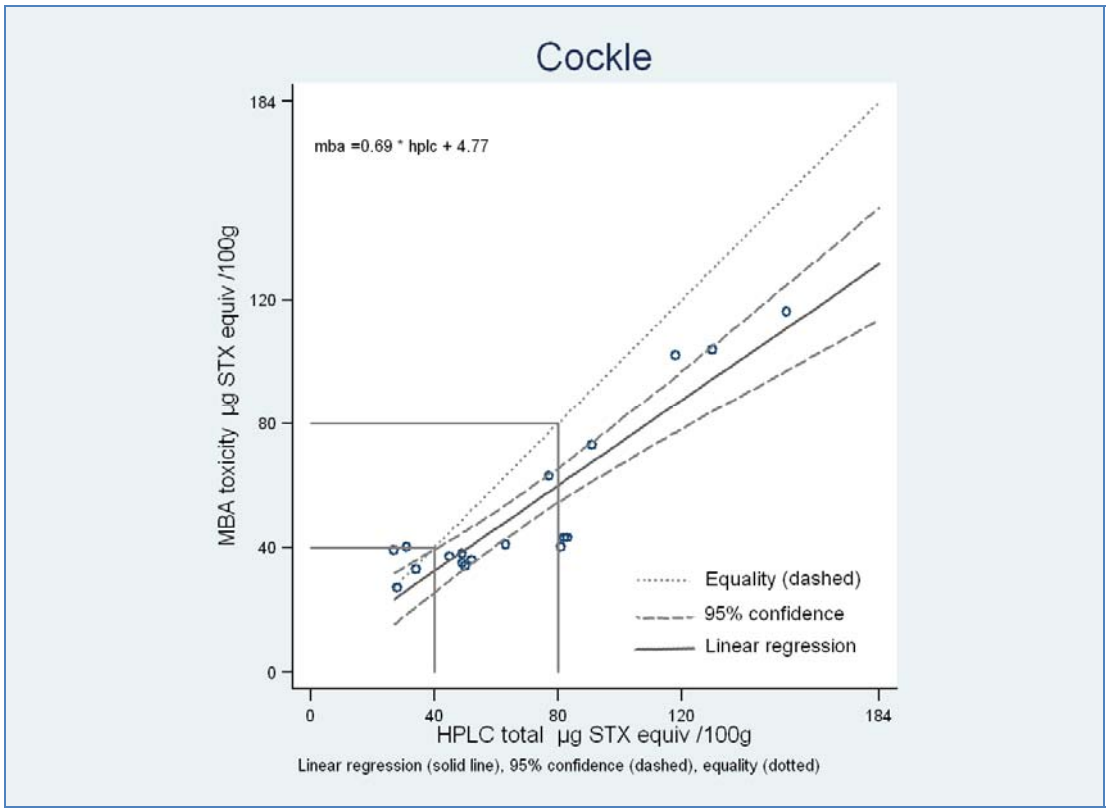

Figure 19. Comparison of total PSP toxicities in cockles* obtained by MBA and quantitative HPLC-FLD, showing linear regression, estimated coefficients and associated 95% confidence intervals. Y=X and 1.0 and 0.5 AL limits are also shown.
* 1 outlier (Co LRM5) removed from regression

Table 32. Summary of number of test results above and below the action level (AL; 80 µg STX eq./100g) from HPLC and MBA analysis of cockles (n=19)

	Number HPLC results below AL	Number HPLC results above AL
Number MBA results below AL	11	5
Number MBA results above AL	0	3

4.3 Pacific oysters

Results for Pacific oysters, illustrated by the correlation in Figure 20 and summarised in table 31, demonstrate a clear positive bias for the HPLC toxicity results as compared with the MBA reference method. A mean HPLC/MBA of 196% (24% RSD; n=14) results in a significant number of samples (>50%) being found lower than the action limit by MBA but higher by HPLC (Table 33).

Given such a high relative bias in the HPLC results, the assumption regarding exclusive use of the highest TEF for each epimeric pair does not fully explain the lack of correlation, even with the relatively high proportion of GTX1,4 present in the samples (Appendix 5d). Use of the lowest TEFs for each epimeric pair would only reduce the mean HPLC/MBA value to 150%. Analyses carried out by CFIA (LC-PCOX-FLD, as above for cockles; Appendix 5c) highlights a good agreement between the pre-column and post-column LC results for Pacific oysters. A slight positive bias in the Cefas HPLC results as compared with the CFIA HPLC results is fully attributable to the assumption in presence of higher TEFs in the former.

The possibility of fluorescence enhancement of PSP toxins in the oyster matrix was investigated given the potential for this to occur as highlighted in section 3.2.9. Experiments carried out previously to assess the repeatability of matrix effects using solvent vs. matrix-spiked calibration standards were undertaken using PSP-free Pacific oysters from the same source as the PSP-positive SAMS oysters. However, the results in section 3.2.9.1 indicate that in this particular oyster matrix, no fluorescence enhancement was measurable, with only a small amount of fluorescence suppression noted for GTX1,4 and NEO (Table 8). As such, there is no evidence for matrix components in these oysters falsely enhancing the toxin signals following LC-FLD analysis.

Another possible factor affecting the correlation between the two methods is the potential difference in toxicity between acetic acid and hydrochloric acid extracts. However, both acidic extracts were quantified by LC-PCOX-FLD at CFIA and showed that the HCl extracts contain on average a slightly higher level of toxicity (mean bias = 110%).

As such, there is an element of positive bias in the Pacific oyster LC-FLD methods as compared with the MBA in results obtained. Without further work being conducted into the source of this bias, the lack of correlation cannot currently be fully explained.

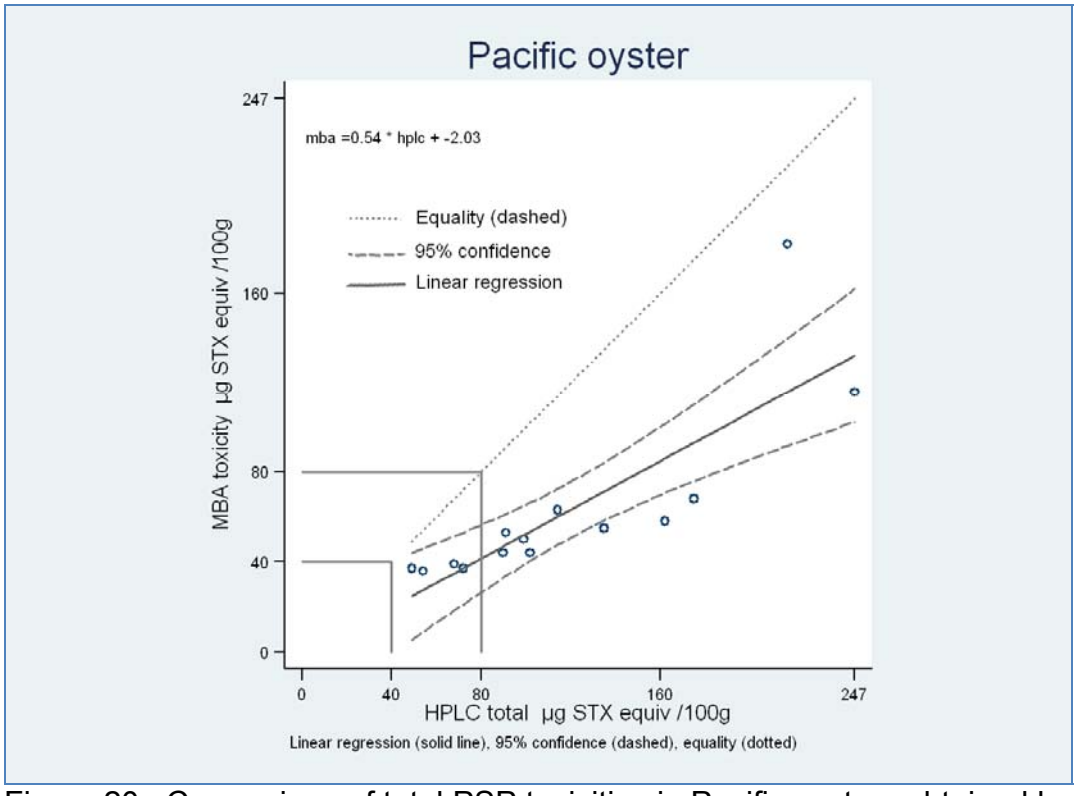

Figure 20. Comparison of total PSP toxicities in Pacific oysters obtained by MBA and quantitative HPLC-FLD, showing linear regression, estimated coefficients and associated 95% confidence intervals. Y=X and 1.0 and 0.5 AL limits are also shown.

Table 33. Summary of number of test results above and below the action level (AL; 80 µg STX eq./100g) from HPLC and MBA analysis of Pacific oysters (n=14)

	Number HPLC results below AL	Number HPLC results above AL
Number MBA results below AL	4	8
Number MBA results above AL	0	2

4.4 Native oysters

Results tabulated in Appendix 5b and summarised in Figure 21 and Table 31 highlight the clear large positive bias in HPLC results for the 15 samples analysed as compared with the MBA. A mean HPLC/MBA of 263% for native oysters resulted in 11 out of the 15 samples being above AL by HPLC whilst below AL by MBA (Table 34). In addition, one sample showed a toxicity of 49 μg STX eq./100g by HPLC but was negative by MBA.

