Cefas contract report C2934 variation 5

Refinement and validation of the AOAC method (2005.06) to improve the determination of N-hydroxylated paralytic shellfish poisoning toxins in king scallops and queen scallops by liquid chromatography and fluorescence detection

FSA Contract Reference: ZB1807 – FS235002

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Final report

June 2011

78 pages

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Executive Summary

The AOAC 2005.06 LC-FLD method for the detection and quantitation of paralytic shellfish poisoning toxins in bivalve shellfish was approved in 2006 by the European Commission as an official control monitoring method. The method involves the use of both a qualitative screening step, for determination of the presence of PSP toxins, and a full quantitation step for determining individual PSP toxin concentrations and total PSP toxicity. The AOAC 2005.06 method was originally subjected to an in-house single laboratory validation at Cefas between July 2008 and July 2009 for whole king scallops (*Pecten maximus*) and whole queen scallops (*Aequipecten opercularis*). The conclusions from this work showed the poor method performance for the determination of the N-hydroxylated toxins in both king and queen scallops. In addition, the periodate oxidation step in general was found to result in poor toxin recovery and subsequent analytical sensitivity. As part of the work, parallel testing was performed whereby a number of scallops samples were analysed by both the mouse bioassay and AOAC LC methodologies. Results indicated a fairly good agreement in toxicity results returned by the methods in king scallops, but with a large under-estimation in PSP toxicity for queen scallops using the LC-FLD method, possibly due to the poor method performance for the N-hydroxylated toxins.

This study details the investigations conducted at Cefas to improve the performance of the AOAC 2005.06 LC-FLD method for the determination of PSP toxins in king and queen scallop samples. Options were initially investigated for refining the method with the aim of improving both the recovery of the N-hydroxylated toxins from the scallop matrices following periodate oxidation and also the subsequent sensitivity of the LC-FLD analysis. Once a suitable method was refined, the goal was to assess the applicability of the refined method to the analysis of PSP toxins in whole king scallops and queen scallops as a potential for use within the UK national biotoxin monitoring program. Validation protocol was applied to the 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 2 and 3 together (GTX2,3), and 5 (GTX5), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl toxins C1 and C2 together (C1,2)). Due to recent availability issues with the N-hydroxylated decarbamoylneosaxitoxin (dcNEO), no work was possible with this toxin.

Refinement work focussed on the potential effects of varying key method parameters. The parameters investigated included the use of additional C18 clean up steps, different ion exchange cartridges, the potential effect of pH on fraction oxidation, the effects of periodate composition and pH, effects of varying oxidation parameters and temperatures, the use of protein precipitation prior to oxidation and the effects of pH during the oxidation reactions. Results indicated that little effect was gained through altering the majority of these parameters, with the exception of the periodate reagent composition. In addition to these investigations, a more thorough study was conducted to examine the potential variability of the scallop matrix effects on the recovery of the GTX1,4 and NEO toxins, with results indicating that in 13 different king scallop samples obtained from around the UK with a variety of temporal and spatial sources, the suppressed recovery effect was still evident, with a variability of recovery between 15% to 19% for each toxin. The problem with the AOAC 2005.06 LC-FLD method therefore was therefore shown to relate to the king scallop samples

sourced from a variety of locations around the UK at different times of the year. Further work was conducted to examine the performance of the official method in other laboratories on the UK scallop samples. A number of king and queen scallop samples were spiked with known concentrations of GTX1,4 and NEO and sent to 3 other experienced biotoxins testing laboratories. Results provided further evidence for the method-related issues with the determination of PSP toxins in both king and queen scallops.

Refinement work continued, focussing on the use of larger analytical injection volumes, optimising the composition of the periodate oxidant, the effects of varying the extraction method and sample dilution, the use and effects of different matrix modifiers for the periodate oxidation reaction and potential opportunities for further reductions in method dilution factors. Experiments conducted indicated the improved performance of the method when utilising a combination of several parameter changes, specifically the use of 100µL injection volumes, the clean-up of larger volumes of extract (1.5 mL), the use of a modified periodate oxidant, and the use of a king scallop matrix modifier for the oxidation of analytical calibration standards. A period of testing was then subsequently conducted to ensure these refined conditions were repeatable within the laboratory and reliably provide a greater level of analytical sensitivity and toxin recovery. Results indicated that the proposed refinements resulted in a significant enhancement in both these factors, with recoveries for GTX1,4 and NEO improving significantly from those reported previously using the normal AOAC 2005.06 method, and the subsequent analytical sensitivity now appearing acceptable. Subsequently, the refined method was taken forward for a full method validation for both GTX1,4 and NEO in king and queen scallops.

The performance of the refined quantitative LC method for king and queen scallops was satisfactory for the N-hydroxylated toxins, which were analysed following refined periodate analysis of C18-cleaned extracts. Toxin recoveries were acceptable, the short, medium and long term precision of the method was also similar to or improved compared to results presented previously for other bivalve species. Importantly, the refined method was shown to be linear over an appropriate range of toxin concentrations and provided good levels of sensitivity in relation to the regulatory limits and the specified target concentration equivalent to 0.2 AL per toxin. Results also showed the method to be robust for the parameters investigated, giving good evidence that the new method is stable and reliable. Method performance characteristics were also shown to fall within the limits detailed in the current UKNRL SOP for the determination of PSP toxins in bivalve shellfish. The validation work conducted enabled the uncertainty of measurement to be calculated for both the N-hydroxylated toxins and for the non-N-hydroxylated toxins following the generation of more up to date long term precision data. Values were found to be similar to those calculated previously for other species.

In terms of comparative performance with the MBA, a total of 25 PSP-negative scallop samples were analysed, and showed no indications of any false negatives. 19 scallop samples shown to be positive following MBA were analysed by the refined LC-FLD method and the overall agreement between the methods was shown to be acceptable. Two queen scallops previously show to give LC-FLD results around 50% of the values determined by the MBA were now found to give an excellent agreement between the MBA and the refined LC-FLD method. A slight positive bias in the LC-FLD results in king scallops as compared with the MBA was found to be attributable to the use of higher toxicity equivalence factors, as found previously in cockles.

As such, the performance characteristics of the refined method for the LC-FLD determination

of PSP toxins in scallops have been shown to be acceptable. Together with the good agreement between the results obtained by LC-FLD and MBA methodologies, the overall recommendation is that this refined method is appropriate to implement into the routine official control monitoring program for the analysis of PSP in both king and queen scallops. It is however important to note that the method is currently only applicable to the analysis of whole scallop samples. Further performance verification testing will be required before the method can be applied to pre-shucked products or any other novel scallop species.

Summary of validation data for LC-FLD analysis of king scallops.

	Linearity	LOD	LOQ	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged -ness	Long term	Standardised
	(r^2)	µg/g STX equiv	µg/g STX equiv	0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL	Stability	Precision	Uncertainty
GTX 1,4	0.999	0.05	0.167	104%	114%	8%	2%	15%	9%	Yes	24%	0.28
NEO	0.996	0.10	0.318	89%	82%	5%	2%	10%	15%	Yes	15%	0.20
dcSTX	0.995	0.007	0.025	61%	63%	5%	3%	7%	8%	Yes	na	0.18
GTX 2,3	0.997	0.09	0.3	67%	67%	8%	4%	13%	14%	Yes	15%	0.21
GTX 5	0.997	0.002	0.008	69%	69%	5%	2%	5%	4%	Yes	20%	0.21
STX	0.997	0.018	0.061	91%	93%	6%	3%	11%	8%	Yes	11%	0.15
dcGTX 2,3	0.990	0.055	0.18	56%	59%	5%	1%	na	na	na	na	na
C 1,2	0.988	0.019	0.063	66%	72%	3%	3%	20%	21%	Yes	13%	0.25
Mean	0.995	0.04	0.14	75%	77%	6%	3%	12%	11%	na	Total = 14%	0.21

na = not analysed. Total = long term precision of total toxicity (%RSD)

Summary of validation data for LC-FLD analysis of queen scallops.

	Linearity	LOD	LOQ	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged -ness	Long term	Standardised
	(r^2)	µg/g STX equiv	µg/g STX equiv	0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL	Stability	Precision	Uncertainty
GTX 1,4	0.999	0.07	0.218	77%	80%	6%	10%	5%	9%	Yes	20%	0.23
NEO	0.982	0.10	0.314	86%	83%	8%	5%	7%	6%	Yes	21%	0.23
dcSTX	0.997	0.004	0.013	72%	67%	2%	5%	22%	6%	Yes	16%	0.24
GTX 2,3	0.991	0.027	0.09	78%	74%	2%	5%	29%	9%	Yes	21%	0.26
GTX 5	0.999	0.002	0.008	77%	72%	4%	7%	22%	9%	Yes	17%	0.26
STX	0.999	0.013	0.043	74%	67%	3%	6%	25%	8%	Yes	18%	0.22
dcGTX 2,3	0.989	na	na	na	na	na	na	na	na	na	na	na
C 1,2	0.987	0.005	0.016	77%	73%	1%	8%	25%	9%	Yes	19%	0.23
Mean	0.993	0.03	0.10	77%	74%	4%	7%	19%	8%	na	19%	0.24

na = not analysed. Total = long term precision of total toxicity (%RSD)

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Glossary

AL	Action Limit
AOAC	AOAC International (formerly Association of Official Analytical Chemists)
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
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	decarbamoylgonyautoxin-2 and 3
dcNEO	decarbamoylneosaxitoxin
dcSTX	decarbamoylsaxitoxin
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
KSc	King scallops
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
QSc	Queen scallops
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

King and queen scallops are filter-feeding bivalve shellfish which may periodically accumulate biotoxins derived from marine phycoplankton. Scallops contaminated with these toxins and subsequently consumed, may impact significantly on human health. One of the most severe groups known to induce human illness [1] is the paralytic shellfish poisoning (PSP) toxins. These Saxitoxin-derived compounds are potent neurotoxins, which may cause severe health effects and even death. Therefore monitoring of scallops is a statutory requirement to ensure consumer protection. The current European Union's (EU) reference method for the detection of PSP toxins is the mouse bioassay (MBA) [2,3]. Cefas and the FSA are committed to moving away from animal assays when valid alternatives are made available. Such methods need to differentiate toxins from non-toxic compounds, separate toxins from each other and operate satisfactorily within a variety of complex matrices [4,5]. One LC 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 has been 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]. Over recent years, Cefas have undertaken the single-laboratory validation of this method for the analysis of PSP toxins in mussels [13], cockles and oysters [14], hard clams and razors [15] and king and queen scallops [16]. Validation experiments followed the requirements described by EC regulation 882/2004 that official control methods should be validated prior to adoption into EU monitoring programmes [17]. Methods need to be characterised by describing the criteria of: accuracy, limits of detection and determination (quantitation), precision, repeatability, reproducibility, recovery, selectivity, linearity, measurement of uncertainty and ruggedness. The validation incorporated a period of parallel testing whereby LC results were compared with those obtained from MBA analysis of mussels obtained from the routine GB biotoxin monitoring programmes. The method was deemed fit for purpose in mussels 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 [18]. Subsequently, in June 2010, the method has been implemented for hard clams, razors and cockles. Validation work for oysters has shown acceptable performance characteristics, but significant differences in method performance between the LC and MBA methods has resulted in a delay to implementation due to the need for additional work on these species. However, work conducted for both king and queen scallops indicated problems with the method performance in these species, specifically issues with the analytical sensitivity and toxin recovery following periodate oxidation of the N-hydroxylated toxins (GTX1,4, NEO and dcNEO) [16,19].

The AOAC 2005.06 method exists at several levels of complexity depending on the PSP toxin within each sample [13]. The method protocol involves the extraction of toxins from shellfish tissue using 1% acetic acid solution in boiling water, followed by the clean-up of extracts using C18 Solid Phase Extraction (SPE) cartridges. Toxins are identified by LC-FLD following periodate oxidation derivatisation to form fluorescent products. This oxidation and analysis step enables samples to be qualitatively "screened" for the presence of the whole suite of PSP toxins. Samples where toxins are detected (LC screen positive) 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 also shown to be present in the screen, the extract is fractionated using ion-exchange SPE cartridges, followed by periodate oxidation of the individual fractions.

Currently, whole king scallops are tested at Cefas with the use of a qualitative screen of HCl extracts, after both periodate and peroxide oxidation, with only PSP-positive samples being fully quantified by MBA. Whole queen scallops as well as pre-shucked (adductor and roe only) scallops are analysed only by MBA.

Given the problems with the method performance in scallops for the N-hydroxylated toxins, the objective of the work presented here was to refine the method to improve the performance. Work was conducted specifically on whole king scallop (*Pecten maximus*) and whole queen scallop (*Aequipecten opercularis*) matrices. Once the method was refined, the objective was to check the performance of the new method, conducting a series of validation tests to define the level of method performance. The validation was to be applied to the two most prevalent N-hydroxylated toxins encountered to date in naturally-contaminated UK bivalve samples, namely GTX1,4 and NEO. Three phases of work were therefore undertaken: Phase 1 involving a series of development and refinement experiments, designed to investigate the potential for improving the method performance for the N-hydroxylated toxins in the two species of scallops. Phase 2 was an in-house single-laboratory validation of the refined method, performed following as closely as possible the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) [4] to obtain information regarding selectivity, linearity, limits of detection, limits of quantitation, recovery, instrumental precision, repeatability, ruggedness and reproducibility. For Phase 3, an assessment was made of the method through testing of scallops obtained from the GB biotoxin monitoring programmes, if available, and from other sources including those contaminated artificially in the laboratory.

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2. Materials and methods

2.1 Overview of the AOAC 2005.06 Method

Whole king and queen scallop samples are shucked and homogenised and the shellfish homogenates extracted with acetic acid, before clean up on C18 Solid Phase Extraction (SPE) cartridges. After pH adjustment, aliquots of the extract are oxidised by periodate reagent in the presence of a matrix modifier, prior to liquid chromatography with fluorescence detection (LC-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.

Both periodate and peroxide oxidation reactions are required as neither oxidant alone will successfully oxidise every toxin to give a suitable level of analytical sensitivity and selectivity. Peroxide oxidation is utilised for the oxidation of all non-N-hydroxylated toxins, whilst the N-hydroxylated toxins, which do not respond to peroxide oxidation, must be oxidised using the periodate reagent. The periodate oxidation method is generally less reliable, due in part to the significant effect of small pH variations [20], 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. Such an approach is believed to result in a more repeatable oxidation (Lawrence, personal communication). This modifier is the C18 SPE cleaned up, acetic acid extract of a Pacific oyster (*Crassostrea gigas*) sample, which has been shown to be free from chromatographic peaks at the same retention times as any of the PSP toxin standards. As described in previous reports [13-16], a quantitation approach was taken to reduce the overall number of analyses per sample to 4 (Table 1; Figure 1).

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

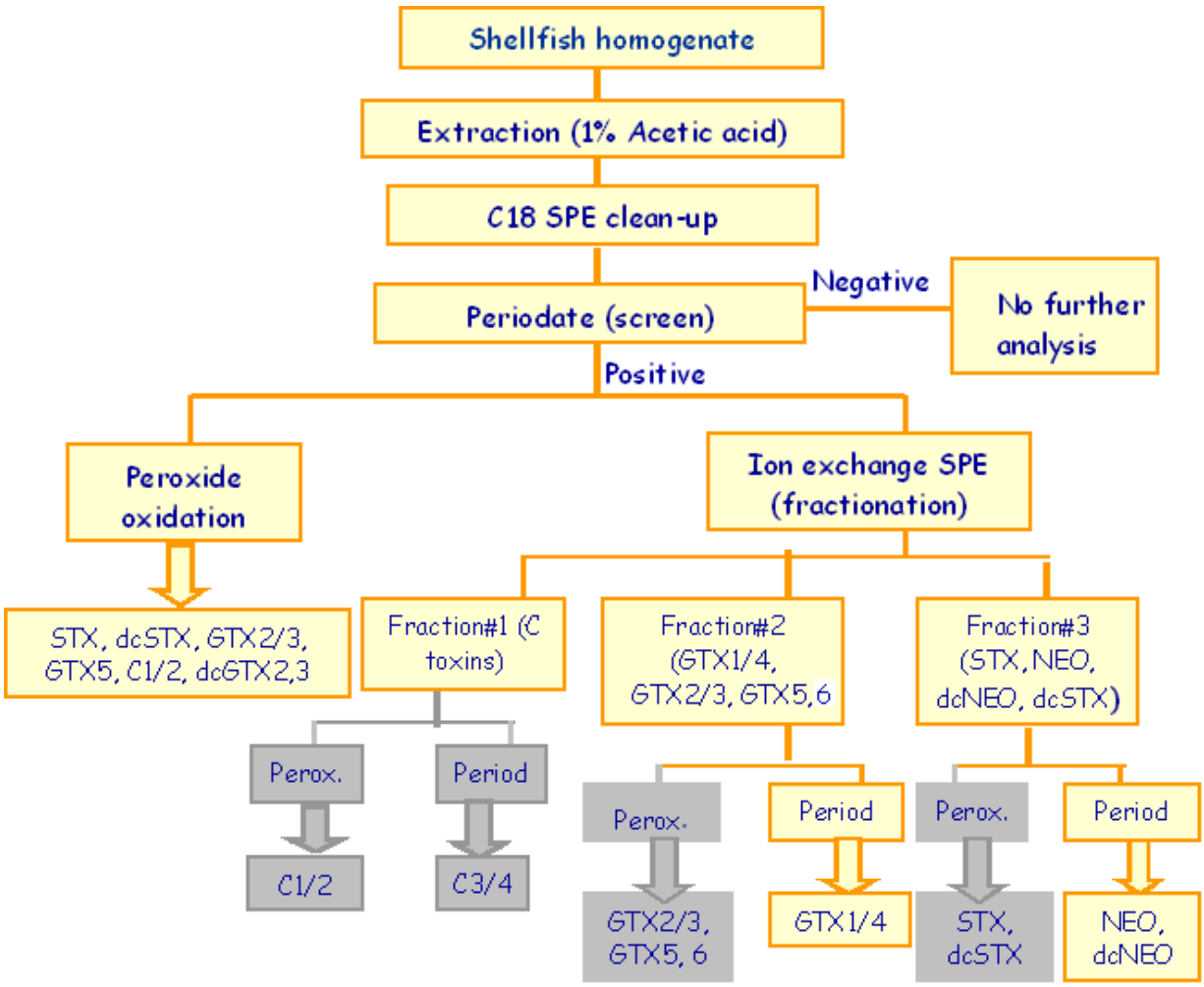


Figure 1. Scheme utilised for screening and quantitation of PSP toxins in scallop samples. *Parts of method in grey not carried out.*

2.2 Laboratory equipment

The following 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), 2 mL 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), cold water 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 was 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 0.1mM 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 the calibration range for each toxin equated to 0.2 to 1.0 AL in terms of STX equivalence. In the case of the isomeric pairs (GTX1,4), the highest toxicity equivalence factor was used for each pair (Appendix 7). Individual toxin results obtained are therefore quoted in terms of μg STX eq./g of flesh and the total PSP toxicity was estimated 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 king scallop samples for use in homogenate and extract spiking studies were obtained from M&J Seafood of Poole, Dorset. Bulk queen scallop samples were obtained from West Coast Sea Products of Kirkcudbright, Dumfries and Galloway. Approximately 0.5 kg each of live king scallops (*Pecten maximus*) and queen scallop (*Aequipecten opercularis*) were shucked and homogenised on arrival, aliquotted into 5.0 (± 0.1g) sub-samples in 50 mL polypropylene centrifuge tubes and stored at –20 °C until use. Frozen 5.0g samples were randomly selected 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. For practical reasons, all validation work involving the spiking of scallops with toxins was carried out on homogenate aliquots taken from the same bulk sample. Due to the low number of naturally contaminated scallops available, king scallop feeding experiments were undertaken to provide further materials for comparative testing (section 5). An additional number of scallop samples (two Atlantic scallops; *Argopecten gibbus*) were obtained from the

Canadian Food Inspection Agency (CFIA) and also analysed using the refined AOAC 2005.06 method. Only two PSP-contaminated queen scallops were available for comparative testing, those which were analysed previously using the normal AOAC 2005.06 method [16].

2.5 Analysis of PSP toxins by Liquid chromatography-fluorescence detection (LC-FLD)

LC was performed on a Gemini C18 HPLC column (150 mm 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. LC 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 LC 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 the suite of available certified toxins (STX, NEO, dcSTX, GTX1,4, GTX2,3, GTX5, dcNEO and C1,2). However, availability issues with dcGTX2,3 prevented the full use of this toxin during the study, with subsequent restrictions being placed on the number of experiments undertaken. The method employed during this study is identical to that described previously for mussels [13], matching closely to that of the original Lawrence method [11,12]. Small deviations from the original procedure have been highlighted previously, specifically the use of an improved ion exchange fractionation step, the use of cooled autosampler and automated solid phase extraction technologies [13-16]. 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 action limit (AL) per toxin (0.16 µg STX eq./g) 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.

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 normal method. Scallops were shucked and homogenised prior to extraction by heating with a 1% acetic acid solution and the supernatant collection. 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) 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 graduated collection tube. The cleaned-up extract was pH-adjusted to pH 6.5 (± 0.5) before diluting the extract with water to a final volume of 4.0mL. Aliquots of this extract were then oxidised prior to LC-FLD analysis. SPE-COOH ion-exchange clean-up was used for all samples containing N-hydroxylated PSP toxins (GTX1,4, dcNEO and NEO). 2mL of cleaned-up extract was passed through an ion-exchange cartridge pre-conditioned with 0.01M ammonium acetate and the eluent 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 [13,18]. 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, dcNEO 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 Phase 1: Refinement of the AOAC 2005.06 method for the improved performance for the N-hydroxylated toxins GTX1,4 and NEO.

In order to determine the way forward with the potential improvement of the LC method for the analysis of N-hydroxylated toxins in scallops, a series of experiments was conducted in order to assess the potential causes of the issues observed. After investigations had been completed, the work was proposed to concentrate on three main areas of work. Firstly, to assess the potential for refining the periodate oxidation method to improve both sensitivity and toxin recovery. Secondly, to examine the effects of varying the extraction and clean-up processes. Thirdly, if none of the above was found to work, then the examination of the potential use of other oxidation reagents with a view to improving the performance of the method would be conducted.

