Cefas contract report C2934 variation 4

Method performance verification for the analysis of minor clam species for paralytic shellfish poisoning toxins by liquid chromatography and fluorescence detection (official method AOAC 2005.06)

FSA Contract Reference: ZB1807 – FS235002

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Executive Summary

A Liquid Chromatography with Fluorescence Detection (LC-FLD) method (AOAC 2005.06) 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 extraction of shellfish flesh with a dilute acetic acid, prior to clean up, oxidation and analysis to quantify PSP toxin (PST) concentrations The AOAC 2005.06 method was previously subjected to an in-house single laboratory validation at Cefas for mussels, cockles, Pacific oysters, native oysters, razor clams and hard clams. More recently, the method has been refined and validated for the analysis of whole king scallops and whole queen scallops. These species represent approximately 95% of the bivalve shellfish samples received at Cefas for routine monitoring of marine biotoxins. Once the LC-FLD method has been implemented for these 8 species, the numbers of bioassays potentially required for the routine monitoring of the remaining species will be small, but still significant. As such, this work describes the additional work carried out at Cefas between January and March 2011, involving the method performance checks carried out for four of the minor bivalve species applicable to the UK monitoring programme. The species investigated were manila clams (*Ruditapes philippinarum*), European otter clams (*Lutraria lutraria*), grooved carpet shell clams (*Ruditapes decussatus*) and surf clams (*Spisula solida*).

The protocol employed for the method performance checks was agreed between the FSA and Cefas prior to the work commencing. The most important aspects of the method, including the assessment of toxin recovery, method sensitivity and precision were assessed in each species for the specific PSP toxins deemed to be both toxic and prevalent in UK samples and also currently available commercially as certified reference standards. Specifically, the protocol was applied to 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)). Additionally, the work was extended to the non N-hydroxylated decarbamoylgonyautoxin-2 and 3 (dcGTX2,3) toxins in surf clams, toxins which were not covered by the official AOAC method, but were previously incorporated into the validation at Cefas for the major bivalve species.

Method verification experiments were conducted to establish the performance characteristics relating to the selectivity/specificity, the method sensitivity, in terms of limits of detection (LOD) and quantitation (LOQ), toxin recovery and method precision. Performance limits were agreed with the FSA before the work commenced. Initial work conducted to investigate the levels of toxin recoveries observed in each of the four species revealed a high degree of toxin conversion to occur within the surf clam matrix. Specifically, the carbamate toxins (STX, GTX1-4, NEO) and the N-sulfocarbamoyl toxins (GTX5, C1,2) were found to convert into their structurally-related decarbamoyl counterparts (dcSTX, dcGTX2,3 and dcNEO). Consequently, method performance checks in surf clams were only conducted using toxins dcSTX and dcGTX2,3, the only two PSP toxins typically found in the limited number of PSP-positive UK surf clam samples received to date.

The performance of the quantitative LC method for the four species of clams was satisfactory in terms of the method selectivity of the analysis. Chromatographic evidence from the analysis of PSP-negative clam samples did not reveal the presence of any matrix co-

extractive components which might potentially interfere with the qualitative or quantitative analysis of the toxins.

Instrumental sensitivity was determined for each of the toxins following the periodate screen in terms of the regulatory action limit, specifically determining whether the screening method was capable of detecting toxins present in each of the clam matrices at concentrations equivalent to the target concentration of 0.16 µg STX eq./g (0.2 AL) per toxin. Predicted limits of detection (LODs) ranged from ~0.02 to 0.15 µg STX eq./g (0.03 to 0.19 AL) for the four species under investigation, thus indicating the suitability of the screening method. It would therefore be suitable to use the periodate oxidation of the C18-cleaned extracts of all clam species as a qualitative screening step prior to the full quantitation of any positive samples.