As with Pacific oysters, a relatively high proportion of GTX1,4 and GTX2,3 is present (albeit lower levels than in Pacific oysters; Appendix 5d), resulting in the potential for over-estimation of total toxicity when using the highest TEF for each epimeric pair. Again, however, this effect does not fully explain the bias seen in the HPLC results, with CFIA PCOX data generated on 5 native oyster samples agreeing closely with Cefas HPLC results (Appendix 5c) and with both HPLC methods showing a large positive bias compared to the MBA.

Low levels of fluorescence enhancement were observed for some toxins in the native oyster matrix (section 3.2) but these enhancements were minor compared to the relative differences in final toxicity results observed here. Appendix 5c also tabulates the PCOX HPLC results obtained in both acetic acid and hydrochloric acid extracts. As with Pacific oysters, the HCl extracts return different toxicities, but these differences do not contribute to any AOAC 2005.06 method bias in all the native oyster samples.

The results described here for native oysters cannot therefore be explained fully with the data generated to date. Both the Cefas AOAC 2005.06 LC-FLD and CFIA PCOX LC-FLD methods are in good agreement, highlighting a positive bias in the HPLC results as compared to the MBA.

Table 34. Summary of number of test results above and below the action level (AL; 80 μg STX eq./100g) from HPLC and MBA analysis of native oysters (n=15)

	Number HPLC results below AL	Number HPLC results above AL
Number MBA results below AL	4	11
Number MBA results above AL	0	0

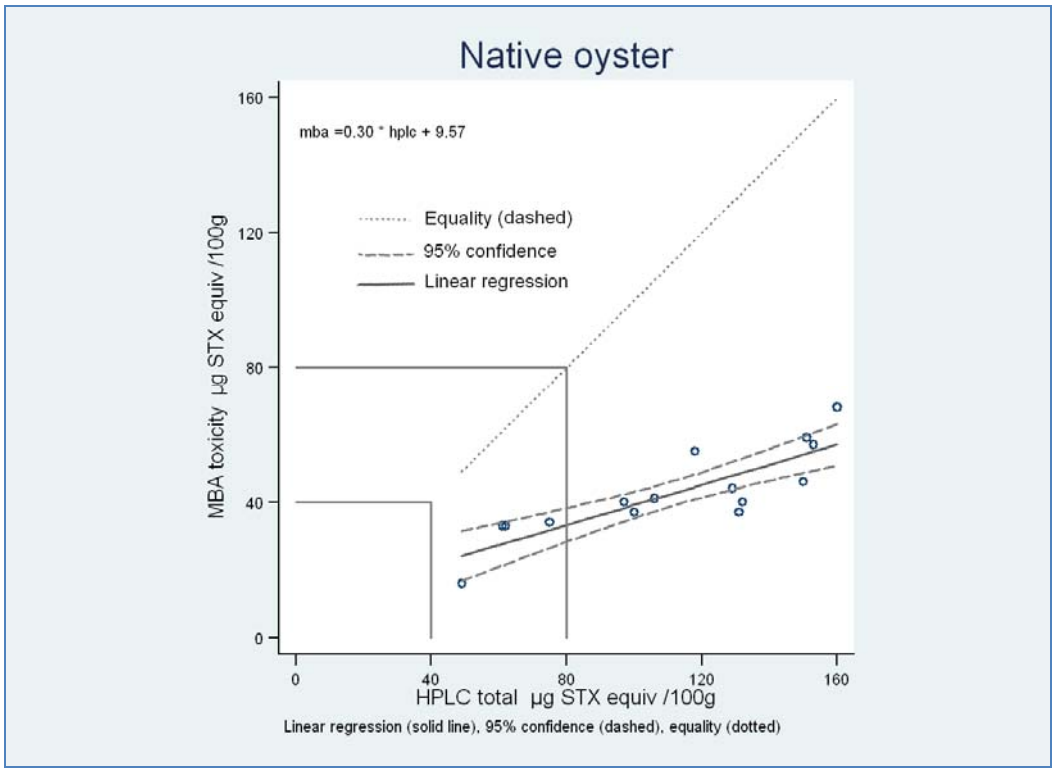

Figure 21. Comparison of total PSP toxicities in native oysters obtained by MBA and quantitative HPLC-FLD, showing linear regression, estimated coefficients and associated 95% confidence intervals. Y=X and 1.0 and 0.5 AL limits are also shown.

4.5 Effect of measurement uncertainty on HPLC results

Expanded uncertainties calculated for each PSP toxin (Table 30) were used to calculate the uncertainty in measurement of estimated toxicity (total saxitoxin equivalence) following HPLC analysis for each PSP-positive cockle and oyster sample. Table 35 summarises the mean and range of uncertainties determined for each species. It is noted that the uncertainties in HPLC results may change due to the variable toxin profiles within each sample and the different measurement uncertainties for each individual PSP toxin. Results indicate total measurement uncertainty for saxitoxin equivalents ranging between 18 and 42%, with Pacific oysters tending to exhibit higher measurement uncertainties than native oysters and cockles. The higher values in Pacific oysters relates to the higher proportions of GTX1,4 in the samples (Appendix 5), a toxin which exhibits higher measurement uncertainty (Table 30) due to the additional ion exchange clean up steps required prior to toxin quantitation. It is difficult to make any comparison between these calculated values of measurement uncertainty with the MU inherent in the MBA, as little data exists on the latter. Specially, there is no data published that provides MU values for the MBA in individual bivalve shellfish species, as is presented here for the HPLC method. Inter-laboratory reproducibility for the MBA has been quoted as ranging from 8 to 40% [25, 26], although strictly this is not directly comparable with the MU values calculated for the HPLC method. In addition, a FAPAS inter-laboratory study in 2003 [27] involving the inter-laboratory analysis by MBA of shellfish fortified with STX at 80 µg STX eq./100g concluded that a statistical evaluation of the results from 9 laboratories was not possible due to the high variability of the results, although this related to a lack of standardised protocol as well as the measurement uncertainty inherent in the method.

Table 35. Summary of measurement uncertainties on total saxitoxin equivalents quantified following HPLC analysis of PSP-positive cockles, native oysters and Pacific oysters.

	Cockles	Native oysters	Pacific oysters
Mean uncertainty in total toxicity	28%	28%	38%
Range of uncertainties	22 – 40%	18 – 34%	31 – 42%

4.6 Summary of phase II results

The aim of this parallel testing, as demonstrated previously for mussels [13,15], was to demonstrate that the HPLC method performs adequately as an analytical procedure for the quantitative analysis of PSP toxins in oysters and cockles, successfully identifies contaminated and non-contaminated samples and identifies positive samples at half or below the regulatory action limit (0.5 AL). For all three species, the qualitative analysis of contaminated and non-contaminated samples was shown to be successful. However, results indicate differences between the results produced by the MBA and HPLC methodologies and that species type is a major factor affecting the relative performance of the two methods.

For cockles, a mean positive HPLC bias was shown for the 19 cockle samples analysed by both HPLC and MBA, but this can mostly be explained (with one exception) by the use of the highest TEF for each epimeric pair during toxicity quantitation. PCOX data confirmed that matrix-induced LC fluorescence enhancement is not responsible for any positive bias seen in the Cefas pre-column oxidation HPLC results.

For both Pacific oysters and native oysters, results strongly indicate a high level of positive bias in the HPLC results as compared with the MBA reference method. Whilst the effects of TEF contribute to the size of this bias to a degree, they do not fully explain the differences in the two methods for these species. Other factors such as fluorescence enhancement and differences in toxicity obtained through different extraction methods do not contribute to the positive bias in any way in this group of samples. Application of measurement uncertainty to the final HPLC toxicity estimations showed the level of uncertainty varying depending on the toxin profile and species type, but with the range of uncertainties comparing similarly to those quoted elsewhere for the MBA.

Further work should be undertaken to understand the causes of the significant differences between the HPLC and MBA results highlighted above in Pacific oysters and native oysters. Results for these two species are of concern as they highlight either the potential for large over-estimation of toxicity by HPLC-FLD, or a gross suppression of toxicity by MBA. Future tests should therefore include the analysis of matrix components present in both biased and well-correlated samples to ascertain if any such components are responsible for the results observed. Given that the MBA is the official EU reference method for PSP toxicity measurement in shellfish, it would not be appropriate to implement the AOAC 2005.06 HPLC method for oysters until the results of this additional work becomes available. Work is currently ongoing at Cefas to further explain these results.

4.7 Comparison with the methods currently in use in the monitoring programme

Since May 2008, the refined AOAC 2005.06 LC-FLD method has been implemented in the UK PSP monitoring programme for mussels. Over this period of time, large numbers (>7,000) of mussels have been extracted, cleaned up, screened and quantified using the HPLC method, the method run alongside the HCl-screening method for oysters, cockles and whole king scallops and no major issues have been encountered. As such, Cefas laboratory staff are well practised in the techniques required for implementation of

the HPLC method for other non-mussel species, and it is not thought that any additional complications or issues will arise once the method is in place for additional species. The ultimate replacement of the HCl-screen / MBA approach for non-mussel species will result in the use of one single HPLC based approach towards PSP screening and quantitation, so if anything will improve efficiencies within laboratory protocol. It is anticipated therefore that the Cefas laboratory could implement the method when required given a month's notice.