2.8.1 Preliminary investigations into the identification of potential causes

Investigations commenced by examining the effects of varying the various protocols and parameters contained within the official LC-FLD method. A range of specific tests were conducted as detailed:

i) The efficiency of the extraction method was first examined to determine whether any physical properties of the scallop matrices were contributing towards the apparent reduced recoveries. This was performed by conducting further extractions on the spiked homogenate material remaining after the normal double extraction process had been completed. Subsequent clean up, oxidation and analysis were then conducted to determine what percentage of toxins remained in the shellfish after the normal extraction procedure. Experiments were conducted using NEO spiked in triplicate into king scallop homogenates at concentrations equal to 0.5 and 1.0 AL (0.4 and 0.8 µg STX eq/g).

ii) The C18 clean-up process was checked, with the use of two separate C18 clean ups for each sample being utilised to assess whether the further removal of hydrophobic matrix co-extractives could result in the reduction of any specific matrix effects. In addition, the current ion exchange clean up was compared against the official AOAC 2005.06 ion exchange method utilising silica based SPE cartridges.

iii) With the awareness of the importance of pH in the oxidation of samples, the pH of fractionated sample extracts containing GTX1,4 and NEO was examined, and pH

changes made, where possible, to assess whether performance could be improved.

iv) Similarly, another experiment was conducted to determine whether the periodate reagent pH could be altered to improve toxin oxidation product response.

v) The composition of the periodate reagent is strictly defined with the official method. However, for the purpose of examining the method in scallops, the relative proportions and concentrations of the components making up the oxidant were also examined to assess whether any such changes would result in any improvement.

vi) In addition, the specific times and volumes of sample, oxidant and acid involved in the oxidation reaction were also examined.

vii) Work conducted on other LC-FLD methods, specifically the single-laboratory validation of a post-column oxidation LC-FLD method (PCOX), reported the satisfactory performance of the method for the determination of the N-hydroxylated toxins in scallops [22]. One specific feature of this method is the high temperature oxidation of the toxins as they elute from the analytical column on-line. As such, the effects of variable temperature were investigated with the aim of improving the performance of the periodate oxidation. Periodate oxidations were performed over a range of temperatures between 20 and 85^{o}C. A thermostatiscally-controlled heating block was used to heat the oxidant, the sample and the tubes and a tube containing H_2O and a thermometer was used to assess the temperature of the reagents.

viii) The PCOX method also stipulates the precipitation of proteins from sample extracts, prior to analysis. Whilst this is primarily done to improve analytical column lifetime [van de Riet, personal communication], this process was investigated to determine whether it may be useful in reducing matrix effects in the AOAC 2005.06 method. The method described in [22] was used as follows. A 3mL aliquot of crude extract from a king scallop sample spiked with a range of PSTs was spiked with 150µL of 30% tricarboxylic acid (TCA) and vortex mixed for 1 minute. The mixture was centrifuged at 16,000g for 5 minutes, after which the supernatant was recovered and adjusted to pH 4.7 using 1.0M NaOH. The total volume of the tube was made up to 4mL with deionised water, vortex mixed and re-centrifuged at 16000g for a further 5 minutes. The resulting supernatant was filtered (0.2µM syringe filter) prior to C18 SPE clean up, duplicate oxidation and analysis alongside untreated C18-cleaned scallop extracts. Results were used to calculate the potential effects of the protein precipitation, and the variable dilution factors employed were used to normalise the calculations.

ix) Issues relating to the potentially variability of the scallops matrix effects were investigated. Thirteen different king scallops flesh samples, received at Cefas as part of the UK official control programmes were removed from frozen storage. The samples chosen were PSP-negative homogenates, sourced from varying regions of the UK at different times of the year and over a period of three years in total (2007 to 2010). Two 5.0g subs-samples of each sample were taken, one spiked with concentrations of GTX1,4 and NEO equivalent to the regulatory action limit (0.8 µg STX eq/g flesh), the other being left un-spiked. Both sub-samples of each sample were extracted following the usual method, C18-cleaned, fractionated at oxidised by periodate oxidation prior to LC-FLD. Results were used to determine both the occurrence of variable interferences in the matrices and to assess the level of variability of the toxin recoveries within scallop samples with different spatial and temporal origins.

x) Finally, the issue of pH was further addressed, with the monitoring of the pH of extracts, oxidants and the components of oxidation products before, during and after periodate oxidation. This monitoring was performed in both mussels and scallops, to assess whether any significant differences were noticeable between the pH conditions of the samples at any stage in the two species, which may account for the differences in toxin recoveries observed.

Results from these preliminary investigations are given in section 3.1.

2.8.2 Inter-laboratory assessment of method performance for scallops using AOAC 2005.06

In order to eliminate the potential for laboratory bias in the performance issues described, a small inter-laboratory study was organised to determine whether the effects described previously and in this report were reproducible. A total of four laboratories, including Cefas, known to have a good level of experience with the AOAC 2005.06 method (CRL, personal communication) were contacted with a view to participate in the analysis of spiked scallop samples for the determination of N-hydroxylated toxin recovery and analytical sensitivity. The external laboratories involved were Agri-Food and Biosciences Institute Northern Ireland (AFBI), the Marine Institute, Ireland and the Institute for Food Safety, in Holland (RIKILT). Given the issues noted for the periodate oxidation of all PSP toxins, one sample was also submitted containing the non-N-hydroxylated toxins. It is important to note that this study does not constitute a full and thorough inter-laboratory validation study. The protocol was designed to provide information to our laboratory on the potential issues encountered in other laboratories, whilst minimising the use and costs of spiking expensive and valuable analytical standards into test samples. As such, the results can only be used as an indication of the method performance externally.

2.8.2.1 Sample preparation and distribution

PSP-free king and queen scallop homogenates prepared previously, were removed from frozen storage and allowed to thaw to room temperature. Aliquots were randomly selected, split into four groups and fortified with toxins following the regime described in Table 4. Once spiked with the toxins, samples were vortex mixed for 60 seconds and placed into frozen storage (-20^{o}C) for 2 days. After this time, each set of samples was packaged into insulated sample boxes containing frozen ice packs and sealed, before shipment to each participating laboratory. The three parcels arrived within one day of shipment and all samples were reported as arriving safely, with samples still in a frozen state. At each laboratory, samples were immediately placed into frozen storage until the day of analysis.

Table 4. Samples prepared for inter-laboratory analysis of scallops.

Sample name	Species	Toxins spiked (concentration in µg STX eq./g)
KSc 1	King scallops	GTX1,4 (0.32); NEO (0.32)
KSc 2	King scallops	GTX1,4 (0.48); NEO (0.32)
QSc 1	Queen scallops	GTX1,4 (0.32); NEO (0.32)
QSc 2	Queen scallops	GTX1,4 (0.48); NEO (0.32)
KSc 3	King scallops	STX, dcSTX, GTX2,3 (0.16); GTX5 (0.04), C1,2 (0.08)

2.8.2.2 Sample analysis and reporting of results

A protocol was provided to each laboratory (Appendix 1) describing the procedure required for sample analysis. A summary of the processing required for each sample is given in Table 5. Analysis was to be conducted and results reported in terms of individual toxin concentrations (µg STX eq./100g). In addition, the signal to noise ratios of each detected

peak were to be reported to enable an assessment of analytical sensitivity in each laboratory. Participants were informed in advance which toxins were present in the samples, but were given no information on toxin concentrations.

Table 5. Samples processing, analysis and reporting required

Sample name	Process required	Analysis required	Results to report
KSc 1	AOAC 2005.06: All extracted in acetic acid and C18 SPE clean up + ion exchange fractionation (collecting F2 and F3)	Periodate oxidation of C18-cleaned extract + Periodate oxidation of fractions F2 & F3	1) Quantitative concentrations of GTX1,4 and NEO in C18, F2, F3 2) Signal to noise ratios of quantitative peaks for GTX1,4 and NEO in C18, F2 and F3
KSc 2			
QSc 1			
QSc 2			
KSc 3	Extract and C18 SPE clean up	Periodate and peroxide oxidation of C18-cleaned extract Periodate and peroxide oxidation of C18-cleaned extract	1) Quantitative concentrations of STX, dcSTX, GTX2,3, GTX5 and C1,2 in C18 following peroxide 2) Signal to noise ratios of quantitative peaks for above toxins in both periodate and peroxide-oxidised extracts

2.8.2.3 Inter-laboratory data interpretation

Analysis was conducted at each laboratory within three weeks of sample receipt, including the Cefas laboratory. Toxin concentrations and signal to noise ratios were provided by participant and results collated and interpreted at Cefas. Results were analysed to assess the general range of toxin recoveries experienced at each laboratory and signal to noise ratios utilised to estimate analytical limits of detection. This then allowed an assessment to be made concerning whether the issues experienced at Cefas were also experienced in other laboratories. Results are given in section 3.2

2.8.3 Investigations conducted to improve method performance

Following the results obtained from the preliminary investigations detailed above, refinement experiments were conducted to examine the effects of the following parameters:

i) Use of variable injection volumes for injection of periodate-oxidised samples. The effects on analytical sensitivity were investigated through the use of higher injection volumes. A range of different toxin standards and cleaned up sample extracts were analysed using both 50 µL and 100 µL injection volumes. The LC-FLD results were compared in terms of closeness of analytical result and also in terms of chromatographic quality, specifically peak width.

ii) The optimisation of the composition of the periodate reagent was conducted, following on from the results obtained from the preliminary investigations. This primarily involved the analysis of the effects of varying the proportions of periodic acid present in the periodate reagent used for oxidation. A series of periodate reagents were prepared containing different proportions of periodic acid and were each used to oxidise spiked scallop extracts containing both GTX1,4 and NEO. The relative chromatographic responses were assessed to determine whether a refined periodate reagent could potentially be utilised to produce a more sensitive method.

iii) The potential effects of variable extraction methodologies and sample dilution on apparent matrix effects were investigated, by assessing the recoveries of toxins when a) higher volumes of extraction solvent were utilised and b) acetic acid, C18-

cleaned and fractionated extracts were subjected to a range of additional dilutions. In addition, recovery results obtained from the various acetic acid extractions were compared against those obtained following extraction in hydrochloric acid (HCl). The following extraction methods were compared:

Table 6. Extraction methods compared for the extraction of GTX1,4 and NEO from fortified scallop samples

Method	**Protocol**	**Total extract volume**	**Extraction dilution factor**
Normal AOAC 2005.06 acetic acid extraction	5g sample with 2 x 3mL extraction in boiling acetic acid (AcH)	10mL (from 5g sample)	2
Double extraction volume method	2 x 8mL extraction in boiling AcH	20mL (from 5g)	4
Triple extraction volume method	3 x 8mL extraction in boiling AcH	30mL (from 5g)	6
Normal AOAC 2005.06 acetic acid extraction, no heat	5g sample with 2 x 3mL extraction in AcH at room temperature	10mL (from 5g)	2
AOAC 959.08 HCl extraction	15mL HCl to 15g sample and diluted to 30mL	30mL (from 15g)	2

iv) An investigation into the use and composition of matrix modifiers was conducted, using a range of different modifiers sourced from difference locations and from different shellfish species. In this study, work is presented involving the comparison of matrix modifiers prepared from both Pacific oysters, following the AOAC 2005.06 official method, and from king scallops. The scallops modifier was prepared with a view to testing the potential for its use to ensure the fluorescence responses of the N-hydroxylated peaks in the analytical standards were similar to the response observed from toxins at the same concentrations present in the scallop samples. With all the evidence for a matrix-related suppression of toxins following periodate oxidation in both the scallop species, use of a scallop matrix modifier in the calibration standards may potentially reduce this effect. As such, matrix modifiers were prepared from both oysters and king scallops and their effects compared on a number of different days using a range of scallops spiked with varying concentrations of GTX1,4 and NEO.

v) In order to potentially increase the sensitivity of the method, other aspects of the method were investigated which may increase the amount of each toxin being injected on-column during each analytical run. Within the method, the three steps which result in sample dilution are the initial extraction to form the crude sample extracts, the C18 SPE clean up and the ion exchange fractionation. The current double extraction method employs a solvent to sample ratio of 2:1, which typically results in an extraction efficiency of 80% [13,14,16]. As such, there is no further room for reduction in this ratio, without significantly affecting the extraction efficiency. The sensitivity has previously been increased, with the reduction in eluant volumes generated during the refined fractionation step [13,14]. There would however be no further capacity for reducing these volumes further, as with the eluant volumes following C18 clean up. Potentially, however, there may be the

capability for the clean-up of larger volumes of sample extract in both the C18 and ion exchange clean up processes. There will be limitations on the sample loading capacity, which relates both to the column type and size as well as the nature of the components present in the samples. However, such an approach was investigated, with the potential for producing a more concentrated cleaned-up extract and subsequently producing a more sensitive methodology.

vi) Finally, experiments were conducted using a combination of all the methodology changes shown to result in the improved method performance for the quantitation of GTX1,4 and NEO in scallops. The aim of this was to demonstrate that the summation of the individual parameter changes proposed would result in the overall improvement of method performance. A range of spiked king and queen samples were extracted, cleaned, oxidised and analysed using the proposed refined methodology. Results obtained from the LC-FLD analysis were used to assess the effects of the method changes on both toxin recovery and method sensitivity. Predicted method limits of detection (LOD) were estimated following the approach detailed in 2.9.2.

Results from these refinement investigations are described in section 3.3

2.9 Phase 2: Validation of the refined AOAC 2005.06 scallops method (extraction, clean-up and LC-FLD analysis) for N-hydroxylated PSP toxins.

In this study, validation was applied to the refined AOAC 2005.06 LC-FLD scallops method, for the quantitation of N-hydroxylated PSP toxins and total saxitoxin equivalents in king scallop and queen scallop homogenates. The aim was to check that the analytical method is fit for purpose over an appropriate range of PSP toxin concentrations in both scallop species. Validation experimental design should ideally describe both the source and size of errors in the analytical method. This should include random measurement error, laboratory effect, method bias and any potential matrix variation. Availability of an appropriate certified reference material, traceable to international standards with a known level of uncertainty, would 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, as is the case for PSP-contaminated scallops, 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 naturally occurring analytes. Specifically for the determination of PSP toxins in scallop 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. As such, it is noted that good recovery of analytes from spiked samples is not a guarantee of method accuracy. 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, cockles and clams, estimation of method bias was previously conducted through the repeat analysis of spiked homogenates [14,15]. As certified reference materials are also unavailable for scallop species, the same approach was used here. 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].

2.9.1 Method selectivity

Homogenised tissues of king and queen scallops were extracted according to the AOAC method and as described above. Extract sub-samples were cleaned-up using C18 SPE cartridges prior to pH adjustment and dilution to volume. Aliquots were further cleaned up using ion exchange fractionation and aliquots of all were analysed using the refined LC-FLD scallops method following periodate oxidation. Results are presented in section 4.1.

2.9.2 Linearity and linear range of the LC-FLD method for N-hydroxylated toxins

In order to determine the linear range of toxin concentrations over which the refined scallop method can be applied, PSP toxins were spiked into king and queen scallop extracts. The following concentrations of GTX1,4 and NEO were prepared and analysed in triplicate (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 and 1.5 AL. Additional concentrations of 2.0 and 2.5 AL were also run for GTX1,4, given the greater tendency for this toxin to be present at higher concentrations in naturally contaminated samples. Spiked extracts were oxidised by periodate prior to LC-FLD analysis and the linearity of the calibrations was assessed. 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 both GTX1,4 and NEO. Linear calibration graphs are presented (section 4.2) along with the associated correlation coefficients, gradients and intercepts. Correlation coefficients were generated and calibration graphs plotted using all triplicate data points. Further examination of the extended data set involved the examination of the residuals after linear regression, graphs for which are also displayed (Appendix 2). Additionally, F-test "Lack of fit" checks, specifically the comparison of the lack of fit against the pure error, was compared against the F-critical (F_{crit}) value at 95% confidence associated with the appropriate degrees of freedom. F was calculated as follows:

F = sum of squares (lack-of-fit) / degrees of freedom =
Sum of squares (pure-error) / degrees of freedom

$$= \frac{\left(\sum_{j=1}^{c}(n_j(\bar{Y}_j - \hat{Y})^2\right) / (c-2)}{\left(\sum_{j=1}^{c}\sum_{i=1}^{n_j}(Y_{ij} - \bar{Y})^2\right) / (n-c)} \qquad [23]$$

where $\bar{Y}$ = mean y value at each concentration (X value)
$\hat{Y}$ = fitted y value from regression $\hat{Y} = mx + b$
n = total number of data points
c = total number of concentration points (X values)

i.e. there are c distinct Xs, X_j (j = 1,2,...c) and there are n_i (i=1,2,...,n_j) observations of Y denoted by Y_{ij}.

F-critical was calculated from the appropriate degrees of freedom (1-α, c-2, n-c). Assumptions of the test include the normal distribution of y replicates, residuals and uniform

variance of the residuals. Any regressions exhibiting lack of fit from this test were subsequently examined visually to demonstrate any significant non-linearity [24].

2.9.3 Determination of limits of detection

The limit of detection (LOD) is taken here as the lowest injected concentration 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. Predicted LODs for both the screening method (following LC-FLD analysis of C18 cleaned extracts) and the quantitation method were subsequently calculated using the following relationship:

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).

Triplicate oxidations for each triplicate spike were used to assess variability and results are presented in section 4.3.1 for the screening method and 4.3.2 for the quantitation method.

2.9.4 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. LOQs were experimentally confirmed with the triplicate spiking and subsequent triplicate analysis of homogenates at the 0.4 AL concentration level per toxin. 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. Results are presented in section 4.3.3.

2.9.5 Determination of accuracy

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 or well-characterised form for scallops, no accuracy studies can be undertaken using reference materials for these particular shellfish matrices.

2.9.6 Assessment of refined method recovery for N-hydroxylated toxins

Assessment of the recovery of GTX1,4 and NEO from king scallop and queen scallop 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 the two toxins to provide, assuming 100 % method recovery, expected concentrations relating to 0.2 and 0.4 AL for each toxin. 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 at least one hour after spiking and analysed, with oxidation and analysis carried out in triplicate. Quantitation of GTX1,4 and NEO concentrations using the refined method involved the comparison of toxin peak area responses obtained from oxidised spiked samples with those obtained from oxidised toxin mix calibration solutions. Recovery results are presented in section 4.4.

2.9.7 Determination of method precision

- Instrumental precision was assessed with the repeated analysis (n=11) over one 30 hour analytical sequence of shellfish extracts containing GTX1,4 and NEO spiked at 2.0 and 1.2 μg STX eq./g (2.5 and 1.5 AL) respectively.
- short term (intra-batch) repeatability was assessed on 3 repeat sample analyses in the same run at both 0.2 AL and 0.4 AL;
- medium term (inter-batch) repeatability was assessed for king scallops on 6 replicates (0.2 AL and 0.4 AL) analysed in 2 batches of 3 samples, more than two weeks apart.
-long term precision for scallops was assessed with the repeated extraction, clean-up and analysis of a king scallop laboratory reference material (LRM) containing both GTX1,4 and NEO toxins and homogenates of queen scallops spiked at 0.2 and 0.4 AL per toxin. 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 1 month, 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 the refined HPLC-FLD method. The acceptability of the precision characteristics of the method was further assessed with the calculation of HorRat values. Precision results are presented in section 4.5.

2.9.8 Refined method ruggedness

Ruggedness of the refined method 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 design was used to analyse the effects of 7 key method parameters on the stability of the refined method for GTX1,4 and NEO, comparing the variability of these effects against method precision. Experimental parameters chosen for the study were similar to those used previously for oysters and cockles [14] and clams and razors [15], but with some changes to focus primarily on the refined oxidation method. Each extract was cleaned eight times by C18 SPE and each cleaned up sample progressed through the ruggedness experiment as described in Table 7. GTX1,4 and NEO were analysed following fractionation of each of the eight C18 extracts. Alongside the ruggedness analysis, eight C18 cleaned extracts and related fractions for each species were analysed following the normal refined method. This enabled the comparison of results against method precision using a significance test (t-test). Results are presented in 4.6.

Table 7. Experimental design for ruggedness testing of N-hydroxylated toxins in scallops

	Experiment number							
Parameter	1	2	3	4	5	6	7	8
Periodate pH	pH 8.15	pH 8.15	pH 8.15	pH 8.15	pH 8.25	pH 8.25	pH 8.25	pH 8.25
Vortex mixing time	4 sec	4 sec	6 sec	6 sec	4 sec	4 sec	6 sec	6 sec
Ambient temp during oxidation	21 °C	24 °C	21 °C	24 °C	21 °C	24 °C	21 °C	24 °C
Oxidation time	55 sec	55 sec	65 sec	65 sec	65 sec	65 sec	55 sec	55 sec
Volume glacial acetic acid	4 μL	6 μL	4 μL	6 μL	6 μL	4 μL	6 μL	4 μL
Matrix modifier pH	pH6	pH7	pH7	pH6	pH6	pH7	pH7	pH6
Periodate composition	101% periodic acid	99% periodic acid	99% periodic acid	101% periodic acid	99% periodic acid	101% periodic acid	101% periodic acid	99% periodic acid

2.10 Completion of repeatability studies for queen scallops for non-N-hydroxylated toxins

The previous validation study for scallops was not completed due to the issues observed with toxin recovery in the scallop species. Specifically, in addition to the issues with the recovery of the N-hydroxylated toxins, the medium-term repeatability work was not completed for the non-N-hydroxylated toxins in queen scallops. Consequently, additional experiments were conducted to determine the recovery of non N-hydroxylated toxins in queen scallops at both 0.2 and 0.4 AL, thereby enabling the completion of the assessment of the medium term precision for this species. Results from this testing are presented in section 4.7.

2.11 Method uncertainty

Results were used from the phase 2 validation studies to calculate an overall value of uncertainty for the measurement of GTX1,4 and NEO toxins in king and queen scallops. Once sources of uncertainty were described, 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" [25]. Measurement uncertainty results are presented in section 4.8. Results for queen scallops were calculated using the long term precision data generated in naturally contaminated king scallops.

2.12 Phase 3. Comparative testing of LC and MBA methods.

Phase 3 of the validation study involved the analysis of naturally contaminated scallops. The aims of this exercise were to demonstrate that the method performs adequately as an analytical procedure for the quantitative analysis of PSP toxins in scallops. A low number of king and queen scallop extracts previously analysed by MBA and archived (≤-15^{o}C), were analysed using the refined LC method. However, these were not re-analysed by MBA, so any comparison between LC and MBA results is based on analysis conducted at different times. In order to comparatively test fresh scallop samples, with no availability of PSP-contaminated scallops from the routine monitoring programs, additional comparative work focussed on the analysis of scallops artificially contaminated within the laboratory by feeding with *Alexandrium*. Once contaminated shellfish were shucked and homogenised, 5g aliquots of each sample were extracted and analysed according to the AOAC 2005.06 method, using the refined scallops method for the quantitation of the N-hydroxylated toxins. 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 0.1 mM 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. Following the recent recommendations from EFSA [21], the EFSA toxicity equivalence factors (TEF) were used in place of those originally suggested by Oshima [26]. Results from the phase 3 testing are given in section 5.