Method LODs for the full quantitation method were calculated as ranging from 0.007 to 0.1 µg STX eq./g per toxin, following implementation of a larger injection volume (100µL) for the analysis of periodate oxidised fractionated extracts for the N-hydroxylated toxins. Consequently, the results indicated the successful verification of the method in terms of its ability to detect toxins at concentrations ≤0.16 µg STX eq./g (0.2 AL). LOQs were found to range from 0.033 to 0.30 µg STX eq./g for all toxins in all species. As such, the LOQs for all toxins are lower than the target concentration level of 0.32 µg STX eq./g (0.4 AL). Consequently, the results indicate the acceptable performance characteristics of the method in terms of its ability to quantify toxins at concentrations ≤0.32 µg STX eq./g in each of the four clam species.

Recoveries calculated for each toxin present in homogenate tissues at 0.2 AL and 0.4 AL showed values falling in the range of 68% to 106% for carpet shell clams, 61% to 91% for manila clams, 64% to 87% for otter clams and 63% to 83% for surf clams. Method recovery was therefore shown to be acceptable, being within the target range of recoveries specified by the FSA, specifically 70%-110% for GTX1,4, GTX2,3 and STX and 60%-120% for all other toxins. These values are also similar to results reported previously in other species and as reported in the official AOAC 2005.06 method.

Analysis of the short-term (within-batch) precision of the method showed RSD% values ≤12% at both 0.2 AL and 0.4 AL for all non N-hydroxylated toxins and ≤15% for the N-hydroxylated toxins subjected to the additional ion exchange clean up steps. The results therefore show that at individual PSP toxin concentrations equivalent to 0.2 and 0.4 AL, the short term repeatability for the LC-FLD analysis of each toxin is within the specified limits for short term repeatability of 15%. Medium-term precision (inter-batch) for the four clam species was also found to be acceptable with RSD percentages all ≤25% for each toxin in each species. Results therefore showed similarities to values generated previously for other species previously and that at each concentration the medium term repeatability is within the specified limits of 25% for each species. Further evidence for an acceptable level of precision was provided by the HorRat values, which are <2.0 for all toxins at both concentration levels, with only dcSTX in surf clams >1.3.

Results obtained from the method performance checks were used to calculate standardised and expanded uncertainties for the LC-FLD method in each of the four clam species. Uncertainty contributions were assessed and included contributions from the uncertainty inherent in the precision, reproducibility and recovery of the method. The combined standardised uncertainties for individual toxins were found to range from 0.08 to 0.26 for all toxins in the carpet, manila and otter clams. In surf clams higher levels of measurement uncertainty were calculated, ranging from 0.33 to 0.34 for the two decarbamoyl toxins, dcSTX

and dcGTX2,3. Expanded uncertainties, calculated using a coverage factor (k) of 2, resulted in a range of values from 0.16 to 0.53 for carpet, manila and otter clams, with expanded uncertainties in surf clams of 0.67 and 0.69 for dcSTX and dcGTX2,3 respectively. Results therefore show a similar range of values for the suite of toxins studied in comparison with the values reported previously for the major bivalve species.

Overall, the work conducted in this study has shown the applicability of the LC-FLD method for the qualitative and quantitative determination of PSP toxins in each of the four clam species. Results fall within the specified performance limits and the overall size of the measurement uncertainty is similar to the values determined for other bivalve species. Consequently, the recommendation is to implement the LC-FLD method into the GB routine monitoring programme for the analysis of PSTs in the four clam species.

Summary of method performance checks for the LC-FLD analysis of carpet shell clams.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
GTX 1,4	y	0.074	0.16	0.188	0.32	98%	106%	70-110	11%	8%	15%	14%	10%	25%	10%	None	0.20
NEO	y	0.082		0.241		72%	68%	60-120	4%	1%		15%	22%		19%		0.26
C 1,2	y	0.011		0.045		90%	80%	60-120	5%	3%		15%	3%		6%		0.16
dcSTX	y	0.009		0.033		91%	83%	60-120	4%	2%		21%	6%		7%		0.21
GTX 2,3	y	0.038		0.156		91%	78%	70-110	5%	2%		23%	3%		6%		0.23
GTX 5	y	0.032		0.125		96%	88%	60-120	4%	2%		7%	4%		4%		0.09
STX	y	0.014		0.055		91%	83%	70-110	5%	2%		12%	4%		8%		0.14
dcGTX 2,3	y	na		na		na	81%	None	na	na		na	na		na		na
Mean	-	0.04		0.12		90%	84%		5%	3%		16%	7%		8%		0.18

na = not analysed.