5. Conclusions – application of the method to the routine monitoring of PSP toxins in oysters and cockles as part of the national biotoxin control program.

In accordance with EU legislation on the use of Official Control Methods [2], the "Lawrence" AOAC 2005.06 HPLC-FLD method was subjected to an in-house programme of validation and applied in tandem alongside the OC MBA. Extensive testing was carried out on Pacific oyster and native oyster tissues using the 2005.06 method to assess the selectivity, linearity, limits of detection and quantitation, accuracy, recovery, precision, repeatability and fitness for purpose of the method. The method validation was extended to include the toxin dcGTX2,3 not included in the AOAC 2005.06 method. In addition, limited availability of certified dcNEO, enabled some limited tests to be conducted, specifically confirmation of LOD and LOQ and recovery tests. The validation was extended further to include cockles, a species not covered by AOAC 2005.06. The aim of the study was an assessment of the overall applicability of the method to the analysis of PSP toxins in cockles and oysters as a potential future part of the national biotoxin monitoring program in the UK. The validation study was conducted on oysters and cockles collected under the GB monitoring programmes and included all PSP toxins currently available as certified reference standards. A comparison of results obtained using the quantitative method with those obtained using the current HCl-screen / MBA was also performed. Quantitative results from each of the validation tasks where appropriate are summarised in Tables 36-38 for each PSP toxin in Pacific oysters, native oysters and cockles and results of this assessment are provided below.

Qualitative chromatographic observations showed that whilst significant matrix components are present in the fluorescence chromatograms of cleaned-up oyster and cockle extracts and fractions, most of these do not interfere with the selectivity of the analysis of individual PSP toxins. Hence, the quantitation of most toxins is not expected to be biased by selectivity effects. The exception to this was the peak of dcGTX2,3 where small levels of matrix interferences were found to be present at the same retention times as toxin quantitation peaks. Such interferences when present could bias accurate quantitation and again supports the need for running unoxidised samples alongside periodate and peroxide oxidised samples as described in the official method and as currently conducted in mussels. Whilst such interference subtraction involves a degree of assumption and uncertainty and significantly adds to the overall turnaround of sample analysis, it reduces the likelihood of false positives.

Investigations of linearity showed that for the suite of PSP toxins studied, the calculated calibration functions demonstrated linearity over the working range of 0 to 1.2 AL per toxin. Evidence for this was obtained through visual inspections of both calibration curves and residual plots, and the use of additional f-test goodness of fit analysis. Some differences were observed between the slopes of calibration curves when comparing matrix (cockle or oyster)-matched and solvent spiked calibration standards. Slope differences were observed in particular for toxins in Pacific oyster extract, where a number of non-N-hydroxylated toxins were found to exhibit significant signal enhancement, although repeat linearity studies using a different source of oysters showed this matrix-enhancement not to be repeatable. No differences in calibration slope were observed for the N-hydroxylated toxins in Pacific oysters and for the non-N-hydroxylated toxins in cockles and native oysters. Conversely, a significant level of toxin suppression was observed for dcGTX2,3 in Pacific oyster and cockle extracts. It was thought that some of these differences may account for enhanced or reduced

recoveries, although there was no definite correlation between these two factors for all toxins in all species. As the relative slope differences varied between toxins in the same matrices, and the matrix effects appear to vary depending on the source of the shellfish, it was noted that it would be impractical to use matrix matched calibration standards. With the exception of GTX1,4 in Pacific oysters, the linearity was shown to extend up to higher concentrations (2.5 to 3.0 AL) in all species.

In relation to regulatory limits, a good level of instrumental sensitivity for most toxins was illustrated for the periodate screen with detection limits of approximately 0.03 to 0.09 µg STX eq./g (0.04 to 0.11 AL) for cockles, 0.03 to 0.18 µg STX eq./g (0.04 to 0.22 AL) for Pacific oysters and 0.02 to 0.18 µg STX eq./g (0.03 to 0.22 AL) for native oysters. Whilst these values are not derived from homogenate spiking experiments, they give an indication of the sensitivity of the screening method when employing acetic acid extracts. Experimentally-determined method LODs ranged from 0.004 to 0.12 µg STX eq./g (~0.005 to 0.15 AL) for all non-N-hydroxylated PSP toxins, with GTX1,4 and NEO exhibiting higher LODs (0.16 µg STX eq./g; 0.2 AL). This illustrates the sensitivity of the method and the ability of the method to detect all toxins at levels less than or equal to 0.2 AL, with most toxins at levels well below this limit. Sensitivities are similar to those quoted in the AOAC 2005.06 method, again with the exception of the periodate-oxidised toxins GTX1,4 and NEO where a poorer sensitivity is described. Limits of quantitation were shown experimentally to vary between 0.01 to 0.40 µg STX eq./g (0.01 to 0.5 AL) for all toxins, with GTX1,4 and NEO in all matrices showing the highest values (0.34 to 0.40 µg STX eq./g) and GTX2,3 showing an LOQ of 0.24 and 0.34 µg STX eq./g in cockles and native oysters respectively. As such, the LOQs for all toxins with the exceptions of GTX1,4, NEO and GTX2,3 in cockles and native oysters are at or lower than the target concentration level of 0.2 AL. These results would therefore suggest that the method can operate with an acceptable level of precision and certainty at less than or equal to 0.2 AL for each of these toxins. The higher LOQ for GTX1,4 and NEO relates to the poorer sensitivity of the instrumental method for the diagnostic peak of these toxins (and most likely a product of inefficient oxidation by periodate). However, the medium term precision data (from spiked homogenate) indicates the precision for these toxins is good at 0.2AL (15% to 17% RSD%; HorRat << 2.0). Thus it can be argued that quantitation for GTX1,4 and NEO can be carried out with a good degree of confidence down to the limit of detection (0.16 µg STX eq./g; 0.2 AL). Such an argument is not unacceptable, given the recognition that analytical measurements below the LOQ are not devoid of information content and may well be fit for purpose [4]. Limited availability of dcNEO enabled the confirmation of LOD and LOQ at 0.16 and 0.32 µg STX eq./g respectively, but with predicted LOD and LOQ well below this level.

Method recovery experiments were undertaken on spiked tissue homogenates at both 0.2AL and 0.5 AL concentrations and for all toxins (dcNEO at 0.4 AL). The results have shown that recovery is similar to the levels described for mussels [13,15] and in the AOAC 2005.06 method, with results demonstrating some differences between the values for each toxin and between different concentration levels. Only dcGTX2,3 and dcNEO in cockles and NEO and dcNEO in Pacific oysters exhibited recoveries <60% and the heightened recoveries for GTX1,4 in cockles and STX in cockles and Pacific oysters are thought to relate to enhancement effects as illustrated by the comparison of solvent and matrix-matched calibrations. Limited availability of dcNEO enabled recovery determination at 0.2 and 0.4 AL for this toxin and results appeared similar to those achieved at 0.2 and 0.5 AL for NEO.

Instrumental precision results showed low variability between toxin peak retention times (RSD = 0.4% to 1.6% in all three matrices; n=10), with the precision of toxin peak area responses also acceptable (mean RSD = 3% to 4% for the three matrices; n=10). Short-term method precision studies involved the triplicate extraction, clean up, fractionation, oxidation and analysis of spiked cockle and oyster tissues at 0.2 and 0.5 AL per toxin. Percentage RSDs calculated were less than 10% for each toxin at 0.5 AL, with the exception of C1,2 (12%) in cockles, dcSTX (13%) in Pacific oysters and GTX1,4 (13%) and NEO (15%) in Pacific oysters. This therefore appears consistent with values quoted previously and in previous studies by the AOAC method authors [9,10,11]. Furthermore, an acceptable level of precision at the 0.2 AL (all HorRat < 2.0) gives a good indication that the method is capable of reliably quantifying all PSP toxins at a concentration level significantly lower than the action limit.

Medium term repeatability was assessed with the replicate (n=6) spiking, extraction, cleanup, fractionation, oxidation and analysis of cockle and oyster tissues spiked at 0.2 and 0.5 AL, all carried out over a period time greater than two weeks and with different analysts. The mean percentage RSD for all PSP toxins was 7% in cockles (0.5 AL) and 12% for Pacific oysters and native oysters (0.5 AL). Mean RSDs at the lower concentration (0.2 AL) were 12%, 11% and 14% for cockles, Pacific oysters and native oysters respectively. Such values represent a significant improvement over the values measured in mussels previously [13]. HorRat values were <2.0 for all toxins at both concentration levels, further evidencing the degree of acceptability associated with the precision of the method. Considering the high variability inherent in such a multi-step method, these values appear reasonable and indicate that the method is repeatable within the laboratory over the medium term.