3. Phase 1: Method refinements: results and discussion

3.1 Preliminary investigations

3.1.1 Extraction efficiency

Recovery data generated from the re-extraction of spiked king scallop homogenates pre-extracted using the normal AOAC 2005.06 extraction method showed some evidence of NEO remaining after the normal extraction process had been completed. In king scallops spiked in triplicate at both 0.5 and 1.0 AL, results showed that 22% ± 11% and 20% ± 4% of NEO remained in the solid sample after the normal double extraction has been completed. As such, the results suggested an 80% ± 10% extraction efficiency for NEO in king scallops. This value compares similarly to the extraction efficiencies determined previously in mussels [13] and oysters [14]. Therefore, there is no indication here that the low NEO recoveries in scallops result from any problems specific to the extraction methodology.

3.1.2 One vs. two C18 clean-up steps

Peak area responses and recoveries of GTX1,4 toxin peaks were compared between the analysis of queen scallop homogenates spiked at 1.0 AL and cleaned up both once and twice with C18 SPEs. The recovery of GTX1,4 from the extract cleaned up once using C18 was 40% in this experiment, as compared to a recovery from the extract subjected to two C18 clean-ups of 33% (with recovery adjusted for additional dilution inherent in second C18 step). As such, there is no evidence for an improvement in toxin recovery with the use of any additional clean-up steps to remove hydrophobic components of the scallop extracts.
In addition, one further experiment was conducted to determine whether there may be any advantage for direct fractionation of the acetic acid extracts, i.e. with removal of the C18 clean-up step prior to the quantitation of the N-hydroxylated toxins. However, results indicated that the ion exchange clean up did not work satisfactorily when the C18 clean-up step was omitted, specifically with a high proportion of GTX1,4 toxins eluting in fraction 1 instead of fraction 2. As a result, this line of investigation was not continued.

3.1.3 Comparison of ion-exchange clean up cartridges

Peak area responses and recoveries of GTX1,4 toxin peaks were compared following the ion exchange clean up of queen scallop homogenates spiked at 1.0 AL using both the polymeric-based Strata-X CW ion exchange cartridges detailed in the refined LC-FLD method [13,18] and the silica-based cartridges originally specified in the AOAC 2005.06 official method [12]. Compared to the 40% recovery of GTX1,4 following fractionation using the Strata-X CW cartridges, the recoveries obtained using the silica based SPEs were found to be 43%, essentially the same using both SPEs. One slight difference was the noticeable carryover of some GTX1,4 into fraction F1 after using the silica-based SPEs, a noted issue when using these types of cartridges [12,20]. As such, the results suggest that the Strata-X CW SPEs are still the preferred option for the ion exchange clean up of shellfish extracts, including scallops.

3.1.4 Effect of pH on oxidation of fractions

Aliquots of fraction F2 obtained following the ion exchange clean up of queen scallop extracts containing GTX1,4 at a concentration equivalent to 1.0 AL, were treated as follows:
Aliquot 1 – untreated sample
Aliquot 2 – F2 adjusted to pH 6.5
Aliquot 3 – F2 adjusted to pH 8.5
It was found that adjustment of the fractions in this manner was very difficult practically due to the relative low presence of matrix co-extractives, which normally provide an efficient buffering capacity when performing pH adjustments. Peak area responses obtained following the LC-FLD analysis of the periodate oxidised aliquots resulted in evidence for lower

recoveries in the pH-adjusted fractions (40%, 33% and 30% in the unadjusted, pH 6.5 and pH 8.5 fractions respectively). Therefore there is no evidence for improved toxin recovery following pH adjustment of fractions.

3.1.5 Effect of variable periodate oxidant pH

Periodate oxidant was prepared following the guidance in the normal AOAC 2005.06 method. Sub-samples of the oxidant were then taken and adjusted to a range of pH values, between pH 7 and pH 10, including the specified optimum value of 8.2. Duplicate samples of queen scallops spiked with 1.0 AL GTX1,4 were analysed by LC-FLD and the toxin recoveries calculated from the toxin oxidation product peak area responses. Figure 2 illustrates the results obtained and shows the clear effect of pH on the oxidation efficiency. Close examination of the data reveals that the currently specified value of pH 8.2 is the optimum value of pH for the oxidation of GTX1,4 in scallops. As such, there is no evidence for any further improvement resulting from pH changes in the oxidation method for the quantitation of GTX1,4 in scallops.

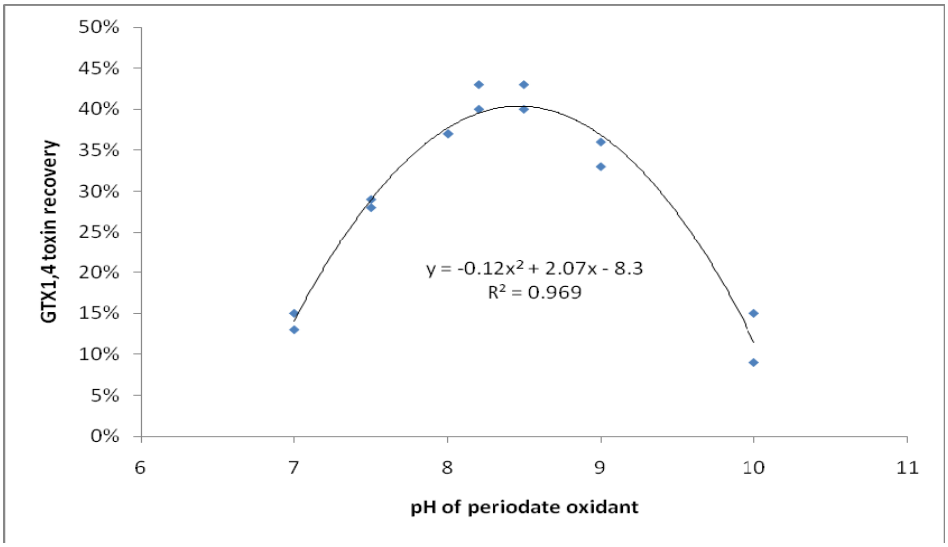


Figure 2. Effect of periodate oxidant pH on the recovery of GTX1,4 from queen scallops.

3.1.6 Preliminary investigations into effects of variable periodate oxidant composition

3.1.6.1 Effect of variable periodic acid volumes

Six different periodate oxidants were prepared, each containing increasingly higher proportions of periodic acid. The exact composition of each reagent and the subsequent calculated recoveries of GTX1,4 from queen scallops spiked at 1.0 AL are given in **Table 8**. Recoveries were calculated by comparing toxin peak area responses against a GTX1,4 calibration curve generated using the normal periodate oxidant. The results indicated some improvement in the response and subsequent toxin recovery following oxidation with higher proportions of periodic acid. As such, this highlighted an avenue for further investigations for potentially enhancing the sensitivity of the toxin analysis in this species.

Table 8. Proportions of reagents utilised for the preparation of different periodate oxidant reagents and subsequent effect on recovery of GTX1,4 from queen scallops

0.03M Periodic acid (mL)	0.3M Ammonium formate (mL)	0.3M Sodium hydrogen phosphate (mL)	Proportion of periodic acid	GTX1,4 recovery
5.0	5.0	5.0	0.33	40%
6.0	5.0	5.0	0.40	50%
7.0	5.0	5.0	0.47	50%
8.0	5.0	5.0	0.53	52%
9.0	5.0	5.0	0.60	53%
10.0	5.0	5.0	0.67	58%

3.1.6.2 Effect of variable concentrations of periodic acid

Six different periodate oxidants were prepared, each containing increasingly higher concentrations of periodic acid. The concentrations prepared and the subsequent calculated recoveries of GTX1,4 from queen scallops spiked at 1.0 AL are given in **Table 9**. Recoveries were calculated by comparing toxin peak area responses against a GTX1,4 calibration curve generated using the normal periodate oxidant. The results indicated little if any improvement in the response and subsequent toxin recovery following oxidation with higher concentrations of periodic acid.

Table 9. Concentration of periodic acid utilised for the preparation of different periodate oxidant reagents and subsequent effect on recovery of GTX1,4 from queen scallops

Concentration of periodic acid in periodate	GTX1,4 recovery
0.03M	41%
0.05M	46%
0.1M	44%
0.15M	42%
0.2M	38%
0.25M	38%

3.1.6.3 Effects of variable concentrations of all reagents

Three different periodate oxidant reagents were prepared using increasingly higher concentrations of all the substituent components. Results obtained following the oxidation of queen scallops (spiked at 1.0 AL GTX1,4) indicated the general decrease in signal with the use of higher concentrations of all three component reagents (**Table 10**).

Table 10. Concentrations of periodate reagent components utilised for the preparation of different periodate oxidant reagents and the subsequent effect on the recovery of GTX1,4 from queen scallops

Concentration of periodate reagents			
Periodic acid	Ammonium formate	Sodium hydrogen phosphate	GTX1,4 recovery
0.03M	0.3M	0.3M	41%
0.05M	0.5M	0.5M	36%
0.1M	1.0M	1.0M	28%

3.1.7 Oxidation process optimisation

3.1.7.1 Effects of addition of variable volumes of periodate oxidant

Queen scallops spiked with GTX1,4 at a concentration equivalent to 1.0 AL were extracted, cleaned up and oxidised by periodate oxidation. In this experiment, the volumes of periodate oxidant were varied to determine if increasing the volume of oxidant would potentially result in any increase in oxidation efficiency as evidence by increased toxin recovery. Calculated recoveries were adjusted to account for the differences in total sample dilution in each oxidised sample. **Table 11** summarises the results and clearly showed the reduction in recovery experienced when using higher volumes of periodate reagent in the oxidation step. As such, no benefit was gained from increasing the amount of periodate oxidant used for the oxidation and quantitation of GTX1,4 in scallops.

Table 11. Volume of periodate reagent utilised for the oxidation of GTX1,4-spiked queen scallops and subsequent effect on toxin recoveries

Volume of periodate oxidant (µL)	GTX1,4 recovery
500µL	40%
600µL	34%
700µL	30%
800µL	29%
900µL	28%
1000µL	28%

3.1.7.2 Effects of addition of variable volumes of glacial acetic acid

C18-cleaned extracts obtained from the extraction of queen scallops spiked with GTX1,4 at 1.0 AL, were used for the repeated periodate oxidation in order to determine any effect of volumes of glacial acetic acid on the apparent toxin recovery. The normal AOAC 2005.06 method specifies the use of 5µL glacial acetic acid for quenching the oxidation reaction. Here oxidation was performed using the increasingly higher volumes of acid as summarised in **Table 12**. The table also summarises the subsequent effects of varying the volumes of acid on the apparent toxin recovery. Results clearly indicated little if any effect on the oxidation efficiency with the use of higher volumes of acid. It is additionally noted that use of higher volumes of glacial acetic acid, can result in subsequent instability of toxin oxidation products, the effects of which may be noticeable over the normal period of a single analytical batch (data not shown). As such, there was no benefit to be gained from changing the volumes of glacial acetic acid.

Table 12. Volume of glacial acetic acid utilised for the quenching of the periodate oxidation of GTX1,4-spiked queen scallops and subsequent effect on toxin recoveries

Volume of glacial acetic acid (µL)	GTX1,4 recovery
5µL	44%
7µL	43%
9µL	44%
11µL	44%
13µL	46%
15µL	46%

3.1.7.3 Effects of varying oxidation times

The effects of varying the time required for periodate oxidation of N-hydroxylated toxins was examined, through comparing peak area responses of both GTX1,4 and NEO

toxins in king scallop matrix oxidised for 1, 1.5 and 2 minutes. Other than the changes to oxidation times, all other oxidation parameters followed exactly those specified in the method. Results indicated that the toxin oxidation product peak areas were found to reduce with the use of longer oxidation times, therefore indicating that no improvement in toxin response would be obtained with longer oxidations (no data shown).

3.1.8 Effects of temperature on periodate oxidation

Results obtained from the oxidation of N-hydroxylated toxins in king scallops at variable temperatures are summarised in **Table 13**. For GTX1,4 the results show very little change in toxin peak area response for temperatures up to 85^{o}C. Results from NEO appear to suggest a slight increase in response at slightly elevated temperatures (30-60^{o}C), before dropping at higher temperatures still (70-85^{o}C). However, such changes are relatively small.

Given the impracticalities associated with conducting the oxidation at higher temperatures, there is no evidence that this line of investigation is worth pursuing.

Table 13. Toxin peak areas for duplicate oxidations of GTX1,4 and NEO-spiked king scallops over a range of ambient temperatures, showing the effects of the temperature on LC-FLD response in comparison with controls

Temp (^{o}C)	GTX1,4 peak areas			% of control	NEO peak areas			% of control
	A	B	Mean		A	B	Mean	
20 (control)	0.29	0.25	0.27	na	0.59	0.58	0.59	na
30	0.23	0.36	0.30	109%	0.76	0.74	0.75	128%
40	0.31	0.29	0.30	111%	0.73	0.74	0.74	126%
50	0.26	0.23	0.25	91%	0.75	0.7	0.73	124%
60	0.28	0.24	0.26	96%	0.69	0.71	0.70	120%
70	0.24	0.26	0.25	93%	0.53	0.48	0.51	86%
85	0.24	0.27	0.26	94%	0.49	0.48	0.49	83%

3.1.9 Investigations into the use of protein precipitation

Following the analysis of king scallop extracts subjected to protein precipitation containing GTX1,4 and NEO toxins at 0.5 AL, the results indicated that the recovery of GTX1,4 following TCA treatment was 74% ± 11% of the recovery in the non-treated samples and the recovery of NEO was 134% ± 14% of that from the non-treated extracts, Given the differences observed, and the lack of any improvement in response for GTX1,4, the results suggest there is no evidence for any positive benefit to be gained from this additional treatment. As a result, no further work was conducted into investigating this approach any further.

3.1.10 Variability of matrix effects

GTX1,4 and NEO toxin concentrations were calculated after the LC-FLD analysis of fractionated extracts from a range of spiked king scallop samples. The results were calculated as percentage toxin recoveries and are summarised in **Table 14**, alongside the results obtained from two additional spiked mussel samples for reference. As expected, data shows a notable degree of suppression for both toxins in all the scallops samples analysed, with the level of suppression varying from sample to sample. However, the overall level of variability of the results is relatively low (RSDs < 20%), indicating that the noted effects with suppression do appear to be present in scallop samples with different spatial and temporal sources. Importantly it shows good evidence for the relative consistency of this effect, which

enables the refinement work to progress with the knowledge that any method optimisation developed will be applicable to all scallop samples obtained through the UK official control monitoring programme.

Table 14. Percentage recovery results of GTX1,4 and NEO spiked into king scallop homogenates with a variety of temporal and spatial origins, as compared with the recoveries of two comparative mussel samples

	% Recovery			% Recovery	
Sample	GTX 1/4	Neo	Sample	GTX 1/4	Neo
KSc 2010/414	43%	40%	Mussel 2011/83	81%	77%
KSc 2009	55%	28%	Mussel 2011/63	65%	74%
KSc 2010/238	49%	38%	Mean mussel	73% ± 12%	75% ± 2%
KSc 2010/239	46%	35%			
KSc 2010/520	35%	47%			
KSc 2010/521	41%	43%			
KSc 2010/522	43%	44%			
KSc 2010/523	60%	39%			
KSc 2010/1045	56%	41%			
KSc 2010/1046	40%	39%			
KSc 2010/1047	52%	56%			
KSc 2010/1048	47%	54%			
KSc 2007	47%	32%			
Mean king scallop	47% ± 7%	41% ± 8%			
RSD%	15%	19%			

3.1.11 Effect of pH during oxidation reactions

A king scallop and queen scallop acetic acid extract containing both GTX1,4 and NEO at concentrations equal to 0.5 AL, were cleaned up using C18 SPE and submitted to ion exchange fractionation. The C18-cleaned extracts and both fraction F2 and F3 were subjected to periodate oxidation alongside a control sample comprising a PSP-positive mussel sample, containing a range of PSP toxins including GTX1,4 and NEO. The pH of each sample was measured before, during and after the oxidations and the results are tabulated in **Table 15**. The results indicated, that even with some differences in pH prior to oxidation, the pH measured both during and after the oxidation reactions are all fairly similar. There is certainly no evidence for any differences in the values measured in the scallops as compared with the mussel sample which could possibly account of the differences in observed toxin recoveries in both the scallop species.

Table 15. pH measurement results from samples before, during and after oxidation.

Sample	Species	Sample pH	pH during oxidation	pH after oxidation
Mus LRM6/527	Mussel	6.91	8.21	4.80
KSc Mix 1 0.5AL – C18	King scallop	6.38	8.19	4.92
QSc Mix 1 0.5AL – C18	Queen scallop	6.90	8.27	4.93
KSc Mix 1 0.5AL – F2	King scallop	4.29	8.23	4.86
QSc Mix 1 0.5AL – F2	Queen scallop	4.40	8.11	4.64
KSc Mix 1 0.5AL – F3	King scallop	4.26	8.15	4.72
QSc Mix 1 0.5AL – F3	Queen scallop	4.07	8.15	4.79

3.2 Inter-laboratory assessment of method performance

3.2.1 N-hydroxylated toxin recovery

Toxin concentrations determined by participating laboratories were supplied and collated. The values determined were used to calculate total method recoveries for both GTX1,4 and NEO at each laboratory. The full toxin concentration results from each participating laboratory are given in Appendix 3 and are summarised as recoveries in Table 16. The results generally indicate notably low recoveries for the two toxins in each sample, although the effect does appear to be variable. The closest agreement is for the determination of NEO in the second queen scallop sample where recoveries determined by all four laboratories returned values between 18% and 31%. Recoveries of NEO in the other samples were also generally low, with most falling between 20% and 50%, but with a couple of higher results returned from Lab 4. A similar situation was seen with GTX1,4 following the periodate oxidation of fraction 2, where for each sample the GTX1,4 recovery was low in 3 out of the 4 sample results, but with one of the four laboratories (Lab 2) returning higher recoveries. Overall, the data suggests that the issues observed at Cefas with low recoveries of N-hydroxylated toxins quantified from scallop samples are also observed in the same samples analysed in other laboratories, although a larger variability is noted in some results. However, without a more detailed investigation involving more participants and a greater number of samples, no statistical assessment of these results is appropriate.

Table 16. Mean toxin recoveries of GTX1,4 and NEO from queen and king scallop homogenates spiked with known concentrations of toxins following periodate analysis of fractionated extracts (GTX1,4 in F2 and NEO in F3).

	KSc1	KSc1	KSc2	KSc2	QSc1	QSc1	QSc2	QSc2
	Mean	**RSD%**	**Mean**	**RSD%**	**Mean**	**RSD%**	**Mean**	**RSD%**
GTX1,4 (F2)	71% ± 28%	40%	68% ± 22%	33%	54% ± 36%	67%	50% ± 30%	60%
NEO (F3)	52% ± 31%	61%	49% ± 31%	62%	36% ± 20%	55%	24% ± 6%	27%

Participant laboratories also supplied results calculated from the quantitation of GTX1,4 and NEO directly from the C18-cleaned extracts. Concentrations calculated from each laboratory are also given in Appendix 3, and results summarised as recoveries in Table 17. The data again indicates that low toxin recoveries are observed following the periodate oxidation of the C18-cleaned scallop extracts. The variability of the results is smaller than for the analysis of the fractions, with mean recoveries showing recoveries between 24% and 53% for NEO and 36% to 54% for GTX1,4.

Table 17. Mean toxin recoveries of GTX1,4 and NEO from queen and king scallop homogenates spiked with known concentrations of toxins following periodate analysis of C18-cleaned extracts.

	KSc1		KSc2		QSc1		QSc2	
	Mean	**RSD%**	**Mean**	**RSD%**	**Mean**	**RSD%**	**Mean**	**RSD%**
GTX1,4 (C18)	54% ± 11%	20%	49% ± 4%	7%	41% ±20%	49%	36% ± 19%	52%
NEO (C18)	46% ± 18%	40%	53% ± 27%	52%	30% ± 9%	30%	24% ± 2%	8%

Overall, the results indicate reduced recoveries of both GTX1,4 and NEO from king and queen scallop extracts, confirming the observations seen within the Cefas laboratory.

3.2.2 Non N-hydroxylated toxin recovery

One additional king scallop homogenate sample was supplied fortified with a range of non N-hydroxylated toxins. This sample was initially analysed by peroxide oxidation in order to determine the recoveries using this oxidation method. The sample was also oxidised by periodate and the laboratories reported the observed signal to noise ratios of the toxin oxidation product peaks. Table 18 summarises the toxin concentrations spiked and determined by the four laboratories and the associated mean recovery of each non N-hydroxylated toxin from the scallop sample. With the exception of the poor recoveries returned by two laboratories for STX, the overall recoveries of the toxins were generally acceptable following the peroxide oxidation, typically being 65% or higher.

Table 18. Toxin concentrations (spiked and determined; µg STX eq./g) of non N-hydroxylated toxins in a king scallop homogenate following peroxide analysis of C18-cleaned extracts..