Summary of method performance checks for the LC-FLD analysis of manila clams.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
GTX 1,4	y	0.076	0.16	0.239	0.32	89%	87%	70-110	8%	6%	15%	16%	14%	25%	15%	None	0.22
NEO	y	0.098		0.259		64%	61%	60-120	7%	7%		12%	7%		7%		0.17
C 1,2	y	0.010		0.046		82%	73%	60-120	6%	2%		13%	5%		5%		0.14
dcSTX	y	0.007		0.035		85%	76%	60-120	4%	9%		16%	6%		6%		0.17
GTX 2,3	y	0.032		0.156		89%	74%	70-110	4%	12%		17%	5%		6%		0.18
GTX 5	y	0.025		0.124		91%	85%	60-120	2%	6%		5%	3%		8%		0.08
STX	y	0.011		0.054		86%	79%	70-110	3%	7%		8%	4%		4%		0.09
dcGTX 2,3	y	na		na		na	68%	None	na	na		na	na		na		na
Mean	-	0.04		0.13		84%	77%		5%	7%		13%	6%		7%		0.09

na = not analysed.

5.

Summary of method performance checks for the LC-FLD analysis of otter clams.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
GTX 1,4	y	0.075	0.16	0.294	0.32	89%	84%	70-110	5%	1%	15%	13%	3%	25%	10%	None	0.15
NEO	y	0.084		0.227		70%	64%	60-120	15%	9%		15%	13%		12%		0.23
C 1,2	y	0.010		0.050		89%	75%	60-120	5%	11%		13%	9%		9%		0.18
dcSTX	y	0.008		0.037		93%	81%	60-120	4%	9%		18%	10%		9%		0.21
GTX 2,3	y	0.033		0.169		96%	78%	70-110	4%	12%		19%	10%		10%		0.22
GTX 5	y	0.029		0.136		97%	87%	60-120	2%	6%		6%	5%		7%		0.10
STX	y	0.012		0.059		95%	83%	70-110	3%	7%		10%	8%		9%		0.14
dcGTX 2,3	y	na		na		na	82%	None	na	na		na	na		na		na
Mean	-	0.04		0.14		90%	79%		5%	8%		14%	8%		9%		0.14

na = not analysed.

Summary of method performance checks for the LC-FLD analysis of surf clams.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
dcSTX	y	0.024	0.16	0.097	0.32	71%	63%	60-120	3%	3%	15%	22%	25%	25%	na	None	0.33
dcGTX 2,3	y	0.006		0.024		88%	83%	60-120	5%	6%		25%	23%		na		0.34
Mean	-	0.015		0.060		80%	73%		4%	4%		24%	24%		na		0.34

na = not analysed.

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Glossary

AL	Action Limit
AOAC	AOAC International (formerly Association of Official Analytical Chemists)
GTX5 (B-1)	Gonyautoxin 5
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
LRM	Laboratory Reference Material
LOD	Limit of Detection
LOQ	Limit of Quantitation
MBA	Bioassay
na	Not analysed
nd	Not detected
Na_p	Not applicable
NEO	Neosaxitoxin
NG (-ve)	Negative
OC	Official Control
PS (+ve)	Positive
PSP	Paralytic Shellfish Poisoning
PSTs	Paralytic Shellfish Toxins
Rt	Retention time
SPE	Solid Phase Extraction
SOP(s)	Standard Operating Procedure(s)
STX	Saxitoxin
µg STX eq./g	Micrograms of STX equivalence per gram of edible shellfish tissue