Long-term precision, or within-laboratory reproducibility assessments were undertaken with the repeat analysis of cockle, Pacific oyster and native oysters contaminated with PSP toxins. Such samples are not readily available from the UK biotoxin monitoring programmes, so cockles and oysters were fed toxic strains of *Alexandrium* species within the laboratory setting. Successful feeding of mass-cultured algae enabled the preparation of laboratory reference materials which when homogenised, aliquoted and stored enabled the long term repeat analysis of PSP toxin concentrations in each of the three shellfish matrices. Results presented showed a good level of reproducibility for the method, with data incorporating variations between analyst, analytical instruments, consumables, reagent and calibration standard batches and laboratory conditions over a period of time greater than six-months.

Ruggedness experiments were undertaken to examine the effects of method parameters on the stability of the method. Results from the analysis of all available PSP toxins in naturally contaminated Pacific oyster extracts, and non-N-hydroxylated toxins spiked into native oyster and cockles showed that the method was robust for all parameters investigated.

The validation results obtained throughout the study were used to calculate standardised and expanded uncertainties for the analysis of PSP toxins in cockles, Pacific oysters and native oysters. The contributions to uncertainty were assessed to ensure all factors were incorporated whilst eliminating duplication. Results showed a combined standardised uncertainty ranging from 0.16 to 0.27 (cockles), 0.14 to 0.34 (Pacific oysters) and 0.11 to 0.33 (native oysters) with expanded uncertainties (k=2) consequently ranging from 0.32 to 0.54 (cockles), 0.28 to 0.68 (Pacific oysters) and

0.23 to 0.66 (native oysters), depending on the toxin. These values therefore appear lower and more consistent than the range of uncertainties reported previously for mussels [13,15].

Following laboratory validation, the analytical method was trialled in comparison with the reference method (MBA), with the analysis of cockle, Pacific oyster and native oyster samples obtained from the GB biotoxin monitoring programme. The samples were analysed in parallel using both the AOAC 2005.06 method and the current HCl HPLC screen and MBA methods. Acetic acid extracts of samples were subjected to both the screening and quantitation elements of the AOAC 2005.06 method and comparisons were carried out between results obtained from the two HPLC screening methods. The correlation between the methods was found to be good, with minor differences between the HCl and acetic acid screening results observed due to the very low levels of PSP toxins present in the samples. No false HPLC positives were recorded using the acetic acid screening method as compared with the MBA. Quantitation of all negative HPLC samples resulted in negative HPLC quantitation results, with no additional toxins detected using peroxide oxidation. Three HPLC positive samples were found to contain minor levels of STX, GTX2,3 and C1,2 toxins. None of the samples analysed were found to be MBA positive.

Due to an absence of PSP-positive cockles and oysters in naturally contaminated samples, batches of shellfish were fed mass-cultured *Alexandrium* algae in order to produce naturally contaminated shellfish within the laboratory. Feeding experiments were conducted at three laboratories using different strains of *Alexandrium* with variable toxin profile. Results for cockles (n=19), showed some positive bias in the HPLC as compared to MBA (mean HPLC/MBA = 143%), but this could on the whole be explained by the use of the highest toxicity equivalence factor (TEF) during the quantitation of co-eluting epimeric toxin pairs. Results for both Pacific oysters (n=14) and native oysters (n=15) showed a much higher positive bias in the HPLC toxicity results as compared to the MBA (196% and 263% respectively). Analysis of the same oyster samples at CFIA (PCOX LC-FLD) gave good additional evidence that an element of bias attributable to the use of the highest TEF could not fully explain the HPLC bias, and that other matrix effects were affecting either the HPLC or MBA analyses. Application of measurement uncertainty to the HPLC results showed the level of uncertainty varying depending on the species and toxin profile, but with the range of uncertainties comparing similarly to those described for the MBA. Whilst the results indicate that it may be safe to implement the HPLC for cockles, the significant disparity between the HPLC and MBA results for both oyster species indicates a strong need to further investigate any issues which may potentially affect either of the two methods.

In terms of method implementation, the logistics of the fully-quantitative HPLC method have been described previously [13]. The AOAC 2005.06 has been implemented for mussels at Cefas since May 5th 2008, and whilst the method is complex and time-consuming, it is not thought that extension of the method to cover additional species will reveal any additional issues current inapplicable to the analysis of mussels. As such, application of this method to the routine monitoring of non-mussel species has the potential to provide further significant reduction in the overall bioassay usage for PSP toxin determination in compliance with the 3Rs (Replacement, Refinement, Reduction). Future work will continue with the validation of the AOAC 2005.06 for other shellfish species (clams and scallops) of importance to the UK marine biotoxin monitoring programme and will be reported separately.

Table 36. Summary of validation data for HPLC-FLD analysis of cockles following AOAC 2005.06.

Toxin	Linearity (r^2)	LOD ug/g STX equiv	LOQ ug/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged-ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL			
GTX 1,4	0.989	0.16	0.34	125%	131%	4%	3%	9%	4%	nd	20%	0.21
NEO	0.99	0.16	0.35	66%	62%	14%	4%	10%	10%	nd	23%	0.27
dcNEO	nd	<0.16	<0.32	71%	58%	3%	5%	nd	nd	nd	nd	nd
dcSTX	0.957	0.009	0.02	93%	85%	4%	7%	12%	7%	Yes	nd	0.21
GTX 2,3	0.916	0.12	0.24	80%	84%	8%	4%	15%	7%	Yes	19%	0.23
GTX 5	0.993	0.004	0.008	92%	90%	5%	7%	12%	7%	Yes	11%	0.16
STX	0.985	0.025	0.05	136%	126%	5%	1%	13%	3%	Yes	18%	0.21
dcGTX 2,3	0.966	0.047	0.09	48%	49%	6%	6%	8%	10%	Yes	nd	0.21
C 1,2	0.986	0.017	0.03	92%	76%	8%	12%	14%	11%	Yes	19%	0.25
Mean	0.973	0.068	0.14	92%	88%	7%	6%	12%	7%	na	Total 10%	na

na = not applicable. Nd = not determined. Total = long term precision of total toxicity (%RSD)

Table 37. Summary of validation data for HPLC-FLD analysis of Pacific oysters following AOAC 2005.06.

Toxin	Linearity (r^2)	LOD ug/g STX equiv	LOQ ug/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged-ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL			
GTX 1,4	0.96	0.16	0.40	88%	97%	18%	8%	17%	16%	Yes	26%	0.34
NEO	0.991	0.16	0.40	51%	61%	24%	7%	17%	7%	Yes	23%	0.32
dcNEO	nd	<0.16	<0.32	58%	54%	4%	3%	nd	nd	nd	nd	nd
dcSTX	0.994	0.004	0.01	82%	77%	6%	13%	16%	21%	Yes	nd	0.27
GTX 2,3	0.991	0.05	0.16	90%	94%	7%	4%	17%	12%	Yes	17%	0.23
GTX 5	0.995	0.002	0.008	85%	85%	4%	2%	14%	10%	Yes	15%	0.20
STX	0.993	0.011	0.05	123%	121%	4%	1%	15%	10%	Yes	5%	0.14
dcGTX 2,3	0.991	0.029	0.09	79%	70%	5%	2%	16%	14%	Yes	nd	0.23
C 1,2	0.994	0.01	0.03	97%	99%	3%	2%	3%	6%	Yes	14%	0.15
Mean	0.989	0.053	0.14	87%	88%	9%	5%	na	na	na	Total 14%	na

na = not applicable. Nd = not determined. Total = long term precision of total toxicity (%RSD)

Table 38. Summary of validation data for HPLC-FLD analysis of native oysters following AOAC 2005.06.

Toxin	Linearity (r^2)	LOD ug/g STX equiv	LOQ ug/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged-ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL			
GTX 1,4	0.964	0.16	0.40	68%	94%	13%	13%	16%	16%	nd	26%	0.33
NEO	0.949	0.15	0.40	59%	82%	8%	15%	16%	16%	nd	19%	0.28
dcNEO	nd	<0.16	<0.32	60%	71%	3%	3%	nd	nd	nd	nd	nd
dcSTX	0.989	0.008	0.03	78%	81%	4%	3%	14%	3%	Yes	nd	0.19
GTX 2,3	0.977	0.1	0.34	73%	78%	6%	3%	11%	12%	Yes	15%	0.19
GTX 5	0.987	0.003	0.01	83%	79%	3%	3%	4%	16%	Yes	18%	0.22
STX	0.983	0.02	0.07	111%	108%	3%	2%	7%	8%	Yes	8%	0.11
dcGTX 2,3	0.988	0.037	0.12	70%	56%	7%	2%	8%	12%	Yes	nd	0.20
C 1,2	0.988	0.017	0.06	82%	68%	8%	4%	13%	10%	Yes	12%	0.18
Mean	0.978	0.062	0.18	78%	81%	7%	6%	11%	12%	na	Total 11%	na

na = not applicable. Nd = not determined. Total = long term precision of total toxicity (%RSD)