	Concentration (µg STX eq./g)					Mean Recovery	RSD%
	Spiked Concentration	Lab 1	Lab 2	Lab 3	Lab 4	Mean	RSD%
C1,2	0.16	0.11	0.08	0.20	0.21	94% ± 41%	44%
dcSTX	0.16	0.11	0.12	0.09	0.12	68% ± 8%	12%
GTX2,3	0.16	0.12	0.15	0.10	0.14	78% ± 14%	18%
GTX5	0.04	0.03	0.03	0.03	0.02	64% ± 13%	20%
STX	0.08	0.06	0.07	0.02	0.02	52% ± 33%	64%

3.2.3 Method sensitivities

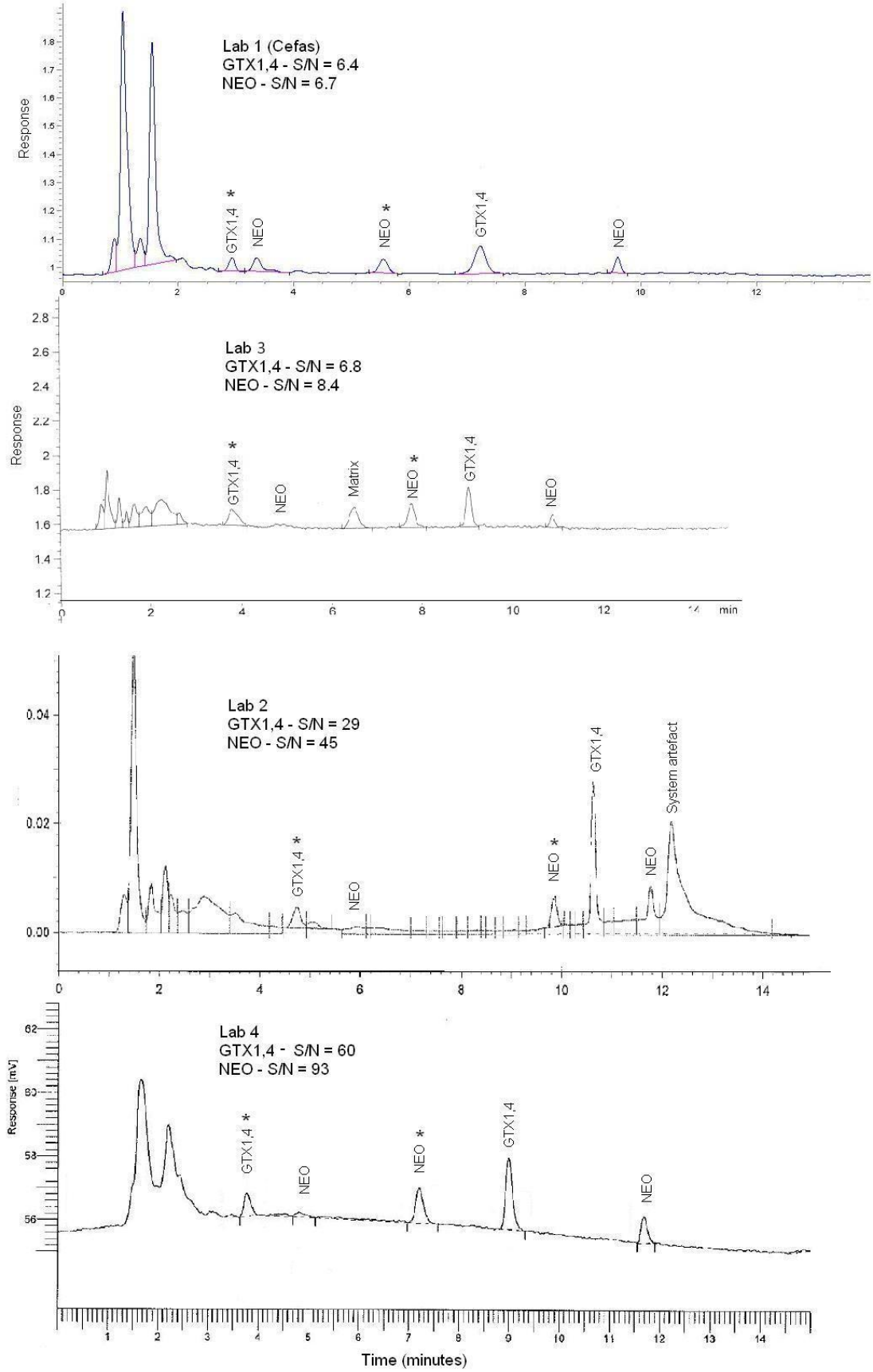
The signal to noise ratios of the toxin peaks observed in the peroxide-oxidised chromatograms of sample KSc3 were typically high. Limits of detection extrapolated from the observed signal to noise ratio and the spiked concentration and using a signal to noise ratio of 3:1 are tabulated in Table 19. LODs range from <0.001 to 0.07 µg STX eq./g per toxin, showing there are no issues with the sensitivity of the peroxide method in the king scallop sample. Table 19 also summarises the calculated LODs from the periodate oxidation of the five scallop samples, with mean values calculated for the estimated LODs for each species. The differences in calculated LODs were found to arise from the use of both different FLD modules and different approaches to calculating signal to noise ratios. Figure 3 shows the chromatograms obtained from the periodate analysis of one sample (C18-cleaned extract of KSc2), showing the peak sizes and differences in peak signal to noise ratios.

Table 19. Estimated LODs (based on S/N = 3) calculated from peroxide oxidation of non N-hydroxylated toxins in one king scallop sample and the periodate oxidation of N-hydroxylated toxins in the three king scallop and two queen scallop samples.

	KSc Lab 1	KSc Lab 2	KSc Lab 3	KSc Lab 4	QSc Lab 1	QSc Lab 2	QSc Lab 3	QSc Lab 4
GTX 1,4	0.20	0.06	0.36	0.06	0.35	0.06	0.38	0.09
NEO	0.35	0.04	0.24	0.03	0.34	0.08	0.41	0.04
C1,2	0.010	<0.0048*	0.022	0.0005	-	-	-	-
dcSTX	0.008	<0.0096*	0.029	0.0007	-	-	-	-
GTX2,3	0.027	<0.0096*	0.032	0.0011	-	-	-	-
GTX5	0.003	<0.0024*	0.009	0.0001	-	-	-	-
STX	0.012	<0.0096*	0.070	0.0010	-	-	-	-

*Value based on minimum S/N ratio of 50

Figure 3. Comparison of chromatograms obtained from 4 participating laboratories for the analysis of the periodate-oxidised C18-cleaned king scallop sample.



In summary, whilst there are differences in the determined recoveries and especially the estimated LODs based on observed signal to noise ratios, there is still clear evidence for

performance issues with the AOAC 2005.06 method for the determination of PSP toxins in both king and queen scallop samples in a number of different laboratories.

3.3 Refinement investigations

3.3.1 Injection volume optimisation

20 toxin standards of varying concentration were analysed over a period of > 3 days using both 50µL and 100µL injection volumes. The peak areas were compared to confirm whether or not doubling the injection volume to 100µL would result in exactly double the peak areas measured following the 50µL injections. Results showed that the peak areas of the 100µL analyses were within 1%of the peak areas expected from doubling the values obtained from the 50µL injections.

In addition, 10 different PSP positive samples, were fully quantified using both 50µL and 100µL injection volumes for the periodate-oxidised, fractionated extracts. Comparison of the results obtained indicated a close similarity in results with concentrations quantified from 100µL injection volumes being 99% ± 3% and 102% ± 4% of the values obtained from 50µL injection volumes for GTX1,4 and NEO respectively.

Finally, a range of king and queen scallop extracts spiked with varying concentrations of GTX1,4 and NEO were extracted, C18-cleaned and subjected to LC-FLD analysis with both 50 and 100µL injection volumes. In all cases the signal to noise ratios of the toxin peaks measured in the 100µL analyses were close to double those of the peaks measured in the 50µL analyses. This subsequently results in the doubling of analytical sensitivity of the method for these toxins.

The peak widths of the GTX1,4 and NEO toxin peaks were also examined and compared between the 50 and 100 µL injection analyses. The peak widths of the latter were found on average to be only 3% higher than those generated from the former. This therefore provides good evidence that the chromatographic efficiency will not be compromised with the use of the higher injection volumes

Overall, therefore, the results have indicated that use of the higher 100µL injection volumes will result in double the sensitivity of the analysis, halving the method LODs and LOQs, whilst not compromising analytical accuracy or chromatographic quality.

3.3.2 Optimisation of periodate reagent

With the slight increases in N-hydroxylated toxin peak area response noted earlier through use of periodate reagents containing higher proportions of periodic acid, further experiments were conducted to assess whether use of a modified periodate oxidant could further enhance the apparent efficiency of the oxidation step. Periodate oxidant was initially prepared using the normal proportions of the three constituent chemicals (100% of the normal volume of periodic acid). Other periodate reagents were subsequently prepared using increasingly higher proportions of periodic acid, specifically made up to 200%, 300%, 400% and 500% of the normal proportions of the acid. The pH of each of the five periodate reagents was adjusted to as close to 8.2 as possible and each used in the oxidation of a king scallop extract containing both GTX1,4 and NEO at concentrations equal to 0.5 AL. **Figure 4** illustrates the effect of the different periodate reagents on the oxidation efficiency of the toxins in the scallop matrix. Results indicate higher toxin oxidation product peak areas for both the GTX1,4 and NEO primary (quantitation) peaks as the proportion of periodic acid increases in the periodate reagent. At the same time, it is evident that the size of the secondary peaks for both toxins decreases with increasing proportions of periodate. As such, results indicate the potential for higher proportions of periodic acid in the periodate reagent to provide a greater peak area response for the quantitation peaks of both the N-hydroxylated toxins, which may provide a greater analytical sensitivity for any refined method when applied to the analysis of scallops.

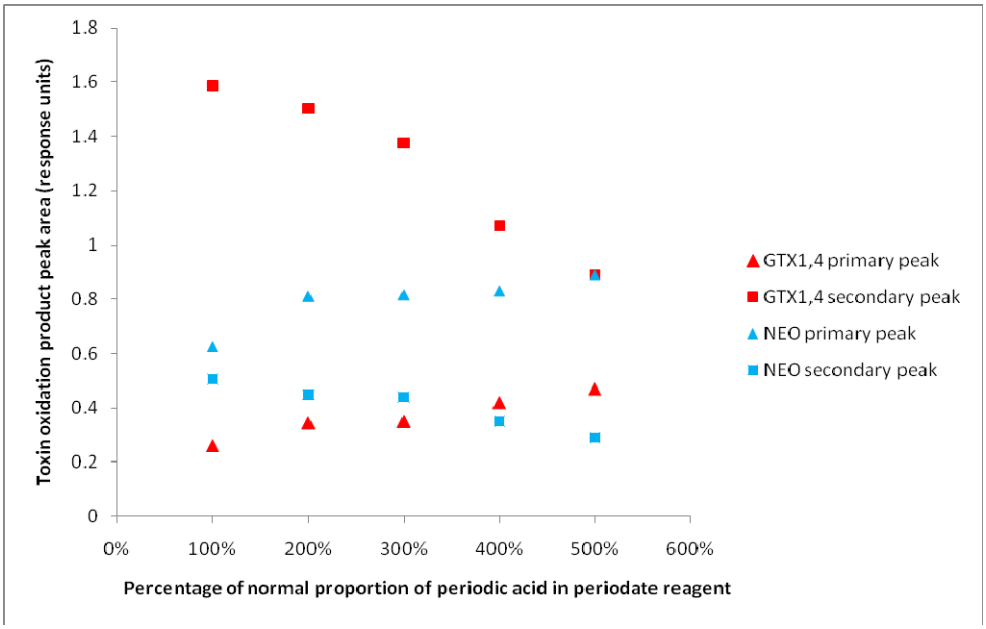


Figure 4. Effect of relative proportions of periodic acid in periodate oxidant GTX1,4 and NEO toxin oxidation product peaks following analysis of fortified king scallop extracts.

During the course of the refinement process in the laboratory, the refined periodate reagent was trialled repeatedly for the oxidation of N-hydroxylated toxins in both scallop samples and in standards. Results indicated that the analytical sensitivity was improved repeatedly, As such, the recommendation was to incorporate the refined periodate into any subsequent validation conducted using the refined methodology for the analysis of N-hydroxylated toxins in scallops.

3.3.3 Effects of extraction method and sample dilution on apparent matrix effects

King and queen scallop homogenates fortified with GTX1,4 and NEO and extracted with a range of different extraction techniques were cleaned up, fractionated and oxidised with periodate prior to LC-FLD. Recovery results and the signal to noise ratios measured for each toxin quantitation peak are tabulated in **Table 20**. The results show that in these particular samples, as observed previously, the recoveries for GXT1,4 in queen scallops and NEO in both scallops species is poor, with only the GTX1,4 recovery in king scallops appearing acceptable [16]. Results also appear to suggest that the recoveries are slightly improved through use of larger extraction volumes, with a significant increase in recovery evident using double and extraction volumes particularly for GTX1,4 in king scallops and NEO in queen scallops. However, the increased dilutions resulting from the higher extract volumes result in a large drop in subsequent method sensitivity as evidenced by the lower signal to noise ratios for each of the toxin peaks. This level of sensitivity in the larger extract volumes, would further decrease the level of sensitivity of analysis. So whilst recovery may be improved, presumably through dilution of the matrix effects present in the extracts, the sensitivity is not. Interestingly, the use of boiling water for the first extraction appears an important parameter for the extraction method. Results indicate substantially lower recoveries of both GTX1,4 and NEO in the spiked samples where no heat was applied. Finally, the results show that there is little difference on average between the recoveries determined using either acetic acid or hydrochloric acid extraction methods, in particular with NEO still exhibiting poor apparent recovery after both extractions.

Table 20. Toxin recoveries for GTX1,4 and NEO in fortified king and queen scallop samples (0.5 AL per toxins) following a variety of extraction protocols (signal to noise ratios of quantitation peaks in brackets)

	KSc	KSc	QSc	QSc
Extraction method	**GTX1,4**	**NEO**	**GTX1,4**	**NEO**
Normal AcH extraction	74% (5.8)	21% (2.4)	34% (5.5)	27% (2.9)
Double AcH extraction	88% (2.8)	24% (1.8)	36% (3.6)	25% (1.6)
Triple AcH extraction	107% (2.4)	17% (1.3)	40% (2.2)	49% (2.7)
AcH extraction no boil	18% (1.4)	0% (0.0)	34% (2.1)	21% (2.1)
Normal HCl extraction	56% (3.4)	22% (2.9)	70% (3.6)	34% (5.7)

Additional work was also undertaken involving the further dilution of crude and cleaned up extracts. Acetic acid extracts were subjected to additional dilutions up to a factor three, as were the C18-cleaned and fractionated extracts. In all cases, there were some slight increases in the apparent recovery for GTX1,4, but the recovery for NEO did not improve, In addition, the related toxin peak signal to noise ratios and calculated LODs became substantially worse. As such, there was no evidence for any advantage with employing additional dilution steps at any stage during the sample preparation protocol.
Overall therefore, with these results, the suggestion is that no changes are made to the current PSP toxin extraction method. Whilst advantages may be apparent with the removal of matrix effects with larger extract dilutions, the negative effect on subsequent analytical sensitivity is too large to make this change a positive one.

3.3.4 Investigations into the use and composition of matrix modifiers

The acetic acid extracts obtained from the extractions of PSP-free king scallop homogenates were cleaned up using C18 SPE and subsequently used as matrix modifiers during the oxidation of N-hydroxylated calibration standards. A number of investigations were conducted to assess whether their use in the periodate oxidation step would result in a level of suppression in the calibration slope which would subsequently produce a higher apparent recovery in the spiked samples. **Table 21** summarises the results obtained from the repeated oxidation of Mix 1 calibration standards (containing GTX1,4 and NEO) in both Pacific oyster matrix modifier (PO-MM) and a king scallop matrix modifier (KSc-MM) on 6 different days over a time period of more than 2 months. The results show the percentage reduction in calibration slope obtained from the KSc-MM calibration as opposed to the slope generated following oxidation of calibrants in the presence of PO-MM. The mean reductions in signal are close to 50% for both toxins, and the effect appears repeatable over the medium term.

Table 21. Percentage suppression in calibration slope gradients for GTX1,4 and NEO when oxidised in the presence of a king scallop matrix modifier (KSc-MM) in comparison with the normal Pacific oyster matrix modifier (PO-MM).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Mean	RSD%
GTX1,4	54%	40%	51%	48%	56%	64%	52% ± 8%	15%
NEO	49%	40%	42%	36%	43%	41%	42% ± 4%	10%

Calibration standards containing KSc-MM were subsequently used to quantify king and queen scallop samples, fortified with GTX1,4 and NEO toxins at concentrations equivalent to either 0.2 or 0.5 AL, With the samples already containing scallop matrix co-extractives, the samples themselves were oxidised in the presence of the normal PO-MM. Oxidations and analysis were again conducted on different days over a period of > 2months. **Table 22** summarises the recoveries obtained. Results indicate more acceptable toxin recoveries for both GTX1,4 and NEO in both the king and queen scallops analysed as compared with the

recoveries calculated using the normal AOAC 2005.06 method (**Table 10**), which are on average 20-30% lower than the values summarised here.

Table 22. Percentage recoveries of GTX1,4 and NEO when quantified against calibration standards containing KSc-MM.

	Day 1	Day 2	Day 3	Day 3	Day 4	Day 4	Day 5	Day 6		
Matrix	**KSc**	**QSc**	**KSc**	**QSc**	**QSc**	**KSc**	**KSc**	**KSc**		
Toxin concentration (AL)	GTX1,4 - 0.5, NEO - 0.2	0.5 AL both toxins	0.5 AL both	0.5 AL both	0.5 AL both	0.5 AL both	0.2AL both	0.5AL both	**Mean**	**RSD%**
GTX1,4	62%	77%	65%	63%	91%	89%	51%	74%	71% ± 14%	19%
NEO	54%	47%	65%	70%	67%	66%	51%	66%	61% ± 9%	14%

In addition to using a king scallop modifier prepared according to AOAC 2005.06, other modifiers were tested including more diluted C18-cleaned king scallop extracts, the extracts obtained post-ion exchange fractionation and extracts containing specific concentrations of salt. **Table 23** summarises the suppressive effects of each of the matrix modifiers prepared. Data indicates the suppressive effects of the KSc-MM are still present, even with additional dilutions (200% and 300%) of the modifier prior to use. However, there is a reduction in the size of the suppressive effect. Notably, the ion-exchange cleaned fractions of the KSc-MM show different effects, with F2 showing a similar effect to the C18-cleaned KSc-MM, where as F3 appears to have no effect. This is interesting particularly, as typically NEO (present in F3) gives lower apparent recoveries than GTX1,4 (present in F2). One potential factor possibly affecting the size of the suppression was the presence of high concentrations of salt (NaCl) in the final fractions. This was further tested with the addition of 2M NaCl to the KSc-MM and was also used independently as a matrix modifier. Results indicate, that the addition of salt has no direct suppressive effect on the oxidation of the toxins.

Table 23. Percentage suppression in calibration slope gradients for GTX1,4 and NEO when oxidised in the presence of a variety of king scallop based matrix modifiers in comparison with the normal Pacific oyster matrix modifier (PO-MM).

	PO-MM	**KSc-MM**	**200% diluted KSc-MM**	**300% diluted KSc-MM**	**KSc-F2 MM**	**KSc-F3 MM**	**KSc-MM + 2M NaCl**	**2M NaCl only**
GTX1,4	100%	51%	57%	60%	50%	110%	48%	112%
NEO	100%	42%	46%	52%	40%	124%	40%	144%

Overall, there is a clear and repeatable suppressive effect of the KSc-MM on the periodate oxidation of both the N-hydroxylated toxins. The effect appears most pronounced in the modifier prepared according to the standard AOAC 2005.06 protocol used at Cefas for the routine preparation of the PO-MM. Whilst the fraction F2 of the KSc-MM following ion exchange also produces similar effects, such an approach would not provide any additional benefits, also being a more costly and time consuming process. As such the recommendation resulting from these studies is the use of a C18-cleaned king scallop matrix modifier to be used in place of the normal Pacific oyster matrix modifier during the oxidation of the calibration standards containing GTX1,4 and NEO toxins. King and queen scallop samples should continue to be oxidised in the presence of the normal PO-MM as per AOAC 2005.06, given the high amount of scallop matrix components clearly already present in the sample extracts. The drawback for this is the consequent requirement to run standards

containing both oyster and scallop matrix modifier when running sample sequences containing both scallop and non-scallop samples.

3.3.5 Investigations into the potential for reducing method dilution factors

Queen scallop extracts containing concentrations of GTX1,4 and NEO equivalent to 0.25 AL, were subjected to a number of C18 and ion exchange SPE clean ups using variable sample loads. The volumes of crude extracts were varied between 1 and 2mL and the volumes of C18-cleaned extracts were varied between 2mL and 4mL. Following LC-FLD analysis of the periodate oxidised fractions following each clean up protocol, the toxin oxidation product peaks were integrated to determine the size of the peaks in terms of signal to noise ratio and calculated toxin recovery. The results are summarised in **Table 24**. Increasing the sample load on the C18 SPE cartridges appears to result in a similar level of toxin recovery, inferring that neither of the toxins are lost due to sample loading on the column. The signal to noise ratios of both the GTX1,4 and NEO quantitation peaks were seen to be noticeable larger following the higher volume clean-ups, with the analysis of the 2mL C18 sample giving rise to peaks at twice the signal to noise ratio than those following the normal 1mL clean up. Use of higher sample loading for the ion exchange fractionation step also seems to improve the signal to noise ratios of the final toxin peaks. However, the results indicate a noticeable reduction in toxin recovery, when either 3mL or 4mL samples are subjected to ion exchange, with recoveries dropping to less than half the values obtained following the normal 2mL clean up. In order to assess whether these effects were repeatable, the use of higher sample volumes for ion exchange clean up was repeated on a different day, using a king scallop extract containing GTX1,4 and NEO at 0.5 AL. Results indicated again, significant losses of toxins when using 3mL or 4mL sample volumes (data not shown).

Table 24. Comparison of the GTX1,4 and NEO recoveries and toxin peak signal to noise ratios (S/N) calculated using a variety of C18 and ion exchange SPE clean up protocols. Oxidation performed using normal AOAC 2005.06 conditions with standards in the presence of PO-MM.

C18 sample load	Ion exchange sample load	% Recovery		% of normal (1mL C18, 2mL ion exchange)		S/N ratio	
		GTX 1,4	Neo	GTX 1,4	Neo	GTX 1,4	Neo
1mL	2mL	40%	21%			2.4	2.6
1.5mL	2mL	39%	20%	98%	95%	3.4	3.8
2mL	2mL	32%	20%	80%	93%	4.6	5.4
1mL	3mL	49%	16%	124%	71%	4.9	3.1
1.5mL	3mL	30%	13%	75%	65%	5.1	4.1
2mL	3mL	36%	14%	89%	69%	6.2	4.6
2mL	4mL	22%	7%	56%	35%	5.5	3.2

From these results, there is good evidence for the potential improvement in method performance to be gained from the C18 SPE clean-up of higher volumes of crude scallop extracts. Specifically, the C18-cleaned extracts are more concentrated, without resulting in any apparent increased level of matrix-related suppression, but resulting in a higher method sensitivity as evidenced by the higher signal to noise ratios. Conversely, the evidence for toxin losses during the ion exchange clean up of larger sample volumes indicates that this approach is not suitable for improving the method performance.

3.3.6 Testing the refined conditions

The work described above has shown evidence for improvement in method performance following a number of modifications to standard methodology. Each of these

experiments was conducted separately so it was important to assess the cumulative effects of combining all the above proposed modifications. A range of fortified king and queen scallops extracts were subjected to clean up, fractionation and periodate oxidation using proposed refined experimental conditions. The samples contained concentrations of GTX1,4 and NEO at concentrations between 0.2 and 0.4 AL per toxin. For each sample, the homogenates were extracted and diluted to 10mL, prior to C18 SPE clean up using 1.5mL of crude extract. 2mL of each C18-cleaned extract were subjected to ion exchange clean up, prior to the refined periodate oxidation. The periodate used for oxidation of all standards and samples was prepared using 0.03M periodic acid, 0.3M ammonium formate and 0.3M sodium hydrogen phosphate with proportions of 5:1:1 respectively, adjusting the pH to 8.2 prior to use. Samples were oxidised in the presence of the normal Pacific oyster matrix modifier, whilst calibration standards were oxidised in the presence of the king scallop matrix modifier. 100µL injection volumes were used for all analyses.