1. Introduction

Clams are filter-feeding bivalve shellfish which are found to accumulate marine biotoxins derived from phycoplankton. Clams contaminated with these toxins may impact significantly on health of the subsequent consumer. Perhaps the most severe group known to induce human illness [1] is the paralytic shellfish poisoning (PSP) toxins. These potent neurotoxins, all structurally-related to the parent compound Saxitoxins, may cause severe health effects and even death [2]. Therefore monitoring of clams and other bivalves is a statutory requirement to ensure protection of the consumer. Whilst the current European Union's (EU) reference method for the detection of PSP toxins is still the mouse bioassay (MBA) [3, 4], Cefas and the FSA have been committed for many years to moving away from animal assays when validated [5] alternatives are made available. One method using Liquid Chromatography with Fluorescence Detection (LC-FLD), commonly referred to as the "Lawrence method", has been developed and gone through single and inter-laboratory validation [6-11]. 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) [3]. Cefas have since conducted single-laboratory validation of this method for the analysis of PSP toxins in mussels [13,14], cockles and oysters [15,16], hard clams and razors [16,17] and king and queen scallops [18]. Validation experiments followed where applicable the requirements described by EC regulation 882/2004 that official control methods should be validated prior to adoption into EU monitoring programmes [20]. Methods, where possible, were characterised according to their performance in terms of accuracy, limits of detection and quantitation, precision, repeatability, reproducibility, recovery, selectivity, linearity, measurement of uncertainty and ruggedness. Where suitable materials were available, the validation incorporated a period of parallel testing whereby LC results were compared with those obtained from MBA analysis of shellfish obtained from the routine GB biotoxin monitoring programmes and/or shellfish contaminated in the laboratory through feeding experiments. The method has to date been deemed fit for purpose in mussels, cockles, hard clams and razors, and is implemented into the routine monitoring programme for these species [16,17]. 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. 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) [18]. As a result, the method has been further refined to improve the method performance for both scallop species, and has recently undergone method validation [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.

Conducting a full method validation experiment is a time consuming and costly process. Although a full method validation for any additional minor species may be inappropriate, given the differences in method performance observed to date between different bivalve species, some form of method performance checks are essential. As such, an agreement was made with the FSA for the method performance to be verified with a series of performance tests on each of the minor species. These tests were designed so as to cover all the major aspects of the LC-FLD method (toxin recovery, sensitivity and precision), whilst using a low number of the scarce and expensive certified reference standards required for the tests.

The protocol agreed between Cefas and the FSA for the method performance checks is summarised in Appendix 1. The tests were applied to the two most prevalent N-hydroxylated toxins encountered to date in naturally-contaminated UK bivalve samples, namely GTX1,4 and NEO, plus the most toxic and/or most commonly encountered non-N-hydroxylated toxins, STX, GTX2,3, GTX5, C1,2 and dcSTX. The work followed the guidelines described by the Analytical Laboratory Accreditation Criteria Committee (ALACC) prepared in tandem with the AOAC International Technical Division for Laboratory Management (TDLM) [21] to obtain information regarding selectivity, limits of detection, limits of quantitation, recovery, precision and repeatability. Results were compared against a list of pre-agreed criteria, which would ultimately enable the verification of method performance for each of the clam species investigated.

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2. Materials and methods

2.1 Overview of the AOAC 2005.06 Method

Clam samples are shucked and homogenised and the 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 Pacific oyster 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 [22], and as such, the AOAC 2005.06 method describes the use of a matrix modifier to be used in all periodate oxidations for both standards and samples, which is believed to result in a more repeatable oxidation (Lawrence, personal communication). This modifier is the C18 SPE cleaned up, acetic acid extract of a Pacific oyster sample, which has been shown to be free from chromatographic peaks at the same retention times as any of the PSP toxin standards. As described in previous reports [13-19], 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