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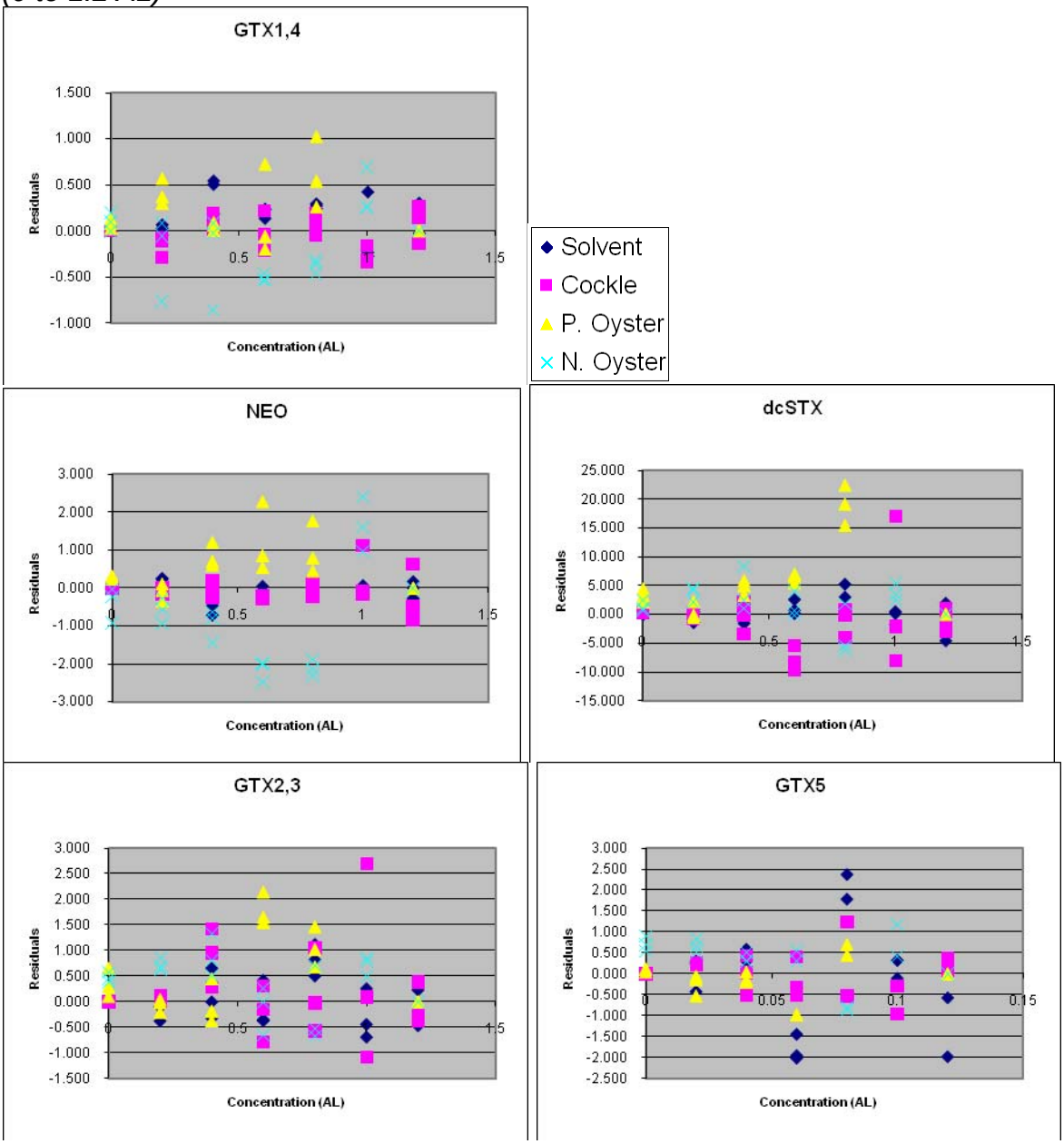
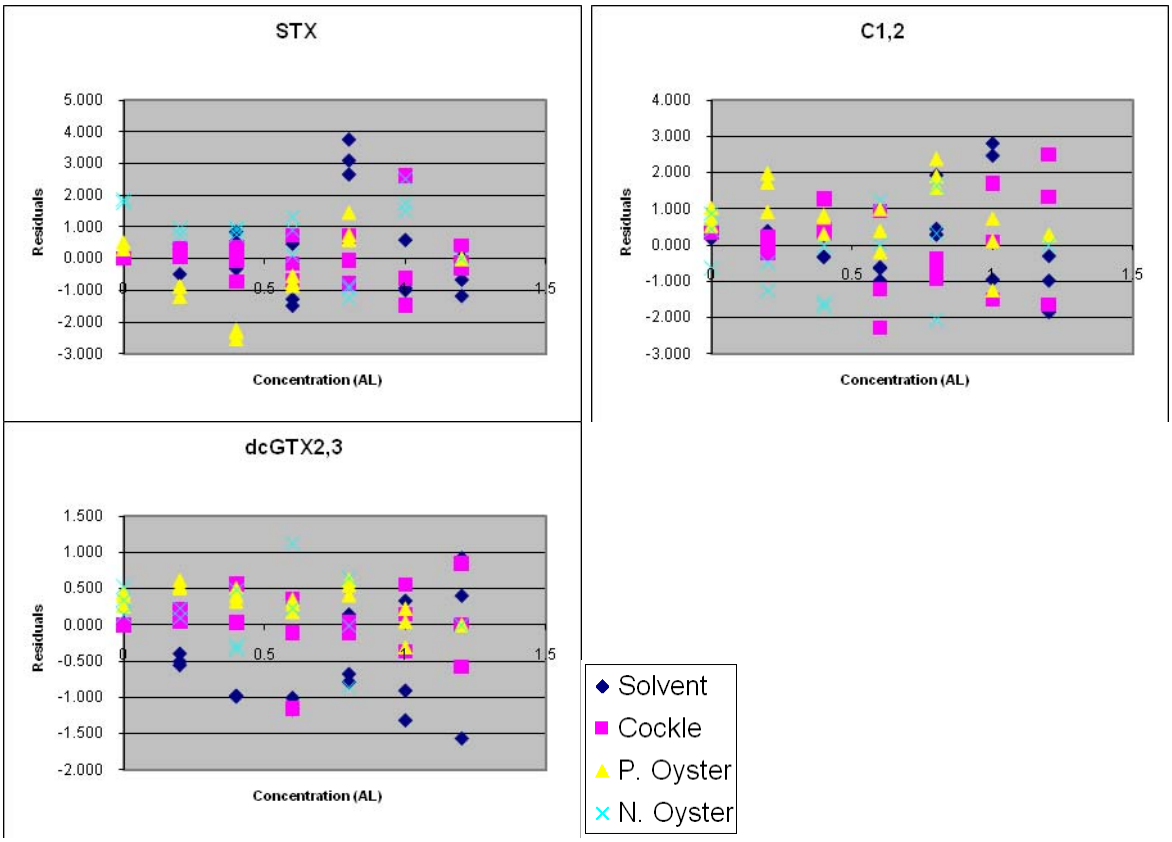
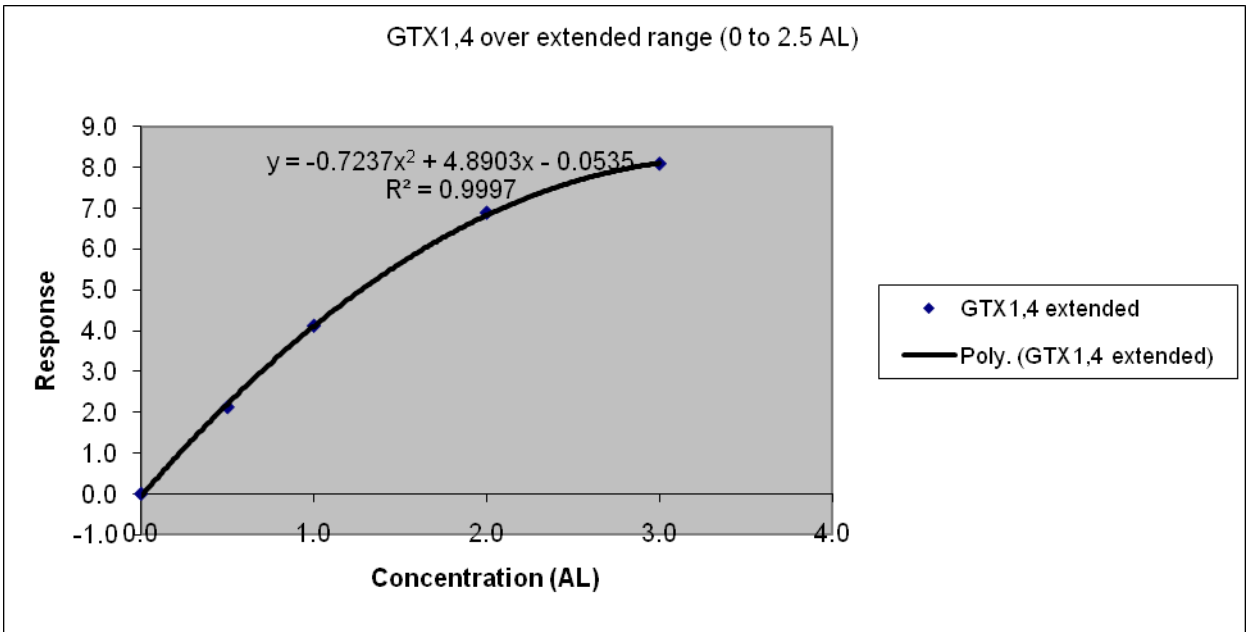
Appendix 1. Residual plots for individual PSP toxins in oyster and cockle extracts, fractions (where applicable) and solvent calibrations over the working calibration range (0 to 1.2 AL)





Appendix 2. Calibration of GTX1,4 in Pacific oyster extract over extended range (0 to 3.0 AL) showing best fit quadratic curve function. Each point plotted from mean of 3 replicate oxidations and analyses.