Table 25. Summary of the GTX1,4 and NEO recoveries and estimated limits of detection calculated using the refined LC-FLD method for king scallops (n=7) and queen scallops (n=6) and analysed in different analytical batches.

	Species	Test	1	2	3	4	5	6	7	Mean	RSD%
GTX 1,4	KSc	Recovery	142%	152%	74%	103%	89%	71%	134%	109% ± 33%	31%
GTX 1,4	KSc	LOD	0.05	0.07	0.11	0.16	0.17	0.11	0.17	0.12 ± 0.05	41%
NEO	KSc	Recovery	40%	50%	66%	72%	66%	61%	79%	62% ± 13%	22%
NEO	KSc	LOD	0.16	0.16	0.07	0.17	0.15	0.07	0.11	0.13 ± 0.4	34%
GTX 1,4	QSc	Recovery	117%	120%	91%	70%	116%	77%	Na	99% ± 22%	22%
GTX 1,4	QSc	LOD	0.07	0.12	0.15	0.17	0.13	0.20	Na	0.14 ± 0.04	32%
NEO	QSc	Recovery	53%	49%	67%	71%	77%	47%	Na	61% ± 13%	21%
NEO	QSc	LOD	0.18	0.17	0.13	0.15	0.06	0.21	na	0.15 ± 0.05	35%

Results indicate (Table 25) that the proposed refined LC-FLD method for the analysis of king and queen scallops results in the enhanced recovery and sensitivity for the quantitation of GTX1,4 and NEO. With the use of KSc-MM in the standards, GTX1,4 recoveries are close to 100%, although the variability from these refinement investigations is fairly high (>30%). However, the variability of all other recoveries is more acceptable, with RSDs for the recoveries determined all < 25%. The recoveries for NEO in both scallop species are close to 60%, so are now similar to those determined previously for the other non-N-hydroxylated toxins in scallops [16].

3.4 Conclusions and recommendations from method refinement studies

A series of investigations into the potential benefits in method performance to be gained from refining the official AOAC 2005.06 method for the analysis of scallops was conducted. This was necessary due to the poor method recovery and sensitivity noted previously for the determination of N-hydroxylated toxins by periodate oxidation and LC-FLD analysis. Results indicated the potential benefits to sensitivity with the use of high injection volumes (100µL), the C18 SPE clean-up of higher volumes of crude extract and the use of a modified periodate reagent containing 5 times the normal relative amounts of periodic acid (**Table 25**). The recovery issues were found to improve with use of a matrix modifier prepared from king scallops in place of the normal Pacific oyster matrix modifier for the oxidation of calibration standards used for quantitation. With all refinements tested together, recoveries were found to range from 61% to 109% for GTX1,4 and NEO in both king and queen scallops, and the mean calculated LODs were all < 0.16 µg STX eq./g (0.2 AL; **Table**

25). As such, these refined conditions will be used for the subsequent validation of a refined method for the periodate oxidation of N-hydroxylated toxins in both queen and king scallops.

4. Phase 2: Validation of refined method - results and discussion

4.1 Selectivity of the method

In order to assess whether components of the scallop matrices may have an effect on the quantitation of PSP toxins following periodate oxidation using the refined methodology, king and queen scallop tissue extracts were analysed after periodate oxidation. Specifically, oxidation was conducted on the C18 SPE-cleaned extracts and fractions F2 and F3 from both species. 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 [19].

Periodate oxidised C18-cleaned extracts

Matrix components were observed (Figures 5 and 6) eluting up to 1.5 minutes in the chromatograms for the periodate oxidation both species, as is typically encountered for all other shellfish species [13-15]. One peak corresponding in retention to the primary toxin oxidation product of dcGTX2,3 was again observed at 2.3 mins in queen scallops. However, following periodate oxidation, the primary toxin peak for dcGTX2,3 is at 2.7-2.8min, so these interference peaks would not compromise the results of the HPLC screen following periodate oxidation. No interfering peaks were observed in the king scallops chromatogram

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

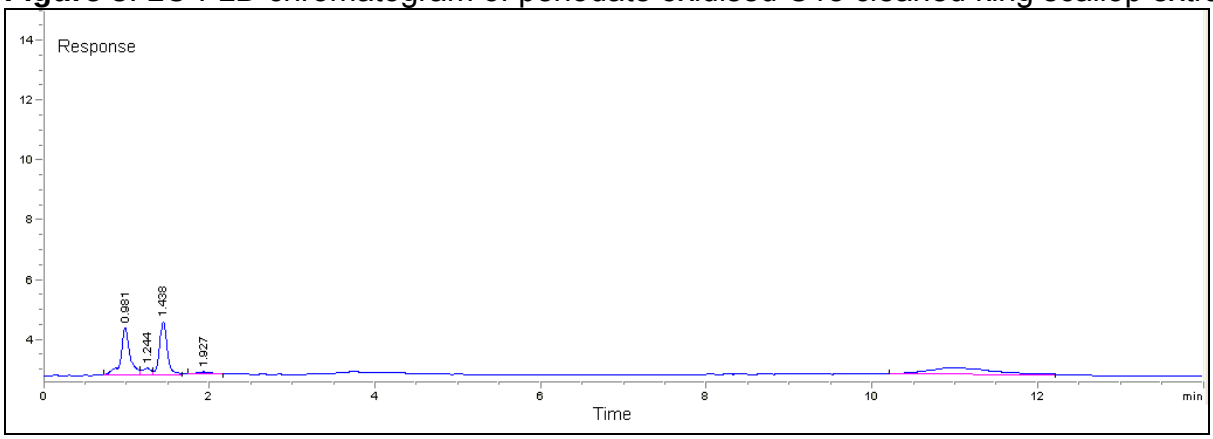
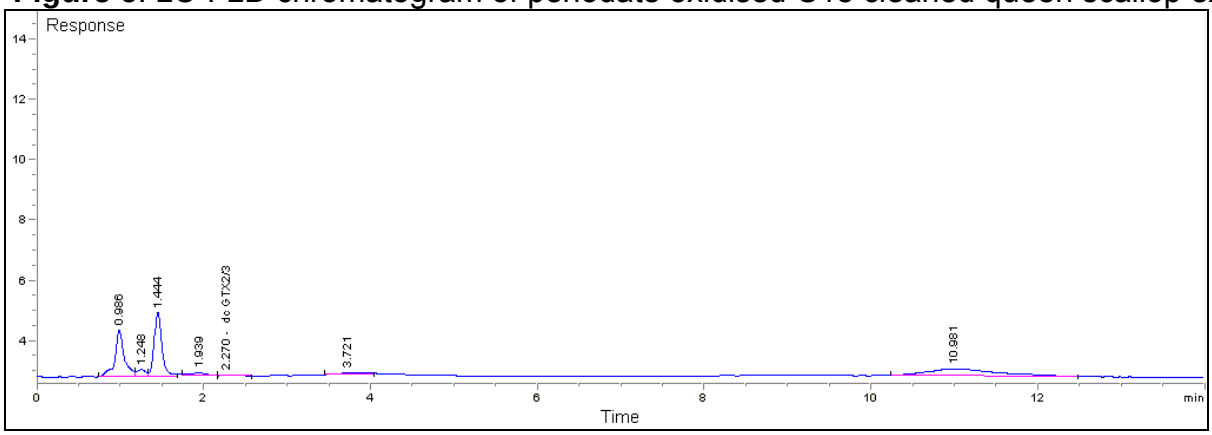


Figure 6. LC-FLD chromatogram of periodate oxidised C18 cleaned queen scallop extract.



Periodate oxidised fractions

Matrix components were again observed eluting up to 1.6 minutes in the chromatograms for the periodate oxidation of both fractions in both scallops species (Figures 7 to 10). Peaks corresponding in retention to the toxin oxidation product of dcGTX2,3 were also observed again at around 2.2-2.3 mins in queen scallops, although not detected in king scallops. Such peaks were however found to be small (signal to noise ratio <3) and would not interfere with the toxin quantitation, as dcGTX2,3 is only quantified following the peroxide oxidation of the C18-cleaned extracts. Whilst the contribution of dcGTX2,3 is removed from GTX1,4, when both are present, this would only be performed if both dcGTX2,3 peaks are present in the chromatogram of fraction F2. As such, there are no apparent issues with the selectivity of the method following the refined periodate oxidation of the king and queen scallop extracts.

Figure 7. LC-FLD chromatogram of periodate-oxidised fraction F2 from king scallop extract.

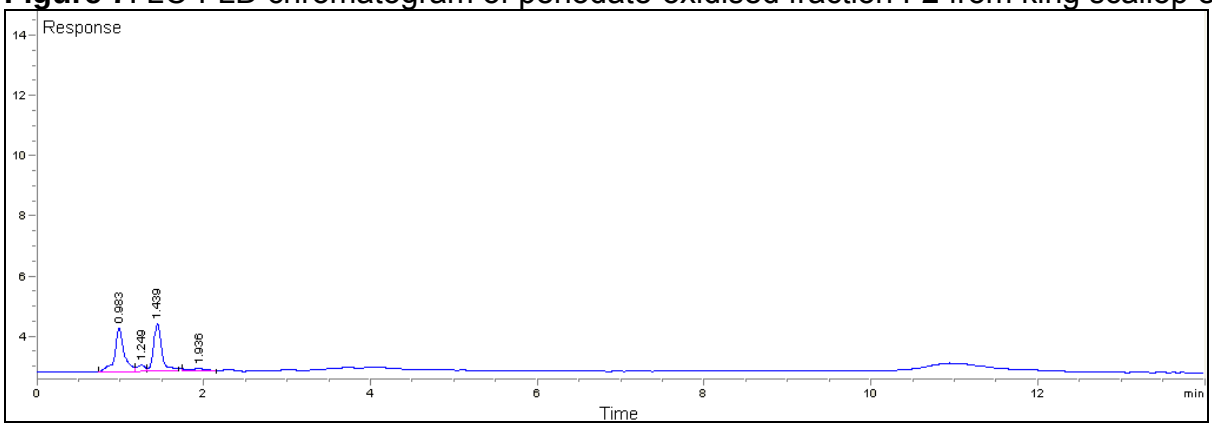


Figure 8. LC-FLD chromatogram of periodate-oxidised fraction F2 from queen scallop extract.

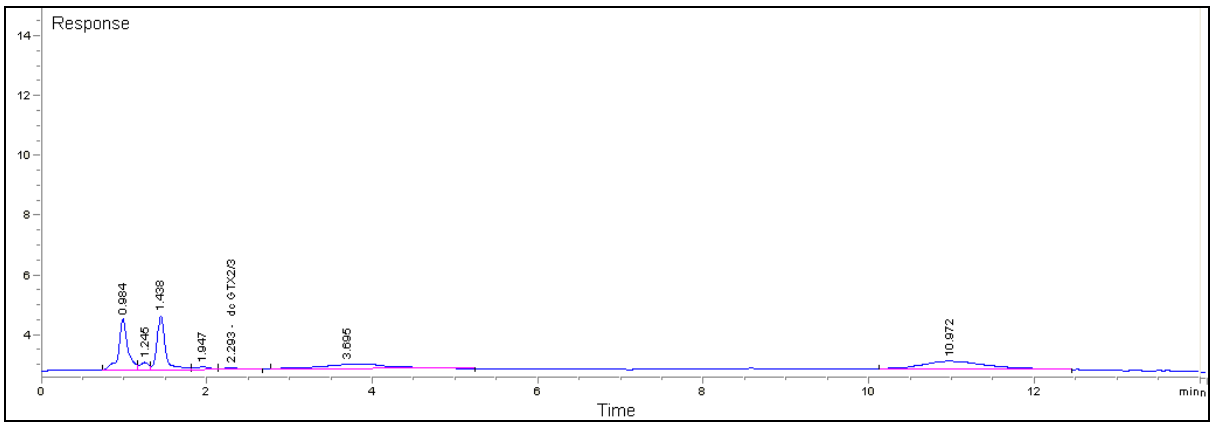


Figure 9. LC-FLD chromatogram of periodate-oxidised fraction F3 from king scallop extract.

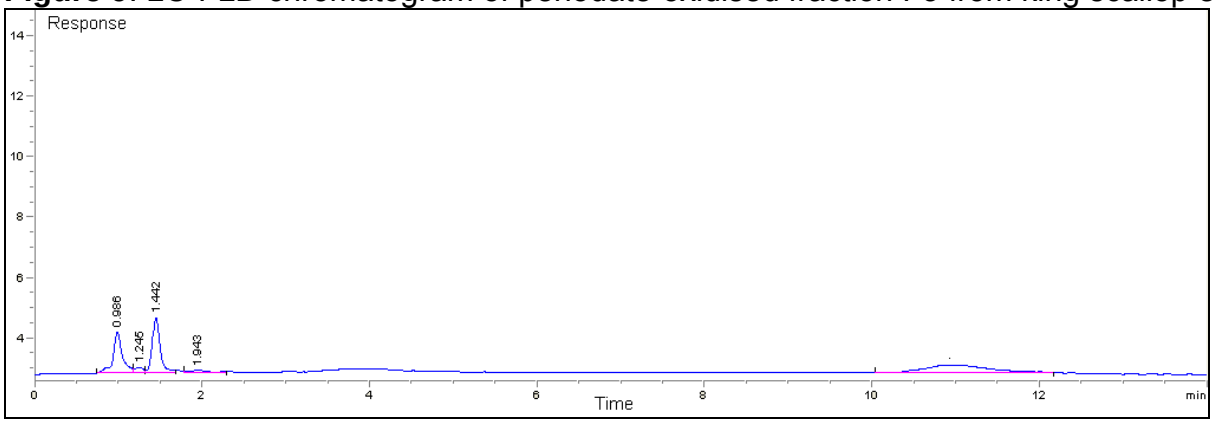
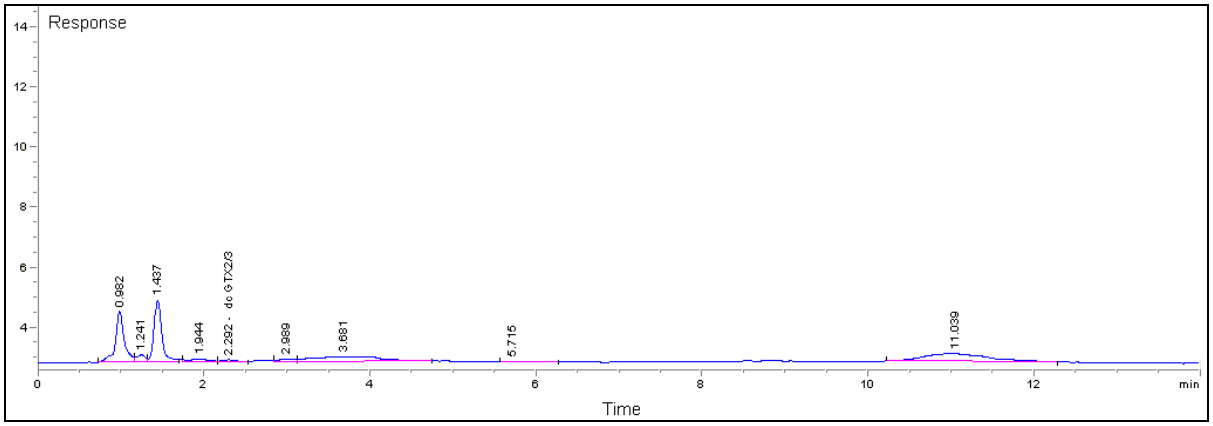


Figure 10. LC-FLD chromatogram of periodate-oxidised fraction F3 from queen scallop extract.



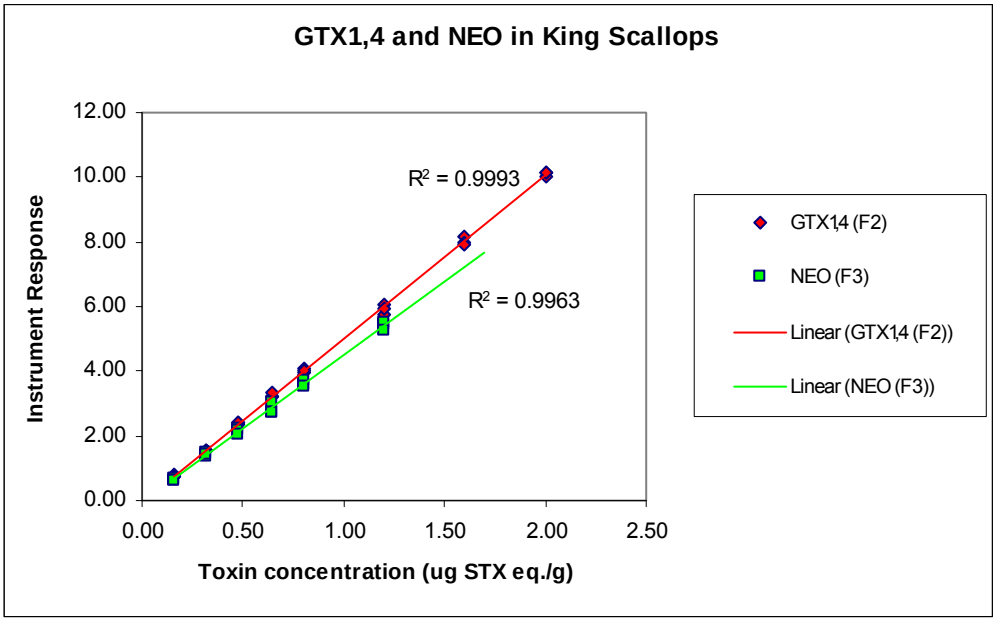
4.2 Linearity of the analytical method

The LC-fluorescence detector was calibrated with individual PSP toxins prepared in both king and queen extracts (after C18 and fractionation by COOH SPE). Results showed that a linear-fit model is the preferred model, with separate slopes for each matrix. A summary of the results is shown in Table 26 and the results for both toxins on both species are presented as follows.

4.2.1 King scallops

Quantitation of GTX1,4 was performed by analysing the presence of this toxin in fraction F2, generated post ion exchange fractionation. NEO was quantified by analysing in the presence of fraction F3. Observation of linearity of both toxins in the calibration graphs (Figure 11) appears acceptable for both toxins, with the correlation coefficient ≥0.996 for each toxin (Table 18). The F-test lack of fit results and F-critical value for the data set and visual examination of the residual plots (Appendix 2) for the fraction plot indicates a linear relationship exists between toxin concentration and analytical response. F-test results are less than F-critical for both toxins (Table 26).

Figure 11. Calibration plots of GTX1,4 and NEO concentration against detector response for standard prepared in king scallop fraction extracts over a calibration range of 0 to 1.5 AL (NEO) and 0 to 2.5 AL (GTX1,4).



4.2.2 Queen scallops

Quantitation of GTX1,4 and NEO was again performed in the presence of fractions F2 and F3 respectively. The linearity (Figure 12) again appears acceptable visually for both matrices, with the correlation coefficient >0.98 for each toxin (Table 26). The F-test lack of fit indicates a linear relationship exists between NEO concentration and analytical response, although the F-test result for GTX1,4 is higher than F-critical. However, this relates to the very low variability of the responses returned by the triplicate results, with visual examination of residual plots (Appendix 2) indicating that there is no apparent systematic deviation of the residuals from zero.

Figure 12. Calibration plots of GTX1,4 and NEO concentration against detector response for standard prepared in queen scallop fraction extracts over a calibration range of 0 to 1.5 AL (NEO) and 0 to 2.5 AL (GTX1,4).

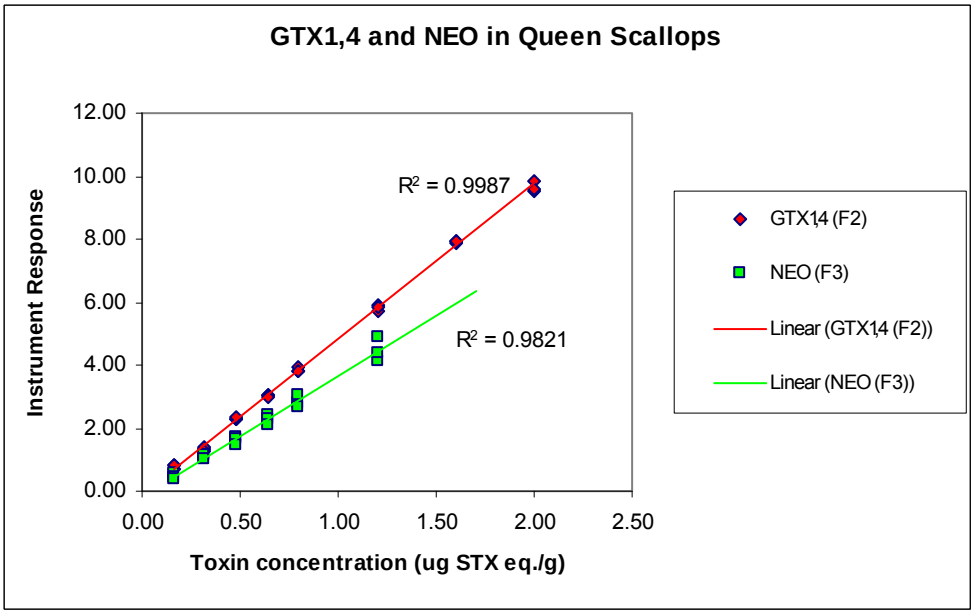


Table 26. Summary of linear regression gradients in scallop fractions, plus relative standard deviations of response factors and F-test lack of fit test results calculated for each PSP toxin.

Toxin	Matrix	Correlation coefficient (r^2)	RSD% of response factors	F-test	F-critical
GTX1,4	King scallops	0.998	3%	1.53	2.51
	Queen scallops	0.999	5%	3.18	2.51
NEO	King scallops	0.996	5%	0.17	2.93
	Queen scallops	0.982	11%	0.26	2.93

In summary therefore, analysis of calibrations over the range of 0 to 1.5 AL (NEO) and 0 to 2.5 AL (GTX1,4), using correlation coefficients and F-test goodness of fit of the residuals, indicates there are no significant systematic deviations from linearity for any of the toxins within either king scallop or queen scallop matrices. RSDs of response factors are generally low (≤11%) indicating an acceptable level of scatter within each of the calibration plots. Visual examination of the regression and residual plots, does not indicate the presence of any systematic deviation of the residuals from zero.