Appendix 3. Linear regression gradients, intercepts and coefficients, plus relative standard deviations of response factors and f-test goodness of fit (f-critical = 2.64) calculated for each PSP toxin in Pacific oyster, native oyster and cockle extracts, solvent and fractions (when applicable) over extended calibration range (0 to 2.5 AL; GTX5 = 0 to 0.25 AL).

Toxin	Matrix	Calibration gradient	Intercept	r2	RSD% of response factors	F-test
GTX1,4	Solvent	3.13	0.017	0.991	8%	1.20
	Cockles	3.88	0.194	0.992	13%	0.35
	P. Oysters	2.69	0.231	0.955	15%	1.82
	N. Oysters	3.32	0.816	0.831	21%	2.23
NEO	Solvent	9.65	-0.249	0.998	4%	1.28
	Cockles	9.53	-0.275	0.997	5%	1.38
	P. Oysters	9.95	1.061	0.987	12%	0.76
	N. Oysters	9.80	-0.007	0.890	22%	1.93
dcSTX	Solvent	75.43	1.146	0.991	5%	2.94
	Cockles	55.44	3.256	0.958	9%	3.78
	P. Oysters	148.64	-0.785	0.999	4%	0.72
	N. Oysters	73.32	0.170	0.994	5%	1.79
GTX2,3	Solvent	7.67	-0.476	0.975	12%	1.59
	Cockles	6.47	0.244	0.939	15%	2.12
	P. Oysters	17.96	-0.223	0.997	3%	2.74
	N. Oysters	8.35	0.356	0.978	12%	1.30
GTX5	Solvent	154.35	-1.210	0.983	16%	0.01
	Cockles	151.42	0.458	0.971	8%	0.04
	P. Oysters	264.26	-0.129	1.000	3%	0.00
	N. Oysters	131.97	0.067	0.992	6%	0.02
STX	Solvent	19.07	-1.150	0.983	12%	1.06
	Cockles	14.94	0.203	0.963	10%	2.90
	P. Oysters	38.87	-0.759	0.999	6%	0.30
	N. Oysters	18.75	0.142	0.992	6%	2.03
C1,2	Solvent	25.01	-0.157	0.991	8%	1.20
	Cockles	22.74	1.456	0.978	8%	2.62
	P. Oysters	25.58	1.427	0.996	6%	0.77
	N. Oysters	33.54	-0.639	0.996	6%	0.87
dcGTX2,3	Solvent	10.33	-0.870	0.944	22%	1.21
	Cockles	4.90	0.347	0.984	7%	2.33
	P. Oysters	5.96	0.393	0.995	7%	0.68
	N. Oysters	10.46	-0.788	0.973	7%	0.85

Appendix 4. Results obtained from ruggedness experiments on Pacific oysters, native oysters and cockles. Results shown in terms of a) concentrations, b) parameter differences and c) parameter difference percentages.

Pacific oysters:

a) Concentration data and RSDs (%) from ruggedness experiments and precision tests (n=8)

	Ruggedness experiments (n=8) (experiment number given)										Precision (n=8)	
Toxin	1	2	3	4	5	6	7	8	Mean	RSD %	Mean	RSD %
GTX 1,4	0.80	0.92	1.03	0.77	0.90	0.95	0.89	0.75	0.88	11%	0.89	9%
NEO	0.16	0.16	0.22	0.15	0.14	0.13	0.15	0.16	0.16	17%	0.17	9%
dcGTX 2,3	0.011	0.009	0.011	0.015	0.015	0.020	0.014	0.010	0.013	26%	0.011	12%
C 1,2	0.33	0.35	0.37	0.45	0.47	0.53	0.48	0.32	0.41	19%	0.36	11%
dcSTX	0.004	0.005	0.004	0.003	0.006	0.003	0.004	0.004	0.004	24%	0.004	11%
GTX 2,3	0.36	0.33	0.35	0.46	0.43	0.54	0.50	0.31	0.41	20%	0.38	11%
GTX 5	0.021	0.021	0.022	0.017	0.021	0.020	0.021	0.018	0.020	8%	0.019	10%
STX	0.37	0.35	0.34	0.30	0.33	0.36	0.37	0.29	0.34	10%	0.38	9%

b) Parameter differences

	A	B	C	D	E	F	G	Key to parameters (non-N-hydroxylated toxins)	Key to parameters (N-hydroxylated toxins)
GTX 1,4	0.0054	0.0296	0.0558	-0.0739	0.0128	-0.1404	-0.0450	A = C18 extract pH	A = C18 extract pH
NEO	0.0252	-0.0228	0.0185	-0.0032	0.0171	-0.0141	-0.0237	B = Peroxide concentration	B = Periodate pH
dcGTX 2,3	-0.0032	0.0013	-0.0006	-0.0040	-0.0003	-0.0007	0.0034	C = Vortex mixing time	C = Vortex mixing time
C 1,2	-0.0709	0.0151	0.0003	-0.0841	-0.0520	-0.0382	0.0699	D = Ambient temperature	D = Ambient temperature
dcSTX	-0.0005	0.0008	0.0009	0.0000	-0.0009	0.0002	-0.0009	E = Oxidation time	E = Oxidation time
GTX 2,3	-0.0712	0.0118	0.0015	-0.0663	-0.0385	-0.0366	0.1099	F = Acetic acid volume	F = Acetic acid volume
GTX 5	0.0003	0.0014	0.0020	0.0001	0.0000	-0.0018	-0.0006	G = NaOH concentration	G = Matrix modifier pH
STX	0.0005	0.0254	0.0288	0.0111	0.0057	-0.0372	0.0244		

c) Parameter difference percentages

	A	B	C	D	E	F	G
GTX 1,4	1%	3%	6%	-8%	1%	-16%	-5%
NEO	15%	-13%	11%	-2%	10%	-8%	-14%
dcGTX 2,3	-29%	12%	-5%	-35%	-3%	-6%	30%
C 1,2	-20%	4%	0%	-23%	-15%	-11%	20%
dcSTX	-13%	20%	23%	1%	-22%	5%	-21%
GTX 2,3	-19%	3%	0%	-18%	-10%	-10%	29%
GTX 5	1%	7%	10%	0%	0%	-9%	-3%
STX	0%	7%	8%	3%	1%	-10%	6%

Native oysters:

a) Concentration data and RSDs (%) from ruggedness experiments and precision tests (n=8)

	Ruggedness experiments										Precision (n=8)	
Toxin	1	2	3	4	5	6	7	8	Mean	RSD	Mean	RSD
dcGTX 2,3	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	17%	0.01	0.15
C 1,2	0.07	0.08	0.08	0.08	0.09	0.10	0.09	0.07	0.08	15%	0.08	0.11
dcSTX	0.18	0.21	0.21	0.18	0.20	0.20	0.20	0.16	0.193	9%	0.21	0.11
GTX 2,3	0.36	0.38	0.40	0.42	0.40	0.51	0.49	0.31	0.41	16%	0.40	0.12
GTX 5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	10%	0.01	0.07
STX	0.18	0.19	0.19	0.16	0.16	0.18	0.20	0.16	0.18	9%	0.21	0.11

b) Parameter differences

	A	B	C	D	E	F	G
dcGTX 2,3	-0.0012	-0.0009	0.0010	-0.0019	-0.0007	-0.0015	-0.0007
C 1,2	-0.0103	0.0025	-0.0007	-0.0125	-0.0076	-0.0132	0.0071
dcSTX	-0.0012	0.0094	0.0095	-0.0090	-0.0086	-0.0263	-0.0046
GTX 2,3	-0.0397	0.0025	0.0067	-0.0481	-0.0270	-0.0725	0.0735
GTX 5	0.0002	0.0000	0.0006	0.0000	-0.0003	-0.0009	0.0000
STX	0.0051	0.0012	0.0108	0.0088	-0.0019	-0.0242	0.0074

c) Parameter difference percentages

	A	B	C	D	E	F	G
dcGTX 2,3	-14%	-11%	12%	-22%	-8%	-17%	-8%
C 1,2	-13%	3%	-1%	-16%	-10%	-17%	9%
dcSTX	-1%	4%	4%	-4%	-4%	-12%	-2%
GTX 2,3	-10%	1%	2%	-12%	-7%	-18%	18%
GTX 5	4%	-1%	10%	0%	-4%	-14%	-1%
STX	2%	1%	5%	4%	-1%	-12%	4%

Cockles:

a) Concentration data and RSDs (%) from ruggedness experiments and precision tests (n=8)

	Ruggedness experiments										Precision (n=8)	
Toxin	1	2	3	4	5	6	7	8	Mean	RSD	Mean	RSD
dcGTX 2,3	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	20%	0.01	0.10
C 1,2	0.06	0.06	0.07	0.08	0.08	0.09	0.08	0.05	0.07	17%	0.06	0.11
dcSTX	0.15	0.16	0.19	0.16	0.19	0.19	0.18	0.14	0.171	12%	0.17	0.09
GTX 2,3	0.36	0.33	0.37	0.45	0.43	0.51	0.49	0.30	0.40	19%	0.38	0.17
GTX 5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	8%	0.01	0.09
STX	0.18	0.20	0.20	0.17	0.21	0.21	0.23	0.16	0.19	11%	0.20	0.08

b) Parameter differences

	A	B	C	D	E	F	G
dcGTX 2,3	-0.0017	-0.0004	-0.0004	-0.0018	-0.0004	-0.0004	0.0022
C 1,2	-0.0077	0.0032	0.0022	-0.0137	-0.0068	-0.0068	0.0109
dcSTX	-0.0109	0.0070	0.0173	-0.0235	-0.0080	-0.0174	0.0029
GTX 2,3	-0.0535	0.0053	0.0120	-0.0704	-0.0432	-0.0399	0.0957
GTX 5	-0.0001	0.0001	0.0005	0.0000	0.0000	-0.0010	0.0002
STX	-0.0124	0.0080	0.0194	-0.0033	-0.0148	-0.0275	0.0064

c) Parameter difference percentages

	A	B	C	D	E	F	G
dcGTX 2,3	-17%	-4%	-4%	-18%	-5%	-4%	23%
C 1,2	-12%	5%	3%	-21%	-11%	-11%	17%
dcSTX	-6%	4%	10%	-14%	-5%	-10%	2%
GTX 2,3	-14%	1%	3%	-18%	-11%	-10%	25%
GTX 5	-1%	2%	6%	0%	0%	-12%	2%
STX	-6%	4%	10%	-2%	-7%	-14%	3%

Appendix 5a. Results obtained from qualitative and quantitative (LC-FLD) analysis of PSP toxins in acetic acid extracts of oyster and cockle samples with those obtained from qualitative (LC-FLD) and quantitative (MBA) analysis of HCl extracts.

Sample code (BTX/2008)	250	319	318	240	313	237	238	248	251	805	806	808	797	799	800	2504	1388	1709*	1113	874	790	796	801
Species	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	PO	PO	PO	PO	PO	PO	Co
GTX 1,4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NEO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dcGTX 2,3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C 1,2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2,3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.02	0.00	0.07	0.00	0.00	0.00
GTX 5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
STX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.00	0.00	0.00
Total HPLC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	0	10	0	0	0
MBA result	No test	No test	No test	No test	No test	No test	No test	No test	No test	No test	No test	No test	No test	No test	No test	Nd	No test	No test	No test	Nd	No test	No test	No test
Original HCl HPLC screen	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Pos	Nd	Nd	Nd	Pos	Nd	Nd	Nd
Acetic acid HPLC screen	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Pos	Nd	Pos*	Nd	Pos	Nd	Nd	Nd

Sample code	1411	1413	1453	1475	1504	1509	1510	1511	1512	1513	1514	1525***	1539**	1502	1437	1438	1443	1450	1461**	1464**
Species	Co	Co	Co	Co	Co	Co	Co	Co	Co	Co	Co	Co	Co	PO	PO	PO	PO	PO	PO	PO
GTX 1,4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NEO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dcGTX 2,3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C 1,2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2,3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
STX	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total HPLC	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MBA result	No test	No test	No test	Nd	No test	No test	No test	No test	No test	No test	No test	Nd	Nd	No test	No test	No test	No test	No test	Nd	Nd
Original HCl HPLC screen	Nd	Nd	Nd	Pos	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Pos	Pos	Nd	Nd	Nd	Nd	Nd	Pos	Pos
Acetic acid HPLC screen	Nd	Nd	Nd	Pos	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd

Individual toxin concentrations given in µg STX eq./g. Total PSP toxin HPLC results and MBA results given in µg STX eq./100 g.
* Acetic acid screen only positive with the presence of three toxin peaks with signal to noise ratio of <3
** Potential toxin peaks detected in acetic acid screen and quantitation, but all below S/N threshold of 3:1
*** HCl screen contained small GTX5 peak only, thought to be interference peak

Appendix 5b. Comparison of results obtained from quantitative (LC-FLD) analysis of PSP toxins in acetic acid extracts of oyster and cockle samples (μg STX eq./g; Oshima TEFs) with quantitative (positive MBA) analysis of HCl extracts.

1) Pacific Oysters (n= 14)

Sample code	RM1	RM2	BTX/2008/1604	RM3	SAMS 1	SAMS 5	SAMS 6	SAMS 7	SAMS 9	SAMS 12	SAMS 13	SAMS 15	SAMS 18	SAMS 19
Sample source	Cefas	Cefas	OCM	Cefas	SAMS	SAMS	SAMS	SAMS	SAMS	SAMS	SAMS	SAMS	SAMS	SAMS
GTX 1,4	0.20	0.88	0.49	0.81	0.44	0.66	0.32	0.41	0.97	0.56	0.33	0.53	1.29	0.48
NEO	0.02	0.19	0.03	0.45	0.25	0.33	0.11	0.17	0.32	0.33	0.21	0.22	0.72	0.32
dcGTX 2,3	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
C1,2	0.25	0.39	0.05	0.10	0.05	0.08	0.03	0.04	0.10	0.07	0.04	0.07	0.14	0.06
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2,3	0.00	0.51	0.19	0.11	0.03	0.06	0.02	0.02	0.09	0.03	0.02	0.05	0.06	0.02
GTX 5	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
STX	0.00	0.16	0.13	0.15	0.14	0.22	0.06	0.08	0.27	0.14	0.09	0.15	0.26	0.12
Total (ug STX eq/g)	0.49	2.17	0.90	1.62	0.91	1.35	0.54	0.72	1.75	1.14	0.68	1.02	2.47	0.99
Total (ug STX eq/100 g)	49	217	90	162	91	135	54	72	175	114	68	102	247	99
MBA result	37 [c]	182 [c]	44	58 [c]	53	55	36	37	68	63	39	44	116	50

2) Native Oysters (n= 15)

Sample code	RM4	SAMS 49	SAMS 50	SAMS 51	SAMS 55	SAMS 60	Cefas 131	Cefas 169	Cefas 170	Cefas 171	Cefas 172	Cefas 173	Cefas 174	Cefas 175	Cefas 176
Sample source	Cefas	SAMS	SAMS	SAMS	SAMS	SAMS	Fresh	Frozen	Frozen	Frozen	Frozen	Frozen	Frozen	Frozen	Frozen
GTX 1,4	0.66	0.55	0.33	0.42	0.50	0.33	0.22	0.18	0.10	0.17	0.11	0.40	0.45	0.35	0.47
NEO	0.51	0.50	0.37	0.48	0.52	0.30	0.06	0.21	0.19	0.16	0.00	0.38	0.34	0.31	0.48
dcGTX 2,3	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C1,2	0.06	0.06	0.05	0.05	0.06	0.04	0.09	0.03	0.03	0.03	0.06	0.05	0.06	0.05	0.07
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2,3	0.09	0.07	0.05	0.05	0.06	0.04	0.07	0.03	0.04	0.06	0.21	0.05	0.06	0.07	0.07
GTX 5	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
STX	0.19	0.41	0.25	0.31	0.36	0.26	0.04	0.18	0.26	0.31	0.94	0.30	0.39	0.22	0.43
Total (ug STX eq/g)	1.51	1.60	1.06	1.31	1.50	0.97	0.49	0.62	0.61	0.75	1.32	1.18	1.29	1.00	1.53
Total (ug STX eq/100 g)	151	160	106	131	150	97	49	62	61	75	132	118	129	100	153
MBA result	59 [c]	68	41	37	46	40	Neg	33	33	34	40	55	44	37	57

3) Cockles (n= 19)

Sample code	Co 21	Co 22	Co 24	Co 29	Co 35	Co 61	Co 62	Co 64	Co 65	Co 66
Sample source	Integrin	Integrin	Integrin	Integrin	Integrin	SAMS	SAMS	SAMS	SAMS	SAMS
GTX 1,4	0.00	0.00	0.00	0.00	0.00	0.36	0.44	0.61	0.51	0.65
NEO	0.00	0.00	0.00	0.00	0.00	0.25	0.27	0.36	0.39	0.50
dcGTX 2,3	0.01	0.02	0.00	0.01	0.02	0.01	0.01	0.01	0.01	0.01
C1,2	0.04	0.04	0.04	0.04	0.05	0.02	0.03	0.05	0.04	0.06
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2,3	0.22	0.27	0.23	0.25	0.37	0.00	0.00	0.03	0.00	0.03
GTX 5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
STX	0.01	0.01	0.00	0.01	0.01	0.12	0.16	0.25	0.23	0.29
Total (ug STX eq/g)	0.28	0.34	0.27	0.31	0.45	0.77	0.91	1.30	1.18	1.54
Total (ug STX eq/100 g)	28	34	27	31	45	77	91	130	118	154
MBA result	27 ***a***	33 ***a***	39	40	37 ***a***	63	73	104	102	116