4.3. Limits of detection and quantitation for PSP toxins in king and queen scallops

4.3.1 Limits of detection for screening method

LODs were calculated for the refined periodate oxidation of each toxin in C18-cleaned king and queen scallop extracts analysed in two separate batches in order to predict the limits of detection for the screening part of the method. This ensures that the sensitivity of the screening method is verified, reducing the risk of false negatives prior to full quantitation. LODs are tabulated (Table 27) in terms of mean ± one standard deviation (sd, n=13) where the standard deviation is calculated from the signal to noise values measured for each result for each individual toxin. Values were calculated for the primary (diagnostic) toxin peak only. For both N-hydroxylated toxins, predicted LODs of ~0.04 to 0.09 µg STX eq./g (0.05 to 0.11 AL) were determined for the periodate screening method of C18-cleaned extracts of shellfish tissue. Whilst these values were not experimentally confirmed with homogenate spiking experiments at the actual LOD concentrations, these values give a good indication of the sensitivity of the screening method. Results clearly indicate the sensitivity of the periodate screen has improved for the N-hydroxylated toxins GTX1,4 and NEO with use of the refined method.

Table 27. Predicted limits of detection (LOD; µg STX eq./g ± 1 sd, n=13) of the LC-FLD screening method for the primary toxin peaks of GTX1,4 and NEO following periodate oxidation of C18-cleaned king and queen scallop extracts.

	LOD (µg STX eq./g) ± sd	
Toxin	King scallops	Queen scallops
GTX 1,4	0.068 ± 0.015	0.090 ± 0.031
NEO	0.040 ± 0.015	0.054 ± 0.021

In addition, LODs were also calculated for the non-N-hydroxylated toxins in C18-cleaned king and queen scallop extracts following the refined periodate oxidation (Table 28). For all toxins, predicted LODs of ~0.02 to 0.09 µg STX eq./g (0.04 to 0.11 AL) were determined. Results therefore indicate that the sensitivity of the periodate screen method has improved for all PSP toxins investigated with use of the refined method.

Table 28. Predicted limits of detection (LOD; µg STX eq./g ± 1 sd, n=3) of the LC-FLD screening method for the primary toxin peaks of the non-N-hydroxylated PSTs following periodate oxidation of C18-cleaned king and queen scallop extracts.

	LOD (µg STX eq./g) ± sd	
Toxin	King scallops	Queen scallops
C1,2	0.034 ± 0.001	0.087 ± 0.02
dcSTX	0.042 ± 0.002	0.085 ± 0.015
GTX2,3	0.018 ± 0.001	0.041 ± 0.008
GTX5	0.076 ± 0.001	0.069 ± 0.034
STX	0.039 ± 0.001	0.091 ± 0.018

The results therefore indicate, that with the use of the refined method, king scallop samples can be safely screened using just periodate oxidation, as opposed to the current protocol whereby both periodate and peroxide oxidation are utilized. This will reduce the number of analyses required for the qualitative analysis of these species.

4.3.2 Limits of detection for quantitation method

Table 29 tabulates the calculated method LODs for GTX1,4 and NEO following the refined periodate oxidation of the fractionated extracts at 0.16 µg STX eq./g (0.2 AL). The data illustrates that the sensitivity of the quantitation method is sufficient to quantify these toxins at below this target concentration of 0.16 µg STX eq./g. Results therefore show that the analytical sensitivity is improved in comparison to the normal AOAC 2005.06 method for these toxins in both scallop species and now acceptable for the purpose of routine quantitation of these toxins in both scallop species.

Table 29. Calculated method limits of detection (LOD; µg STX eq./g ± 1 sd) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions of C18-cleaned king and queen scallop extracts.

	LOD (µg STX eq./g) ± sd	
Toxin	King scallops	Queen scallops
GTX 1,4	0.050 ± 0.016	0.065 ± 0.026
NEO	0.095 ± 0.031	0.094 ± 0.022

4.3.3 Determination of the limit of quantitation of the method

The results from the experimental confirmation of LOQ are summarised in Table 30. LOQs are confirmed at <32 µg STX eq./g for both N-hydroxylated toxins therefore significantly improved as compared with the values reported using the normal AOAC 2005.06 method. As such, it is clear from these results that the method is now fit for purpose for the

detection and quantitation of N-hydroxylated toxins in either king scallop or queen scallop matrices.

Table 30. Calculated method limits of quantitation (LOQ; µg STX eq./g ± 1 sd) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions of C18-cleaned king and queen scallop extracts.

	LOQ (µg STX eq./g) ± sd	
Toxin	King scallops	Queen scallops
GTX 1,4	0.167 ± 0.054	0.218 ± 0.09
NEO	0.318 ± 0.10	0.314 ± 0.08

4.4 Determination of the recovery of N-hydroxylated PSP toxins from spiked shellfish tissues

Recoveries were calculated in terms of expected mean recovery for each toxin in each of the three spiked, extracted, cleaned and oxidised samples. Table 31 presents the mean recovery percentages of GTX1,4 and NEO from king and queen scallops spiked at 0.4 AL and 0.2 AL per toxin with RSDs calculated from the mean recovery of the triplicate oxidations of each of the replicate (n=6) spikes.

Table 31. Mean percentage recoveries (RSDs of replicate spikes, n=6) of PSP toxins from king and queen scallop homogenates spiked at expected concentrations of 0.4 AL and 0.2 AL.

	King scallops		Queen scallops	
	0.4 AL	0.2 AL	0.4 AL	0.2 AL
GTX 1,4	114% (9%)	104% (15%)	80% (9%)	77% (5%)
NEO	82% (15%)	89% (10%)	83% (6%)	86% (7%)

Table 31 shows the mean recoveries of both N-hydroxylated toxins spiked at 0.2 and 0.4 AL falling in the range of 82% to 89% for NEO and 77% to 114% for GTX1,4 after analysis with the refined scallops method. RSDs associated with the recoveries of most toxins indicate a good degree of repeatability associated with such measurements. It therefore appears that in both scallop species, the recoveries for both toxins are acceptable, being similar or improved to results reported previously in mussels [13,18], oysters and cockles [14] and clams [15]. The recoveries show a marked improvement over the values reported previously using the normal AOAC 2005.06 method, when values were poor [16]. Use of the refined method has therefore been shown to improve the method performance in these species and gives a good indication that the method is more suitable and applicable to the routine analysis of N-hydroxylated toxins in both scallop species.

4.5 Determination of the precision of the method

4.5.1 Estimation of instrumental precision

Instrumental precision of toxin peak retention times following the repeat analysis (n=11) of king scallops and queen scallops is presented in Table 32. The table shows that the level of precision of chromatographic retention times is high over a lengthy sequence of 30 hours (RSD ≤ 2.0%), hence a high degree of confidence can be placed upon the toxin peaks consistently eluting at repeatable retention times. The table also displays the results obtained from the same analysis for the precision (n=11) of toxin peak area responses for both GTX1,4 and NEO. Relative standard deviations calculated from the replicate analyses were shown to range between 2.0% and 2.9% for each toxin in both scallop species. These values

therefore demonstrate an acceptable level of instrumental precision for the refined periodate oxidation method for both GTX1,4 and NEO in king and queen scallop samples.

Table 32. Instrumental precision, showing variability (RSD%) of toxin retention times and peak area responses in king and queen scallops over a 30-hour analytical sequence.

	Toxin peak area precision (RSD %; n=11)		Retention time precision (RSD %; n=11)	
Toxins	King scallops	Queen scallops	King scallops	Queen scallops
GTX 1,4	2.3%	2.0%	1.6%	1.7%
NEO	2.5%	2.9%	2.0%	1.9%

4.5.2 Estimation of short-term repeatability

Table 33 shows the concentrations calculated for triplicate homogenate spikes of king and queen scallops at 0.2 AL and 0.4 AL for both GTX1,4 and NEO following single batch analysis using the refined scallops periodate method. Standard deviations calculated from the resulting concentrations illustrate an acceptable level of short-term method repeatability for most of the toxins. RSD% values are less than or equal to 10.3% at 0.4AL for both toxins therefore suggesting that at the toxin concentrations equal to 0.4 AL, the short term repeatability for the LC-FLD analysis using the refined method is reliable. Values are also acceptable at the lower concentration of 0.2 AL, with the RSD% values in both scallops <8%. On the whole, these values compare well with those generated previously for mussels [13, 18] and oysters and cockles [25], with equivalent or improved levels of precision. It is noted that the short term repeatability is improved in comparison with the normal AOAC 2005.06 method for the quantitation of scallops, where previously values as high as 34% were observed [16].These results therefore provide evidence for the improved performance of the refined method for the analysis of the N-hydroxylated PSP toxins in both scallops species.

Table 33. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of triplicate spiked king and queen scallop homogenate at 0.2 AL and 0.4 AL per toxin showing estimations of short-term method repeatability in terms of percentage relative standard deviation (n=3; same batch).

Toxin	King scallops				Queen scallops			
	0.4 AL		0.2 AL		0.4 AL		0.2 AL	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
GTX 1,4	0.35 ± 0.01	1.8%	0.16 ± 0.01	7.9%	0.27 ± 0.03	10.3%	0.12 ± 0.01	5.4%
NEO	0.29 ± 0.01	2.2%	0.15 ± 0.01	4.7%	0.26 ± 0.01	4.7%	0.13 ± 0.01	7.5%

4.5.3 Estimation of medium-term repeatability

Table 34 shows the medium-term precision for king and queen scallops following the analysis of six replicate spiked homogenates (both 0.2 and 0.4 AL) performed over a longer period of time (2 weeks). RSD percentages range from 5% to 15% at both concentration levels for both toxins. As such, the degree of precision associated with the analysis of GTX1,4 and NEO in both scallop species appears acceptable at both concentration levels. Further evidence for an acceptable level of precision is provided by the HorRat values, which are <1.0 for both toxins at both concentration levels.

Table 34. Calculated mean concentrations (µg STX eq./g +/- 1 sd) of six replicate spiked king and queen scallop homogenates at 0.2 AL and 0.4 AL per toxin showing estimations of medium-term method repeatability in terms of percentage relative standard deviation (n=6)

	King scallops					
	0.4 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.37 ± 0.03	9%	0.47	0.17 ± 0.02	15%	0.70
NEO	0.26 ± 0.04	15%	0.77	0.14 ± 0.01	10%	0.47
	Queen scallops					
	0.4 AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
GTX 1,4	0.26± 0.02	9%	0.47	0.12 ± 0.01	5%	0.24
NEO	0.26 ± 0.01	6%	0.30	0.14 ± 0.01	7%	0.34

4.5.4 Estimation of long-term repeatability

Concentration data from the extraction, clean up and analysis of a king scallops reference material was generated over a period of one month by different analysts, using different batches of reagents and consumables and with analysis on different LC-FLD instruments with different toxin calibrations. The data realistically describes the within-laboratory reproducibility of the method, incorporating variable changes such as different working calibration solutions, instruments, analysts and other laboratory conditions experienced over the longer term. The king scallop LRM used for the testing was generated with the laboratory feeding of king scallops with mass cultured *Alexandrium*. The material was homogenised and aliquoted prior to long term analysis. Results obtained from the repeated analysis of the LRMs are summarised in Table 35.

Table 35. Mean concentration +/- sd and %RSD data generated from long term extraction, clean-up, fractionation, oxidation and analysis of a king scallop LRM and spiked queen scallop homogenates using the refined scallops LC method. Toxins present at concentration levels < 0.2 AL are shaded.

Toxin	King scallop LRM (n=11)			Queen scallop spiked homogenates (both 0.2 and 0.4 AL; n=12)
	Mean (µg/100g STX equiv)	%RSD	HorRat	%RSD
GTX 1,4	152 ± 36	24%	1.58	20%
NEO	66 ± 10	15%	0.89	21%
GTX 2,3	31 ± 4.5	15%	0.77	na
GTX5	0.34 ± 0.07	20%	0.54	na
STX	38 ± 4.1	11%	0.58	na
C1,2	17 ± 2.2	13%	0.61	na
Total	306 ± 43	14%	1.03	na

na = not analysed

Results indicate an acceptable level of long-term precision for both N-hydroxylated toxins in the scallop LRM studied. Long-term repeatability RSDs for non-N-hydroxylated toxins (analysed following peroxide oxidation of the C18 cleaned extracts) exhibited values between 11% and 20%, similar to those reported previously [16], whereas toxin quantitation requiring the additional fractionation step (GTX1,4 and NEO) exhibited slightly higher variability (up to 24% for GTX1,4). All HorRat values calculated were < 1.6. These values are similar to those

generated previously for oysters and cockles [14], clams [15] and for mussels [13,18] and do not appear to worsen at the lower concentration levels. Given the absence of a contaminated queen scallop samples, the size of the within lab reproducibility was estimated from the variability of the toxin concentrations calculated from 12 spiked homogenates over a period of more than 2 weeks, using a combination of both low and high toxin concentrations. The RSDs for GTX1,4 and NEO are both close to 20%, which seems acceptable for the method given the number of steps required for quantitation of these toxins. Overall, the results therefore indicate a good level of within-laboratory reproducibility of the refined quantitation method for scallops.

4.6 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 36-37 show the t-test results for each parameter in each matrix.

Table 36. T-test results (n=8, t-critical = 2.37) from ruggedness experiment of N-hydroxylated toxins (king scallops)

Toxin	Periodate pH	Vortex time	Ambient temp	Oxidation time	Acetic volume	pH Matrix modifier	Periodic acid composition
GTX 1,4	0.67	0.64	1.11	1.03	1.01	1.18	-0.27
NEO	-1.46	0.32	-0.11	1.07	1.40	-0.70	-1.19

Table 37. T-test results (n=8, t-critical = 2.37) from ruggedness experiment of N-hydroxylated toxins (queen scallops)

Toxin	Periodate pH	Vortex time	Ambient temp	Oxidation time	Acetic volume	pH Matrix modifier	Periodic acid composition
GTX 1,4	1.42	0.95	-0.01	0.35	0.59	0.56	-0.43
NEO	0.08	0.41	1.95	0.42	0.93	0.34	0.39

Results show that all t-test values were lower than t-critical (n=8, 95% confidence) for both king scallop and queen scallop ruggedness experiments. 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. Importantly, this infers that any small changes to the parameters involved in the refined periodate oxidation do not seem to have any negative effect on the performance of the method.

4.7 Completion of validation studies for the performance of the AOAC 2005.06 method for the quantitation of non-N-hydroxylated toxins in queen scallops following peroxide oxidation.

Quantitative analysis was conducted on the range of non N-hydroxylated PSTs in queen scallop matrices, spiked at concentrations equivalent to both 0.2 and 0.4 AL. Results were used to calculate the recovery of the method for these toxins and together with the initial recovery results reported previously [16] were used to calculate medium/long term precision of the method for these toxins in queen scallops. It is noted that due to the timing of the experiments conducted to determine the recoveries of the 2nd batch of samples, the analysis of the total sample set was conducted more than 18 months apart. Table 38 summarises the total mean recoveries determined for each non-N-hydroxylated toxin and the repeatability in terms of percentage RSD. At both toxin concentrations, the mean recoveries are all acceptable (67% - 78%). As such there is good evidence still for the acceptable

performance of the method following peroxide oxidation of queen scallop samples. The repeatability of the method over the time period shows very low variability in recoveries determined at the higher concentration level, with RSD% ranging from 6% to 9%. These values compare very well to those determined previously in other species. All HorRat ratios were found to be < 0.8. The variability at the lower concentration is higher than expected, with some HorRat values > 1.3. However, the variability is still similar to values determined previously at this concentration in other species.

Table 38. Mean toxin recoveries and associated long-term precision (non-N-hydroxylated) for the AOAC 2005.06 method in queen scallops

	Queen scallops					
	0.5 AL & 0.4AL			0.2 AL		
Toxin	Mean	RSD	HorRat	Mean	RSD	HorRat
dcSTX	67%	6%	0.36	72%	22%	1.17
GTX 2,3	74%	9%	0.55	78%	29%	1.45
GTX 5	72%	9%	0.74	77%	22%	1.59
STX	67%	8%	0.45	74%	25%	1.18
C 1,2	73%	9%	0.68	77%	25%	1.68

4.8 Measurement of uncertainty

Uncertainty of measurement associated with the method is assessed through the propagation of standard uncertainties. These include uncertainty of measurement inherent in the precision, assessment of recovery and repeatability/reproducibility. 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, as with the assessment for other bivalve species [13-16]. The uncertainty of measurement has been presented previously for the standard AOAC 2005.06 method and is updated here using the refined method for the quantitation of the N-hydroxylated toxins in both scallop species, together with the updated results from the recovery and precision determination of non-N-hydroxylated toxins in queen scallops.

4.8.1 Precision – Repeatability

The measurement 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 [25, 27]. Uncertainties are calculated at two concentration levels (0.2 AL and 0.4 AL) for medium term precision and RSDs are pooled to give total standardised precision uncertainties in king and queen scallops (Table 39):

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

Where:

$u_c(y)$ = pooled uncertainty of precision uncertainty components

a,b = RSDs of components at each concentration

n = number of replicates used in precision studies for each concentration

Table 39. Revised precision values (RSDs) and pooled uncertainties calculated for PSP toxins in king scallops.

	King scallops			Queen scallops		
	Medium term		"Pooled"	Medium term		"Pooled"
Toxin	0.2 AL	0.5 AL	Uncertainty	0.2 AL	0.5 AL	Uncertainty
GTX 1,4	0.15	0.09	0.12	0.05	0.09	0.07
NEO	0.10	0.15	0.13	0.07	0.06	0.07
dcNEO	0.12	0.05	0.09	na	na	na
dcSTX	0.08	0.07	0.08	0.22	0.06	0.16
GTX 2,3	0.14	0.13	0.13	0.29	0.09	0.21
GTX 5	0.04	0.05	0.04	0.22	0.09	0.17
STX	0.08	0.11	0.10	0.25	0.08	0.18
C 1,2	0.21	0.20	0.21	0.25	0.09	0.19

na = not analysed (due to toxin unavailability)

4.8.2 Within-lab reproducibility or long-term repeatability

The uncertainties associated with long term precision (Table 40) were estimated for king scallops from the precision data generated by the repeated extraction, clean-up, fractionation, oxidation and analysis of the king scallop LRM. For toxins not present in the current LRMs (dcSTX and dcNEO), uncertainties were estimated from the mean of all other toxins present. For the determination of the uncertainty associated with the reproducibility in queen scallops where no contaminated matrix material was available, the repeatability data for the N-hydroxylated toxins GTX1,4 and NEO quantified after the refined periodate oxidation, was calculated from the repeat analysis of spiked homogenates. Reproducibility data for the peroxide analysis of the non-N-hydroxylated toxins was taken from the data generated in the king scallop LRMs, probably the most realistic approach given the similarity in performance characteristics reported earlier.

Table 40. Within-lab reproducibility uncertainties calculated from repeat analysis (>2 months) of a king scallop LRM

	King scallops	**Queen scallops**
GTX 1,4	0.24	0.20**
NEO	0.15	0.21**
dcNEO	0.16*	0.16^{+}
dcSTX	0.16*	0.16^{+}
GTX 2,3	0.15	0.15^{+}
GTX 5	0.20	0.20^{+}
STX	0.11	0.11^{+}
C 1,2	0.13	0.13^{+}

* Mean values from other toxins present in LRMs
** Values calculated from repeat analysis (n=12) of spiked homogenates
$^{+}$Values for non-N-hydroxylated toxins taken from repeat analysis of KSc LRM

4.8.3 Uncertainty in recovery estimation

Recovery was calculated previously using the levels spiked into the tissues as the expected values. The uncertainties present in the determination of recovery were estimated by calculating the standard deviation for each toxin at each concentration, thus generating information on the uncertainty in recovery determination. Values are tabulated for each toxin at a low (0.2 AL) and high (0.4 AL) concentration in Table 41 below. Pooled uncertainties are

calculated for each toxin using the same formula as above and are shown to be of relatively small magnitude.

Table 41. RSDs and pooled uncertainties associated with determination of recovery in king and queen scallops.

	King scallops			Queen scallops		
	Concentration		"Pooled"	Concentration		"Pooled"
Toxin	Low	High	Uncertainty	Low	High	Uncertainty
GTX 1,4	0.08	0.02	0.06	0.05	0.10	0.08
NEO	0.05	0.02	0.04	0.08	0.05	0.06
dcNEO	0.12	0.05	0.09	na	na	na
dcSTX	0.03	0.05	0.04	0.01	0.04	0.03
GTX 2,3	0.04	0.08	0.07	0.01	0.03	0.03
GTX 5	0.02	0.05	0.04	0.02	0.05	0.04
STX	0.03	0.06	0.05	0.02	0.04	0.03
C 1,2	0.03	0.03	0.03	0.01	0.05	0.04

na = not analysed (due to toxin unavailability)

4.8.4 Calculation of combined standard uncertainty

Preliminary combined standardised uncertainties for each PSP toxin in king and queen scallops (Table 42) were 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 = standardised uncertainties for precision component
u_2 = standardised uncertainties for reproducibility component
u_3 = standardised uncertainties for recovery component

Table 42. Combined uncertainties calculated from validation data for king and queen scallops showing uncertainties as (a) standardised uncertainty and (b) expanded uncertainty (k=2).

	Standardised uncertanties		Expanded uncertainties (k=2)	
Toxin	KSc	QSc	KSc	QSc
GTX 1,4	0.28	0.23	0.55	0.46
NEO	0.20	0.23	0.40	0.46
dcNEO	0.21	na	0.41	na
dcSTX	0.18	0.24	0.36	0.48
GTX 2,3	0.21	0.26	0.42	0.52
GTX 5	0.21	0.26	0.42	0.53
STX	0.15	0.22	0.31	0.43
C 1,2	0.25	0.23	0.49	0.46

na = not analysed (due to toxin unavailability)

The values for uncertainty of measurement reported in Table 42 are preliminary as further work on the method, generation of additional LRMs, and long term use of such materials within the monitoring programme will build up further data on long term repeatability of the method for king scallops. The results above show a range of combined standardised uncertainties for individual toxins, ranging from 0.15 to 0.28 in king scallops and 0.22 to 0.26

in queen scallops. Expanded uncertainties, calculated using a coverage factor (k) of 2, result in a range of values from 0.31 to 0.55 for both species. 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 [27] and as assumed previously for other bivalve species [13-16]. Results show a similar range of values for the toxin suite compared with values reported previously for mussels, oysters, cockles and clams [13-15]. Values are similar to the range of values reported for oysters and cockles [14] and clams [15]. Standardised uncertainties could not be calculated for the toxin dcGTX2,3, due to low availability of the dcGTX2,3 reference standard and the absence of the toxin in the contaminated samples during the lifetime of this study.

5 Phase 3. Analysis of naturally contaminated scallop samples

Since the previous validation work undertaken during 2009 on scallop samples, no further scallops have been received at Cefas containing any concentrations of PSTs above the reporting limit. As a result of this shortage, the study was supplemented with additional samples generated following in-house feeding of king scallops with toxic *Alexandrium* species.