Sample code	Co LRM5 *b*	Co RM6a	Co RM6b	Co RM6c	Co RM6d	Co RM6e	Co RM7a	Co RM7b	Co RM7c
Sample source	Cefas	Cefas	Cefas	Cefas	Cefas	Cefas	Cefas	Cefas	Cefas
GTX 1,4	0.99	0.29	0.28	0.20	0.17	0.17	0.31	0.23	0.18
NEO	0.36	0.03	0.04	0.00	0.00	0.00	0.00	0.00	0.00
dcGTX 2,3	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.02	0.00
C1,2	0.12	0.16	0.15	0.10	0.10	0.10	0.17	0.12	0.11
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2,3	0.17	0.21	0.21	0.12	0.13	0.13	0.22	0.15	0.12
GTX 5	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
STX	0.20	0.12	0.11	0.07	0.07	0.07	0.12	0.09	0.07
Total (ug STX eq/g)	1.84	0.82	0.81	0.52	0.50	0.49	0.83	0.63	0.49
Total (ug STX eq/100 g)	184	82	81	52	50	49	83	63	49
MBA result	58 ***c***	43 ***c***	40 ***c***	36 ***c***	34 ***c***	38 ***c***	43 ***c***	41 ***c***	35 ***c***

Results highlighted in red are > 80μg STX eq./100g

[a] For MBAs where only 1 mouse was positive, MBA result taken as mean of 1 mouse + 2nd mouse at half LOD (16 ug STX eq/100g)

[b] HPLC result = mean of multiple extractions and analyses (long term LRM)

[c] Samples were frozen prior to shipment and MBA analysis at external laboratory

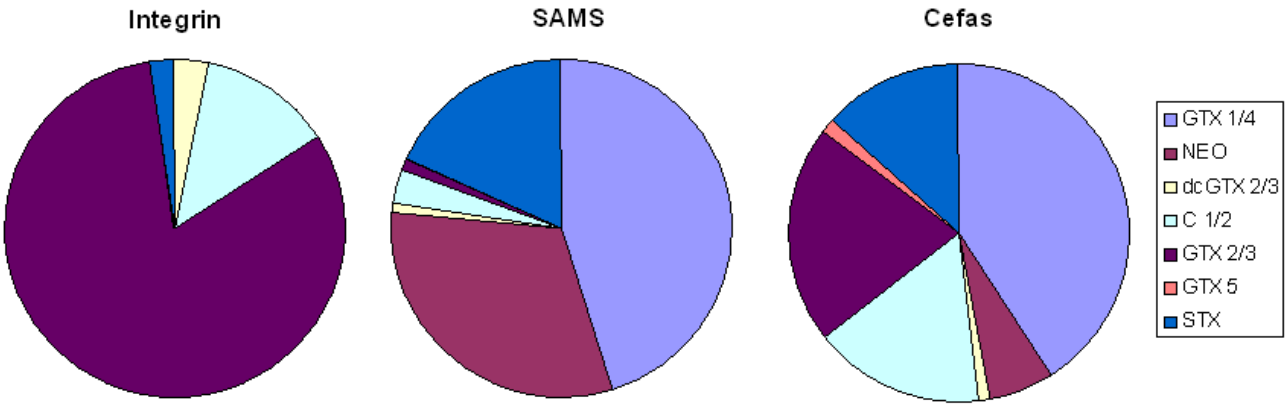
Source: Cefas, Integrin, SAMS or OCM (official control monitoring programme)

Appendix 5c. Comparison of results obtained from quantitative (LC-FLD) analysis of PSP toxins in extracts of oyster and cockle samples (μg STX eq./g; Oshima TEFs) at Cefas with quantitative (LC-PCOX-FLD) analysis at CFIA.

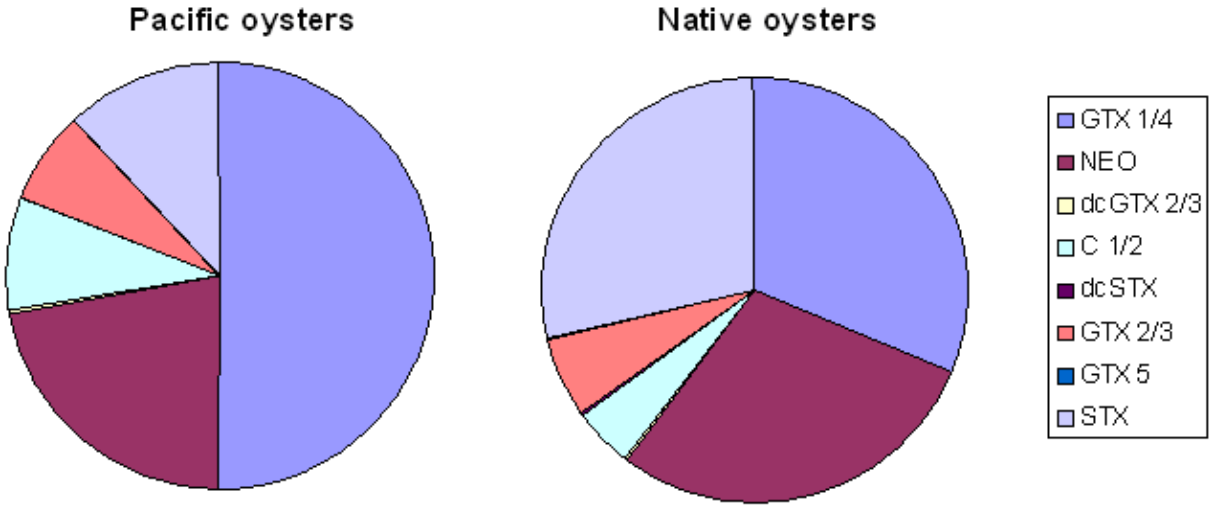
			Cefas		CFIA	
Species	Source	Sample	MBA	HPLC	HCl HPLC	HAC HPLC
P. Oyster	SAMS	PO1	53	91	112	85
		PO5	55	135	113	110
		PO6	36	54	43	41
		PO7	37	72	55	49
		PO9	68	78	124	120
		PO12	63	114	102	101
		PO13	39	68	67	58
		PO15	44	102	80	86
		PO18	116	247	184	175
		PO19	50	99	96	77
	OCM	BTX1604	44	90	na	68
	Cefas	PO RM2	182	217	na	155
N. Oyster	SAMS	NO 49	68	160	147	121
		NO 50	41	106	91	76
		NO 51	37	131	68	121
		NO 55	46	150	128	106
		NO 60	40	97	87	108
Cockle	Integrin	Co 21	27	28	na	16
		Co 22	33	34	na	24
		Co 24	39	27	na	23
		Co 29	40	31	na	19
		Co 35	37	45	na	30
	SAMS	Co 61	63	77	62	58
		Co 62	73	91	107	87
		Co 64	104	130	125	101
		Co 65	102	118	125	92
		Co 66	116	154	167	140
	Cefas	Co LRM5	58	184	na	136
		Co RM6a	43	82	na	42
		Co RM6b	40	81	na	50
		Co RM6c	36	52	na	36
		Co RM6d	34	50	na	31
		Co RM6e	38	49	na	33
		Co RM7a	43	83	na	65
		Co RM7b	41	63	na	50
		Co RM7c	35	49	na	39
Mussel	Cefas / OCM	Mus LRM 126	na	51	na	30
		Mus LRM 161	na	56	na	51
		Mus LRM 126	na	56	na	52

Appendix 5d. Mean relative proportions (as percentage of total μg STX eq.) of individual toxins present in the cockles, Pacific oysters and native oysters

i) Cockles



ii) Pacific oysters and native oysters (mean of all contaminated materials from all sources)



Appendix 6: Relative toxicity factors for PSP toxin analogues (based on Oshima, 1995)

Toxin	Relative toxicity	Toxins	Relative toxicity used
GTX1	0.994	GTX 1.4	0.994
GTX4	0.726		
dcNEO	0.513	dcNEO	0.513
NEO	0.924	NEO	0.924
dcSTX	0.513	dcSTX	0.513
GTX 2	0.359	GTX 2,3	0.638
GTX 3	0.638		
GTX 5	0.064	GTX 5	0.064
STX	1.000	STX	1.000
dcGTX 2	0.154	dcGTX 2,3	0.377
dcGTX 3	0.377		
C 1	0.006	C 1,2	0.096
C 2	0.096		

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Head office
Centre for Environment,
Fisheries & Aquaculture Science
Pakefield Road, Lowestoft,
Suffolk NR33 0HT UK

Tel +44 (0) 1502 56 2244
Fax +44 (0) 1502 51 3865
Web www.cefas.co.uk

Centre for Environment,
Fisheries & Aquaculture Science
Weymouth Laboratory,
Barrack Road, The Nothe, Weymouth,
Dorset DT4 8UB

Tel +44 (0) 1305 206600
Fax +44 (0) 1305 206601

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