5.1 Samples found PSP negative by MBA or HCl screen LC-FLD

Appendix 5 tabulates the results of the quantitative LC-FLD results from 13 UK-harvested king scallop samples found to be PSP negative following the HCl-screen LC-FLD analysis and 12 queen scallops found to be negative by LC-FLD and MBA. Overall, the agreement between the results was excellent, with all MBA and HCl-screen LC-FLD negative samples also found to contain no or very low levels of PSP toxins. Once toxin concentrations and PSP toxicities were fully quantified by LC-FLD, all samples showed toxicities significantly lower than the MBA detection limit, with a maximum toxicity of 1.7 μg STX eq./100g, well below the LC-FLD quantitative reporting limit of 16 μg STX eq./100g.

5.2 Samples found PSP positive by MBA

Appendix 6 tabulates the quantitative LC-FLD (showing both total PSP toxicities and individual toxin concentrations) and MBA results obtained from the analysis of 19 PSP-positive scallops. In total, 15 whole king scallops, 2 queen scallops and 2 Atlantic scallop samples were analysed using the two quantitative methods. These included 3 archived PSP-positive king scallop and 2 queen scallop extracts, previously analysed by MBA and subsequently archived. These were analysed using the refined LC-FLD method, but were not re-analysed by MBA. Only 12 freshly generated king scallop samples were analysed by MBA and the refined LC-FLD method at the same time. Figure 13 shows the visual comparison of toxicity results, 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. A summary of these results is also given in Table 43.

Table 43. Summary of results from HPLC and MBA analysis of naturally contaminated scallops (total toxicities in μg STX di-HCl eq./100g flesh)

	KSc	QSc	ASc	All scallops
Number of samples	15	2	2	19
Mean LC toxicity	158	44	121	142
Mean MBA toxicity	114	45	119	107
Mean LC/MBA	141%	97%	104%	133%
RSD of LC/MBA ratios	14%	6%	8%	25%
Correlation coefficient (r)	0.85	na	na	0.87
Linear regression slope equation	y=0.62x+17	na	na	y=0.61x+19

KSc = whole king scallops; QSc = whole queen scallops, ASc = whole Atlantic scallops. All scallops includes all samples together in one data set.
na = not analysed given low number of data points

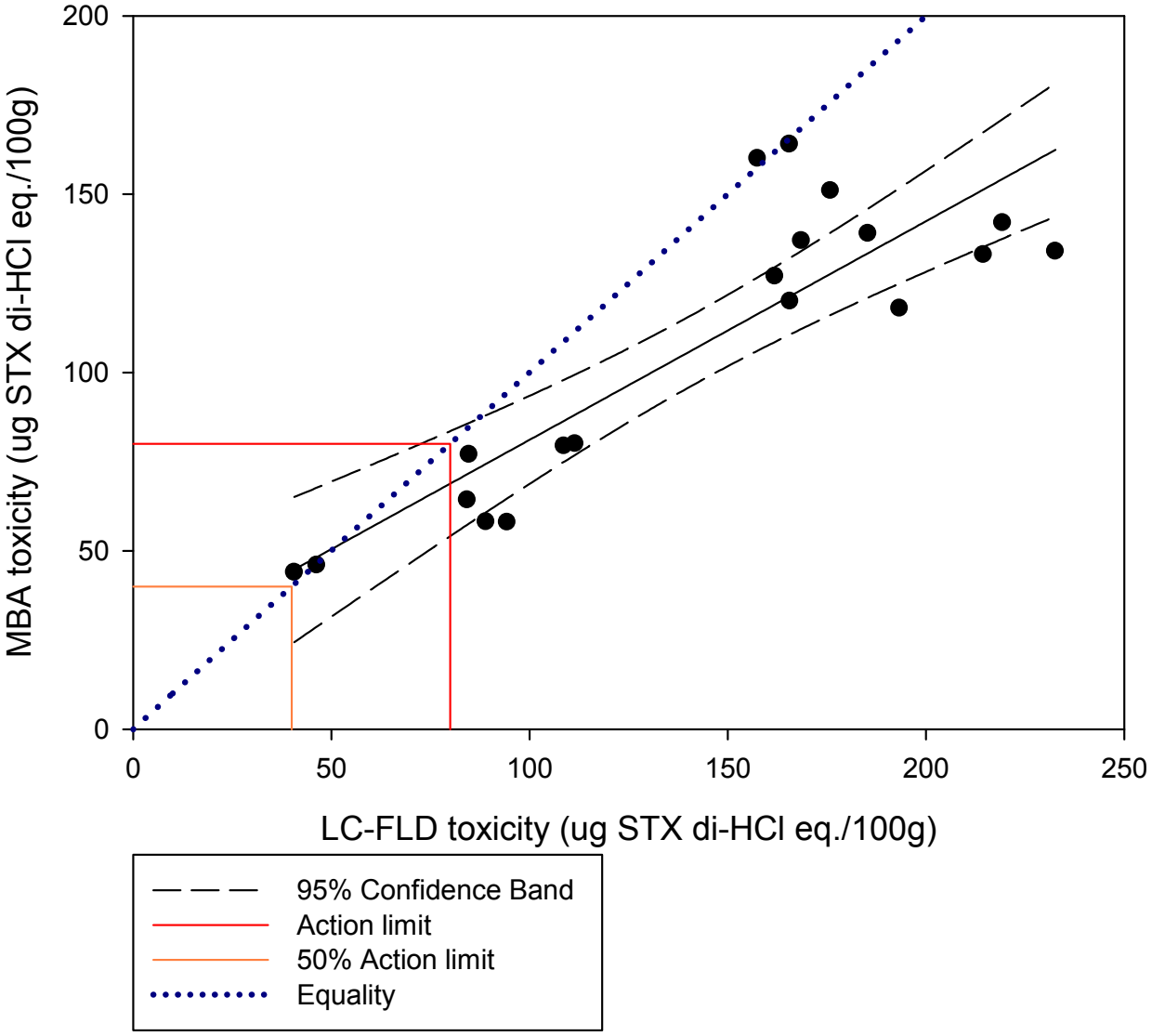


Figure 13. Comparison of total PSP toxicities in scallops obtained by MBA and quantitative HPLC-FLD, showing linear regression, estimated coefficients and associated 95% confidence intervals. Y=X and 1.0 and 0.5 AL limits are also shown.

The mean LC/MBA ratio for all the 19 scallop samples combined is 133%, showing some similarity between the results returned by the two methods but with evidence for a slight positive bias in the LC results. This relationship is also shown visually in Figure 17. The correlation between the results is also acceptable (Pearson correlation coefficient=0.87). Table 43 also shows the results listed for each individual species, specifically showing that the positive bias in the LC results is observed primarily in the king scallop samples, with the mean LC/MBA ratios being closer to unity for both the queen and Atlantic scallops (97% and 104% respectively). The excellent agreement between the results from the two methods for queen scallops is especially important given the significant under-estimation in toxicity returned by the LC method previously when using the normal AOAC 2005.06 quantitation method [16]. Here, use of the refined LC-FLD method for scallops has returned a much closer agreement between the results. The level of bias observed in the king scallop samples is almost identical to the size of the bias observed previously in the analysis of PSP-contaminated cockles [14]. As before, this level of bias can be attributed to the use of the

highest toxicity equivalence factor (TEF) for the quantitation of each epimeric pair. The strains of Alexandrium used to contaminate the king scallops contained relatively high levels of the toxins GTX1,4, C1,2 and GTX2,3 (Appendix 6), quantitation of which requires the assumption that the highest toxicity isomer is exclusively present. Results recalculated using the lowest TEF for each epimeric pair result in a mean LC bias of 103% for all scallops analysed, thereby showing an excellent agreement between the two methods.

Overall, results from all scallop samples show 5 out of the 19 samples exhibiting LC toxicities above the action limit (80μg STX eq./100g) with the MBA below AL (Table 44), although all samples are close to AL (Appendix 6). More importantly, with use of the refined method, there are no samples exhibiting toxicities above the action limit by the MBA which are showing results below the action limit by LC. As such, there is no longer any evidence for the LC method under-estimating PSP toxicity through use of the refined LC-FLD scallops method.

Table 44. Summary of number of test results above and below the action level (AL: 80 μg STX eq./100g) from LC and MBA analysis of scallops (all species; n= 19)

	Number LC results below AL	Number LC results above AL
Number MBA results below AL	2	5
Number MBA results above AL	0	12

5.3 Effect of measurement uncertainty on HPLC results

Expanded uncertainties calculated for each PSP toxin (Table 45) were used to calculate the uncertainty in measurement of estimated toxicity (total saxitoxin equivalence) following LC analysis for each PSP-positive scallop sample. Table 36 summarises the mean and range of uncertainties determined for each species. It is noted that the uncertainties in LC 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 17 and 32%, comparing well with those determined previously for oysters, cockles, clams and razors [14,15]. Whilst these values compare similarly to values of between-lab reproducibility published for the bioassay [27,28], no direct comparison of measurement uncertainty is possible with the absence of any published MU data in the MBA for either species of scallops.

Table 45. Summary of measurement uncertainties on total saxitoxin equivalents quantified following HPLC analysis of PSP-positive scallops (all species combined).

	All scallops
Mean uncertainty in total toxicity	25%
Range of uncertainties	17 – 32%

5.4 Summary of phase 3 results

As with previous studies [13-16], the aim of this parallel testing was to demonstrate that the LC-FLD method performs satisfactorily as an analytical procedure for the quantitative analysis of PSP toxins in king and queen scallops. It should successfully identify contaminated and non-contaminated samples and identify positive samples at half or below the regulatory action limit (0.5 AL). In addition, previous parallel analysis indicated

the potential for under-estimation of PSP toxicity in queen scallops when using the standard AOAC 2005.06 LC-FLD method [16].

For the scallop samples analysed to date with the refined LC-FLD method validated in this study, the qualitative determination of the presence of PSP toxicity in both contaminated and non-contaminated samples was shown to be successful. Quantitative analysis by both LC and MBA methods indicates some visual correlation between the two methods, but with some variations noted between the different scallop species. Specifically, there appears to be an excellent agreement between the results returned by the two methods in both Queen and Atlantic scallops, with the large under-estimation in the LC method observed previously with use of the standard AOAC 2005.06 method, now being corrected with the use of the refined LC method. Comparison of the results obtained in king scallops shows some degree of positive bias in the LC results as compared with the MBA. The size of the bias was found to be nearly identical to that observed during the analysis of cockle samples, and as with this species, the cause was found to relate primarily to the assumptions made with the sole presence of the most toxic toxin for each epimeric pair. Application of measurement uncertainty to the final LC toxicity estimations showed the level of uncertainty varying depending on the toxin profile and scallop species, but with the range of uncertainties comparing similarly to those described for other bivalve species. As such, there is further good evidence for the suitability of the refined scallops method for the quantitation of PSP toxins in both king and queen scallops in the routine official control monitoring programme.

6. Conclusions – application of the method to the routine monitoring of PSP toxins in king scallops and queen scallops as part of the national biotoxin control program.

The AOAC 2005.06 LC-FLD "Lawrence" method was previously subjected to an in-house programme of validation for whole king scallops and whole queen scallops in accordance with EU legislation on the use of Official Control Methods [2], and applied in tandem alongside the bioassay (MBA) EU reference method [16]. Results indicated whilst the performance of the official method was acceptable on the whole for the quantitation of the peroxide-oxidised non-N-hydroxylated toxins, the performance was poor, in terms of toxin recovery and sensitivity, for the quantitation of the N-hydroxylated toxins following periodate oxidation. Consequently, a period of study was begun looking at options for refining the official AOAC 2005.06 to improve the recovery and sensitivity of analysis of periodate oxidation step in the PSP method for scallops. Once refined, validation studies were applied to king scallop and queen scallop matrices to assess the selectivity, linearity, limits of detection and quantitation, recovery, precision, repeatability, reproducibility, ruggedness and fitness for purpose of the method. Low availability of certified dcGTX2,3 and dcNEO standards during the project enabled only a limited number of tests to be undertaken for those toxins. The study goal was to assess the applicability of the refined method to the analysis of PSP toxins in whole king scallops and queen scallops as a potential for use within the UK national biotoxin monitoring program. Results obtained from PSP contaminated scallops using the LC method were compared with those obtained using the current HCl-screen / MBA methodology. Quantitative results from each of the validation tasks are summarised in Tables 46-47 for each available PSP toxin in both scallop species and a summary of these results is provided below.

Initial studies focussed on the potential for improving the methodology by altering a number of the key method parameters. A large number of factors were investigated, including the use of double C18 clean up steps, use of different ion exchange cartridges, the potential effect of pH on fraction oxidation, the effects of periodate composition and pH effects of varying oxidation parameters and temperatures, the use of protein precipitation prior to oxidation and the effects of pH during the oxidation reactions. Results indicated that little effect was gained through altering the majority of these parameters, with the possible exception of the composition of the periodate reagent, where higher proportions of periodic acid appeared to improve the peak area response of the N-hydroxylated toxins in the subsequent analysis. In addition to these investigations, a more thorough study was conducted to examine the potential variability of the scallop matrix effects on the recovery of the GTX1,4 and NEO toxins, with results indicating that in 13 different king scallop samples from a variety of temporal and spatial sources, the suppressed recovery effect was still evident, with a variability of recovery between 15% to 19% for each toxin. The problem therefore did appear to relate to all the king scallop samples sourced from a variety of locations around the UK at different times of the year. Further work was conducted to examine the performance of the official method in other laboratories on the UK scallop samples. A number of king and queen scallop samples were spiked with known concentrations of GTX1,4 and NEO and sent to 3 other laboratories known to provide reliable and accurate PSP quantitation in other proficiency testing schemes [Community Reference Laboratory for Marine Biotoxins, personal communication]. Results obtained from the other laboratories showed that on average, problems with toxin recovery were evident, following the periodate oxidation of GTX1,4 and NEO. As such, further evidence was generated for the problem being method-related rather than just laboratory-specific.

Work continued with a focussed series of refinement investigations, targeting the potential effects of using larger analytical injection volumes, optimised periodate oxidant composition,

the effects of varying the extraction method and of subsequent sample dilution, the use and effects of different matrix modifiers for the periodate oxidation reaction and potential opportunities for further reductions in method dilution factors. Experiments conducted indicated the improved performance of the method when utilising a combination of several parameter changes, specifically the use of 100µL injection volumes, the clean up of larger volumes of extract (1.5 mL), the use of a modified periodate oxidant, comprised of 500% the normal proportions of periodic acid and the use of a king scallop matrix modifier for the oxidation of analytical calibration standards. A period of testing was then subsequently conducted to ensure these refined conditions were likely to be repeatable within the laboratory and reliably provide a greater level of analytical sensitivity and toxin recovery. Results indicated that the proposed refinements resulted in a significant enhancement in both these factors, with evidence for acceptable toxin recovery and with sensitivity improving so that the target LOD of 0.2 AL per toxin could easily be achieved. As a result of this refinement work, the refined method was taken forward for a full method validation for both GTX1,4 and NEO in both king and queen scallops.

Very low levels of some matrix components were observed in the LC chromatograms of cleaned-up king and queen scallop extracts, most of which were low in abundance and did not interfere with the chromatographic elution of PSP toxins. The exception to this was for the early eluting toxin dcGTX2,3, where a small interference peak was found to be present at the same retention times as the primary toxin quantitation peak. However, these peaks were present at very low intensity, so would not interfere with the qualitative detection or quantitation of PSP toxins in either species. As such, the refined method appears to have a good level of selectivity.

Linearity was demonstrated for PSP toxin calibrations over the working range of 0 to 1.5 AL for NEO and 0 to 2.5AL for GTX1,4 in both king scallop and queen scallop matrices. Visual and statistical evidence was obtained, including inspections of calibration curves, residual plots and the use of f-test “lack of fit” analysis.

Sensitivity was determined for both the N-hydroxylated toxins following the periodate screen in terms of the regulatory action limit and found to be much improved in comparison to the official AOAC 2005.06 method for scallops. Detection limits ranged from 0.04 to 0.09 µg STX eq./g (0.04 to 0.11 AL) for both toxins in both species, therefore well within the target concentration of 0.2 AL (0.16 µg STX eq./g). In addition, spiking and analysis was conducted to determine the LODs of the refined periodate screening method for the non-N-hydroxylated PSP toxins, which were also previously found to be poor. Data generated showed an improved performance of the refined method, with LODs all <0.1 µg STX eq./g for all toxins in both species. The results therefore show that use of the refined periodate screen would enable the safe qualitative screen of scallop samples without the need to resort to the additional use of peroxide oxidation.

Method LODs for the full quantitation of GTX1,4 and NEO following ion exchange fractionation were found to range from 0.05 to 0.10 µg STX eq./g in both species, illustrating an acceptable sensitivity for the quantitation method of N-hydroxylated toxins following the refined periodate oxidation and the ability of the method to detect these toxins at levels less than 0.2 AL. Limits of quantitation were experimentally confirmed at <0.32 µg STX eq./g (0.4 AL) for both of the N-hydroxylated toxins, with LOQs ranging from 0.167 to 0.318 µg STX eq./g. Values determined fall within the guidelines defined within the current UKNRL SOP for performance characteristics of the PSP LC-FLD method [28].

Method recoveries determined at concentrations equivalent to 0.2AL and 0.4 AL per toxin in spiked tissue homogenates were found to vary between 82% and 114%, with the highest values observed for the recovery of GTX1,4 in king scallops. With the use of the refined method, the recovery of GTX1,4 and NEO in both king scallops and queen scallops is now acceptable and within the limits required by the UKNRL [28]. With the regular occurrence of GTX1,4 and NEO in the strains of Alexandrium commonly found in UK waters and the subsequent likelihood of PSP-positive scallops containing these toxins, this provides further evidence that the refined method is safe to implement as a routine methodology for the determination of PSP toxicity in UK scallops.

Results from instrumental precision tests showed low variability between toxin peak retention times (RSD = 1.7% to 2.0% in both matrices; n=11), with the precision of toxin peak area responses also acceptable for each toxin (RSD = 2.0% to 2.9% in both matrices; n=11). Short-term method precision studies involved the triplicate extraction, clean up, fractionation, oxidation and analysis of spiked scallop homogenates at 0.2 and 0.4 AL per toxin. Percentage RSDs calculated were less than 8% for each N-hydroxylated toxin at both concentration levels, giving good evidence for the improved performance of the refined method, presumably in part due to the larger analytical peaks measured and the lower effects of background noise on the repeatable integration of toxin oxidation product peak areas.

Replicate (n=6) spiking, extraction, cleanup, fractionation, oxidation and analysis (time period > 2 weeks) was used to assess the medium term repeatability of king and queen scallop tissues spiked at 0.2 and 0.4 AL. All RSDs for both PSP toxins were ≤15% at both concentrations in both species, with values (ranging from 5% to 15%) which are generally improved compared to those determined previously in other species. HorRat values were <1.0 for all toxins at both concentration levels, further evidencing the degree of acceptability associated with the precision of the method.

An assessment of within-laboratory reproducibility (long-term precision) was undertaken with the repeat analysis (> 2months) of king scallops contaminated with PSP toxins through laboratory feeding experiments. No contaminated queen scallops were available for testing, so additional data was obtained from the repeat analysis of spiked queen scallop homogenates. Reproducibility of the refined method was shown to be good for all toxins studied (HorRat < 1.5), even those present at concentrations significantly lower than 0.2 AL. Values were found to be highest for the quantitation of N-hydroxylated toxins GTX1,4 and NEO, as expected given the additional ion-exchange fractionation step required prior to quantitative analysis. All precision and repeatability data was found to be within the limits described by the UKNRL SOP [28} for the quantitation of PSTs.

Ruggedness experiments were conducted to examine the stability of the refined method following deliberate modification of method parameters. Parameters were chosen which were thought to best reflect the most important variables within the refined periodate oxidation step. Results generated showed that the method was robust for all parameters investigated. In particular, it was noted that there were no apparent adverse effects with method instability resulting from small changes to the composition of the new periodate reagent utilised in the refined method.

Work was also undertaken to complete the validation of the AOAC 2005.06 method for the quantitation of non-N-hydroxylated toxins in queen scallops, following the peroxide oxidation of C18-cleaned extracts. The analysis of homogenates spiked at concentrations equivalent to 0.2 and 0.4 AL per toxin (GTX5 at 1/10 of the concentration) was conducted and the recovery

of the method shown to be acceptable. The data generated was compared with the recovery data generated during the original validation project and used to calculated long-term precision data for the non-N-hydroxylated toxins. These were all shown to range from 5% to 9% at 0.4 AL and from 22% to 29% at 0.2 AL. The precision is therefore acceptable at the higher concentration, and even at the lower concentration results in HorRat values mostly <1.6, comparing well to data generated previously in other shellfish species.

Results obtained during this study were used to calculate standardised and expanded uncertainties for the analysis of PSP toxins in king and queen scallops. As with other species previously, uncertainty contributions were assessed ensuring all factors were incorporated whilst taking care to eliminate duplication. Results showed combined standardised uncertainties of measurement ranging from 0.15 to 0.28 for king scallops and 0.22 to 0.26 for queen scallops with expanded uncertainties (k=2) subsequently ranging from 0.31 to 0.55 and from 0.43 to 0.52 respectively. These values therefore appear similar or improved compared to those reported previously for other species [13-16] and as currently used in reporting results from the official control monitoring programme.

Following the single laboratory validation, the refined LC method was tested for scallop samples in comparison with the official control MBA. Both PSP-positive and PSP-negative samples were chosen for analysis. Due to the absence of naturally contaminated king scallops, the sample set was supplemented with two additional scallop samples from Canada (Atlantic scallops) and with UK king scallops fed toxic *Alexandrium* in the laboratory. In addition, two PSP-positive queen scallops were obtained from the official control monitoring program which had been analysed previously [26] and subsequently held in storage at -20^{o}C. The scallop samples were analysed in parallel using both the AOAC 2005.06 method and the current MBA method. Acetic acid extracts of samples were analysed using both the periodate screen and the full LC quantitation method. For samples shown to be PSP negative by HCl-screen or MBA (13 king scallops and 12 queen scallops), the correlation between the two approaches was excellent with no false LC positives recorded using the acetic acid screening method. Full quantitation of all 25 samples, showed all samples with toxicities significantly lower than the MBA detection limit, with a maximum toxicity of 1.7 μg STX eq./100g.

Nineteen PSP-positive scallops were analysed using both LC and MBA methods, including fifteen whole king scallops, 2 queen scallops and 2 Atlantic scallops. Previous work, using the normal AOAC 2005.06 LC method had shown a good agreement in the results reported compared with the MBA for the Atlantic scallops, but with data showing a 50% under-estimation in PSP toxicity using the LC method in queen scallops. Results here show that the agreement between the refined LC method and the MBA is excellent, with a mean LC/MBA ratio of 97%, albeit for just the two samples. The LC/MBA ratio for the two Atlantic scallops is also still good (104%). As such, use of the refined clean up and oxidation steps in the new LC method has resulted in an improved agreement between the two method for the samples previously showing a poor correlation. Results for the whole king scallops show a good level of agreement but with an element of positive bias in the LC results (mean LC/MBA ratio = 141%, RSD = 14%, r = 0.85). The size of this bias was found to be almost identical to that observed previously for cockles [14], and as with this species, was found to be attributable to the assumptions made regarding the sole use of the highest toxicity equivalence factor for each epimeric pair. Application of measurement uncertainty to the final HPLC toxicity estimations showed the level of uncertainty varying depending on the toxin profile and scallop species, but with the range of uncertainties comparing similarly to those described for other bivalve species. Overall therefore, the comparison between the LC and MBA method for the

scallop samples analysed to date shows a good agreement between results and there is no evidence for any high levels of bias in either method which may be of concern.

Table 46. Summary of validation data for LC-FLD analysis of king scallops following AOAC 2005.06.

Toxin	Linearity (r^2)	LOD µg/g STX equiv	LOQ µg/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged -ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL			
GTX 1,4	0.999	0.05	0.167	104%	114%	8%	2%	15%	9%	Yes	24%	0.28
NEO	0.996	0.10	0.318	89%	82%	5%	2%	10%	15%	Yes	15%	0.20
dcSTX	0.995	0.007	0.025	61%	63%	5%	3%	7%	8%	Yes	na	0.18
GTX 2,3	0.997	0.09	0.3	67%	67%	8%	4%	13%	14%	Yes	15%	0.21
GTX 5	0.997	0.002	0.008	69%	69%	5%	2%	5%	4%	Yes	20%	0.21
STX	0.997	0.018	0.061	91%	93%	6%	3%	11%	8%	Yes	11%	0.15
dcGTX 2,3	0.990	0.055	0.18	56%	59%	5%	1%	na	na	na	na	na
C 1,2	0.988	0.019	0.063	66%	72%	3%	3%	20%	21%	Yes	13%	0.25
Mean	0.995	0.04	0.14	75%	77%	6%	3%	12%	11%	na	Total = 14%	0.21

na = not analysed. Total = long term precision of total toxicity (%RSD)

Table 47. Summary of validation data for HPLC-FLD analysis of queen scallops following AOAC 2005.06.

	Linearity (r^2)	LOD µg/g STX equiv	LOQ µg/g STX equiv	Recovery %		Short term precision RSD%		Medium term precision RSD%		Rugged -ness Stability	Long term Precision	Standardised Uncertainty
				0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL	0.2 AL	0.4AL/ 0.5AL			
GTX 1,4	0.999	0.07	0.218	77%	80%	6%	10%	5%	9%	Yes	20%	0.23
NEO	0.982	0.10	0.314	86%	83%	8%	5%	7%	6%	Yes	21%	0.23
dcSTX	0.997	0.004	0.013	72%	67%	2%	5%	22%	6%	Yes	16%	0.24
GTX 2,3	0.991	0.027	0.09	78%	74%	2%	5%	29%	9%	Yes	21%	0.26
GTX 5	0.999	0.002	0.008	77%	72%	4%	7%	22%	9%	Yes	17%	0.26
STX	0.999	0.013	0.043	74%	67%	3%	6%	25%	8%	Yes	18%	0.22
dcGTX 2,3	0.989	na	na	na	na	na	na	na	na	na	na	na
C 1,2	0.987	0.005	0.016	77%	73%	1%	8%	25%	9%	Yes	19%	0.23
Mean	0.993	0.03	0.10	77%	74%	4%	7%	19%	8%	na	19%	0.24

na = not analysed.

7. Final recommendations

The results presented in this study show that the performance characteristics of the refined LC method for the determination of N-hydroxylated toxins in king and queen scallops are acceptable. The poor performance characteristics reported previously for the qualitative and quantitation determination of GTX1,4 and NEO in both scallops species has been improved greatly using the refined conditions. As a result both the sensitivity and the recovery of the refined method have been shown to be acceptable and both the precision and ruggedness of the method are also within acceptable limits. The comparison of the results obtained from both LC and MBA methodologies has shown there is no large difference between the toxicity results generated when scallop samples were analysed for PSP toxicity, and with a noticeable improvement in the agreement between the LC and MBA results for the determination of toxicity in the two naturally contaminated queen scallop samples analysed. With both the peroxide and refined periodate oxidation parts of the method now shown to perform to an acceptable level for the analysis of PSP toxins in both king and queen scallops, from the data generated to date it is recommended to implement of the refined LC method for the routine monitoring of both scallop species for PSP toxins in the official control monitoring programme.

It is noted that the refinements made to the AOAC 2005.06 LC-FLD method for the determination of PSTs in scallops represent a level of deviation from the official method. However, without these refinements, the work has shown that the official method would not be suitable for use as a routine monitoring tool in either of the scallop species. The refinements made involve small changes to the composition of the periodate reagent, the amount of extract cleaned up by C18 SPE and the use of a king scallop matrix modifier for the oxidation of the calibration standards. As such, it is believed that the changes made do not deviate greatly from the official method, representing a refinement rather than a major change to the method. It is also important to note that the refinements currently apply only to whole king and whole queen scallops. Further checks of the method will be required before the method may be used for the determination of PSP toxins in pre-shucked products.

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Appendix 1. Protocol provided to participating laboratories for the inter-laboratory analysis of spiked scallop samples

Scallop samples provided for testing by external laboratories – protocol

Many thanks indeed for kindly agreeing to help out with the analysis of some UK scallop samples spiked with a range of PSP toxins. The reason for this testing is to assess whether the problems we are finding with toxin recovery and method sensitivity for the AOAC 2005.06 method in scallops is also found when analysed in other laboratories. Previous work conducted at Cefas has shown the analytical sensitivity following peroxide oxidation to be generally acceptable, but problems appear to relate to the analysis of toxins following periodate oxidation. This particularly affects the quantitation of the N-hydroxylated toxins which can only be analysed following periodate oxidation, but also affects the sensitivity of all toxins following the screen if only the periodate oxidation is used. These problems do not appear to relate to pH issues before, during or after oxidation and exhibit themselves through evidence of low recovery, high limits of detection and matrix-related suppression.

Please note – this is not a full interlaboratory study – merely a few additional tests being run in a small number of laboratories. The results will not be used for any full statistical assessment of performance – merely as an indication as to method performance outside of our laboratory. I will of course feedback the results to you once all are received. Please let me know if you are happy for your lab names to be included in this, or if you would rather this is done anonymously.

I am therefore providing you with **5 samples** to extract, clean up and analyse. The first four contain just the two major N-hydroxylated toxins (GTX1,4 and NEO), whilst the 5th sample contains the major non-N-hydroxylated toxins. Once you have provided the data on both quantified PSP toxin concentrations and the observed signal to noise ratios on each of the quantified peaks, it will help me determine whether the recoveries you experience are similar to ours, or if we are experiencing some within-lab problems with the analysis of PSP toxins in these species.

Analytical protocol

Samples provided
The package will contain the following **five** samples which are spiked with the following toxins:

> **King scallops: KSc1**, **KSc2** – 5g homogenates spiked with **GTX1,4** and **NEO** toxins only
> **Queen scallops: QSc1**, **QSc2** – 5g homogenates spiked with **GTX1,4** and **NEO** toxins only
> **KSc3** – 5g homogenate spiked with **STX**, **dcSTX**, **GTX2,3**, **GTX5** and **C1,2** only

Sample extraction and clean up

> Please extract each of the **5 samples** in the same batch using the standard AOAC 2005.06 extraction method making the total volume of final extract up to 10.0mL
> Next, clean up each of the five samples in the same batch using C18 SPE clean up as per the official method, diluting cleaned up extracts to 4.0mL
> For samples KSc1,2 and QSc1,2, please also fractionate using your current ion exchange clean up procedure, collecting fractions F2 and F3

For sample KSc3, no fractionation is required

Oxidation and analysis

Samples **KSc1,2 and QSc1,2**: Please oxidise by **periodate** each of the C18 cleaned extract plus fractions F2 and F3 from all 4 samples

For **KSc3**, please conduct **periodate** and **peroxide** oxidation on the C18 cleaned extract

Please quantify against your normal calibration standards, using standards for both N-hydroxylated standards (GTX1,4 and NEO) and non-N-hydroxylated standards (STX, dcSTX, GTX23, GTX5 and C12)

Please use your own in-house Pacific oyster matrix modifier in all solvent-based standards and samples as per the method. No matrix matching is required for the standards.

Results to report

For each analysis please report both the:

Actual quantified toxin concentration (in µg STX di-HCl eq./100g flesh)

The signal to noise ratio of each quantified peak

Please report these values for each of the C18, F2 and F3 analyses conducted on samples KSc1,2 and QSc1,2 and also both the periodate and peroxide-oxidised C18 cleaned KSc3 sample

Please also report what your calibration standards toxin concentrations are (whatever units you use is fine – just let us know what the units are!), and what peak areas/signal to noise ratios you obtain from the analysis of those standards during your sequence

Note, for sample KSc3, just report the toxin concentrations following peroxide oxidation (i.e. quantify against your normal peroxide-oxidised standards). For the periodate-oxidised KSc3 sample, just report toxin peak areas and signal to noise ratios

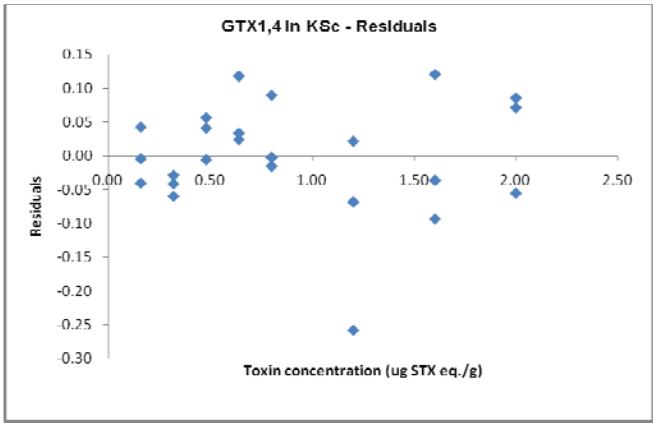
Summary of work required

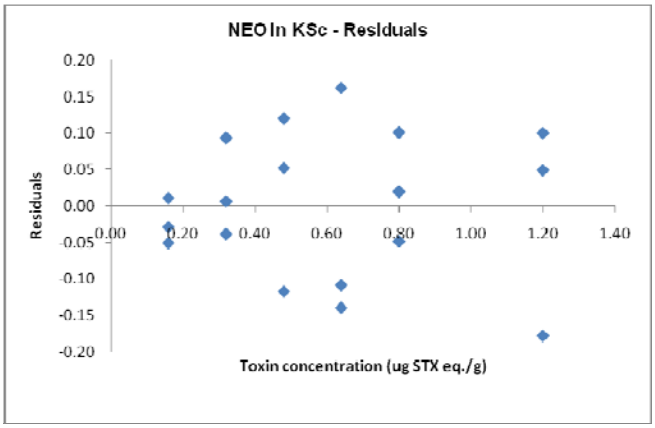
Sample name	Process required	Analysis required	Results to report
KSc 1 KSc 2 QSc 1 QSc 2	AOAC 2005.06: All extracted in acetic acid and C18 SPE clean up + ion exchange fractionation (collecting F2 and F3)	Periodate oxidation of C18-cleaned extract + Periodate oxidation of fractions F2 & F3	1) Quantitative concentrations of GTX1,4 and NEO in C18, F2, F3 2) Signal to noise ratios of quantitative peaks for GTX1,4 and NEO in C18, F2 and F3
KSc 3	Extract and C18 SPE clean up	Periodate and peroxide oxidation of C18-cleaned extract Periodate and peroxide oxidation of C18-cleaned extract	1) Quantitative concentrations of STX, dcSTX, GTX23, GTX5 and C12 in C18 following peroxide 2) Signal to noise ratios of quantitative peaks for above toxins in both periodate and peroxide-oxidised extracts

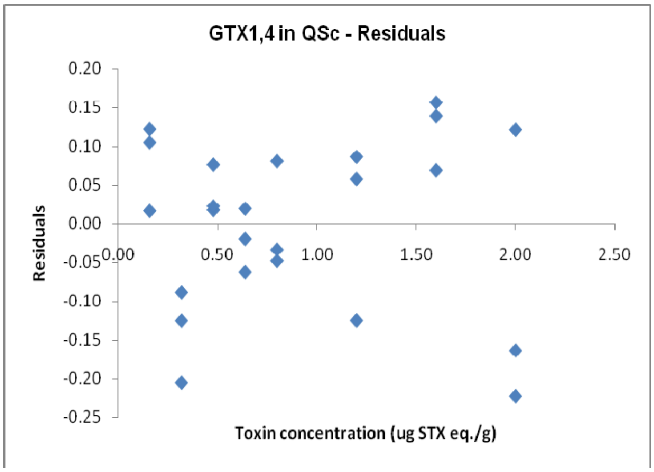
Results reporting template
Please fill in the attached excel worksheet with your results – reporting each toxin concentration in µg STX di-HCl eq/100g flesh and give the signal to noise ratio of each peak. There is also space to fill in toxin standard concentrations, peak areas and signal to noise ratios. Please could you also indicate the volumes of each extract, C18 and fraction you collect (space provided) and how your standards are prepared.

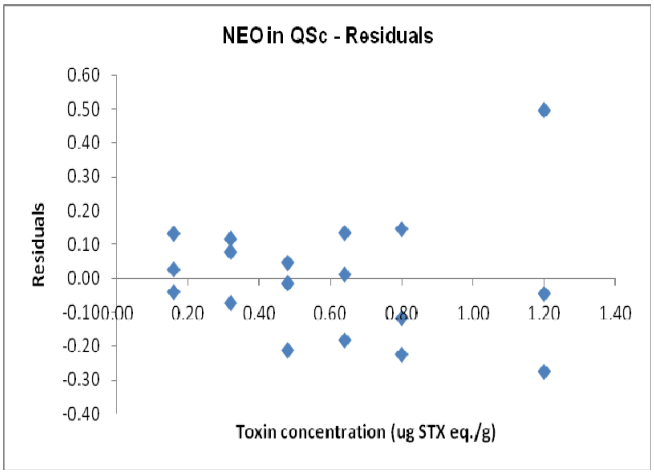
Andy Turner
Cefas
Jan 2011

Appendix 2. Residual plots for GTX1,4 and NEO in king scallop and queen scallop fractions over the respective calibration ranges.









Appendix 3. Results obtained from the inter-laboratory analysis of king scallop and queen scallop samples spiked with known concentrations of GTX1,4 and NEO toxins.

Sample	**KSc1**				**KSc2**				**QSc1**				**QSc2**			
Toxin spike & concentration	**0.32 ug STX eq/g (GTX1,4 & NEO)**				**0.32 (NEO) & 0.48 ug STX eq/g (GTX1,4)**				**0.32 ug STX eq/g (GTX1,4 & NEO)**				**0.32 (NEO) & 0.48 ug STX eq/g (GTX1,4)**			
Lab no.	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**
GTX1,4 (F2)	0.28	0.33	0.18	0.13	0.38	0.45	0.27	0.14	0.12	0.32	0.20	0.05	0.15	0.44	0.27	0.11
NEO (F3)	0.07	0.18	0.12	0.30	0.07	0.15	0.11	0.30	0.09	0.09	0.08	0.21	0.06	0.09	0.06	0.10
GTX1,4 (C18)	0.19	0.17	0.21	0.13	0.16	0.16	0.16	0.14	0.14	0.10	0.22	0.07	0.11	0.10	0.20	0.06
NEO (C18)	0.14	0.13	0.10	0.23	0.14	0.12	0.11	0.30	0.08	0.08	0.08	0.14	0.07	0.08	0.07	0.08

Appendix 4. Results obtained from ruggedness experiments on king and queen scallops. Results shown in terms of a) concentrations, b) parameter differences and c) parameter difference percentages.

King scallops:

a) Concentration data (μg STX eq./100g) and RSDs (%) from ruggedness experiments and precision tests (n=8)

	Ruggedness experiments (n=8)										Precision (n=8)	
Toxin	1	2	3	4	5	6	7	8	Mean	RSD	Mean	RSD
GTX 1,4	56.2	52.5	53.1	51.6	53.2	51.3	51.9	53.6	52.93	3%	53.3	3%
NEO	14.9	15.2	15.1	13.8	15.0	15.4	15.1	15.9	15.06	4%	14.6	4%

b) Parameter differences

Toxin	A	B	C	D	E	F	G
GTX 1,4	0.8215	0.7858	1.3514	1.2604	1.2336	1.4441	-0.3327
NEO	-0.6010	0.1305	-0.0469	0.4385	0.5770	-0.2896	-0.4900

c) Parameter difference percentages

Toxin	A	B	C	D	E	F	G
GTX 1,4	2%	1%	3%	2%	2%	3%	-1%
NEO	-4%	1%	-0.3%	3%	4%	-2%	-3%

Queen scallops:

a) Concentration data (μg STX eq./100g) and RSDs (%) from ruggedness experiments and precision tests (n=8)

	Ruggedness experiments (n=8)										Precision (n=8)	
Toxin	1	2	3	4	5	6	7	8	Mean	RSD	Mean	RSD
GTX 1,4	81.6	79.3	76.9	75.4	75.2	73.9	69.9	75.2	75.93	3.50	78.7	4.7%
NEO	26.3	19.8	23.5	19.6	23.0	21.3	23.1	21.2	22.23	2.20	23.0	2.5%

b) Parameter differences

Toxin	A	B	C	D	E	F	G
GTX 1,4	4.7086	3.1519	-0.0383	1.1612	1.9524	1.8758	-1.4419
NEO	0.1468	0.7340	3.4863	0.7523	1.6698	0.6055	0.6973

c) Parameter difference percentages

Toxin	A	B	C	D	E	F	G
GTX 1,4	1.42	0.95	-0.01	0.35	0.59	0.56	-0.43
NEO	0.08	0.41	1.95	0.42	0.93	0.34	0.39

Appendix 5. Results obtained from quantitative (LC-FLD) analysis of PSP toxins in acetic acid extracts of whole king and queen scallop samples. Samples analysed were those found negative by either MBA (QSc) or HCl screen LC-FLD (KSc).

	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc
	BTX/10/414	BTX/2009/Sc1	BTX/10/238	BTX/10/239	BTX/10/520	BTX/10/521	BTX/10/522	BTX/10/523	BTX/10/1045	BTX/10/1046	BTX/10/1047	BTX/10/1048	BTX/2007/Sc2
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
dcNEO	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
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
C 1/2	0.00	0.00	0.00	0.00	0.00	1.14	1.53	1.35	0.94	1.38	0.84	1.83	1.53
dcSTX	1.78	1.78	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
GTX 5	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
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
Total	1.78	1.78	0.00	0.00	0.00	1.14	1.53	1.35	0.94	1.38	0.84	1.83	1.53

	QSc	QSc	QSc	QSc	QSc	QSc	QSc	QSc	QSc	QSc	QSc	QSc
	BTX/10/1801	BTX/10/2192	BTX/10/2282	BTX/10/2346	BTX/10/2463	BTX/10/2563	BTX/10/2826	BTX/10/2908	BTX/10/2980	BTX/10/2981	BTX/10/2982	BTX/10/2983
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
dcNEO	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
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
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
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
GTX 2/3	0.00	0.00	0.00	0.00	0.00	1.30	1.01	1.71	0.41	0.00	0.00	0.00
GTX 5	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	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	0.00	0.00	0.00	0.00	0.00	1.30	1.01	1.71	0.41	0.00	0.00	0.00

Appendix 6. Comparison of results obtained from quantitative (LC-FLD) analysis of PSP toxins in acetic acid extracts of scallop samples (μg STX eq./g; Oshima TEFs) with quantitative (positive MBA) analysis of HCl extracts.

	QSc*	QSc*	ASc**	ASc**	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc	KSc
Toxin	BTX/1469	BTX/1425	080507-1	080507-2	116RM b	117RM c	118 RM d	11 A	11 B	11 C	11 D	11 E	11 F	11 G	11 H	11 I	11 J	11 K	11 L
GTX 1,4	5.3	10.5	25	22	32	28	29	68	72	98	94	78	73	85	41	51	105	110	86
dcNEO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NEO	9.2	8.6	25	12	25	20	20	43	42	59	34	33	49	56	23	18	34	25	24
dcGTX 2,3	1.63	0.91	0.73	0.00	0.85	0.91	0.84	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	4.0	3.4	0.8	0.4	7.5	6.5	5.3	10.1	9.9	14.2	8.9	9.1	11.6	13.3	5.6	7.5	14.6	9.8	9.4
dcSTX	0.0	0.0	0.0	0.3	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GTX 2,3	9.0	8.6	43	21	16	13	11	22	22	31	19	20	26	29	12	17	33	21	21
GTX 5	0.16	0.18	0.30	0.15	0.15	0.14	0.13	0.15	0.14	0.15	0.14	0.13	0.15	0.15	0.05	0.11	0.18	0.17	0.09
STX	11	14	63	29	27	21	17	22	23	31	21	21	26	30	13	18	33	27	25
Total	41	46	157	85	109	89	84	166	169	233	176	162	185	214	94	111	219	193	166
MBA	44	46	160	77	**79**	**58**	**64**	164	137	134	151	127	139	133	58	80	142	118	120
HPLC/MBA ratio	92%	101%	98%	110%	137%	153%	131%	101%	123%	174%	117%	127%	133 %	161%	163%	139%	154%	164%	138%

QSc = queen scallops, KSc = king scallops, ASc = Atlantic scallops
KSc Samples from in-house contamination of king scallops with *Alexandrium*
* QSc Samples sourced from GB biotoxin monitoring programme (BTX code numbers)
** ASc Samples sourced from Canadian waters (supplied by CFIA)

Appendix 7: Relative toxicity factors for PSP toxin analogues (based on EFSA, 2009)

Toxin	Relative toxicity	Toxins	Relative toxicity used
GTX1	1.0	GTX 1.4	1.0
GTX4	0.7		
dcNEO	1.0	dcNEO	1.0
NEO	1.0	NEO	1.0
dcSTX	1.0	dcSTX	1.0
GTX 2	0.4	GTX 2,3	0.6
GTX 3	0.6		
GTX 5	0.1	GTX 5	0.1
STX	1.0	STX	1.0
dcGTX 2	0.2	dcGTX 2,3	0.4
dcGTX 3	0.4		
C 1	-	C 1,2	0.1
C 2	0.1		

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