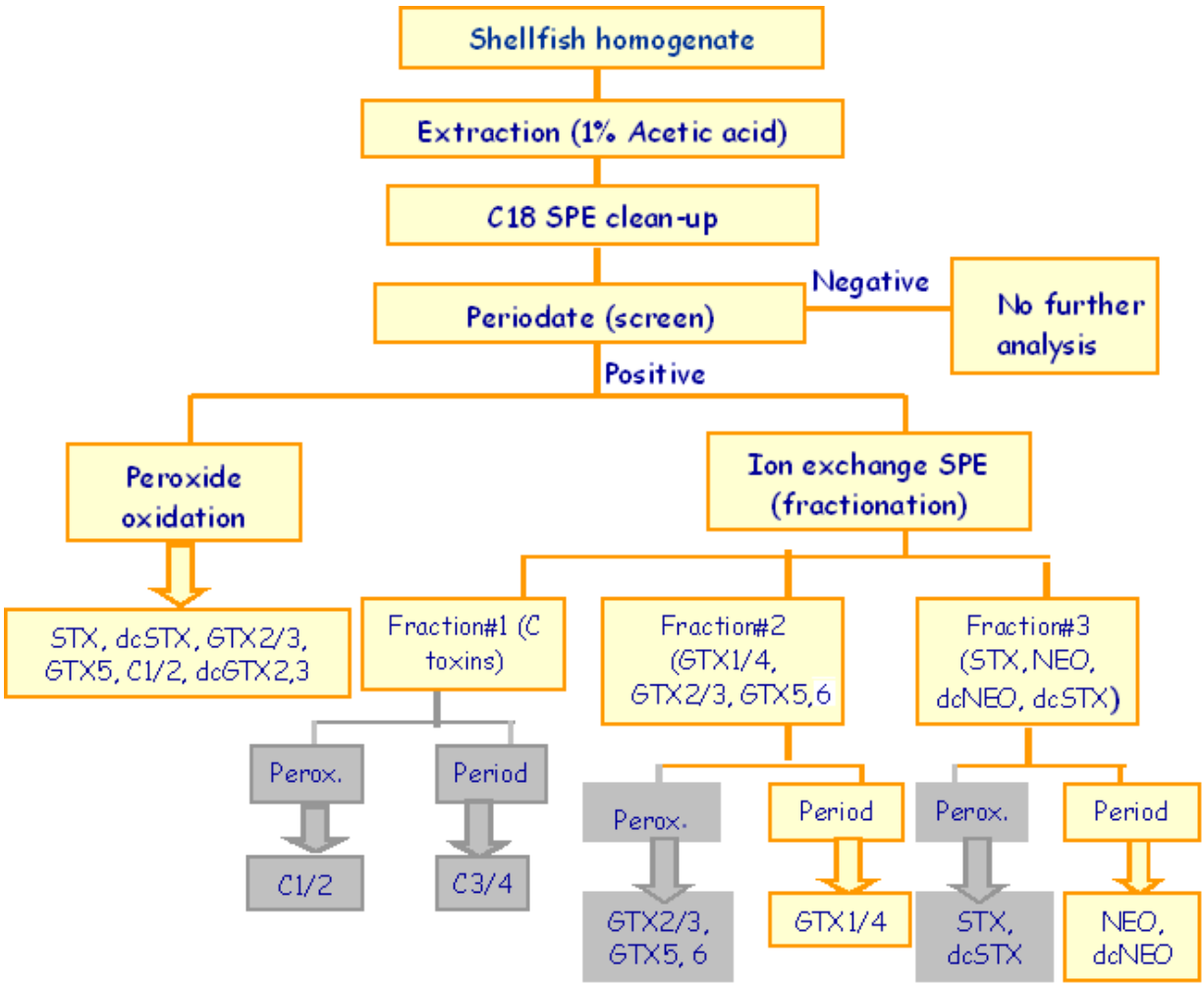


Figure 1. Scheme utilised for screening and quantitation of PSP toxins in clam samples. *Parts of method in grey not carried out.*

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 [23] 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 2). 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 carpet shell clams (*Ruditapes decussatus*) for use in homogenate and extract spiking studies were obtained from the Fish Society of London (http://www.thefishsociety.co.uk/fish-detail_byname_clams_219_0_9.html). Otter clams (*Lutraria lutraria*), manila clams (*Ruditapes philippinarum*) and surf clams (*Spisula solida*) were all obtained through the GB official control biotoxin monitoring programmes. Pacific oysters used for the preparation of the matrix modifier were those sourced previously from M&J Seafood of Poole, Dorset [13]. Approximately 0.5 kg each of clam species were shucked and homogenised, 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 clams with toxins was carried out on homogenate aliquots taken from the same bulk sample.

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

LC-FLD 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 Selection of method performance checks

The method performance criteria chosen were selected following a period of discussion between Cefas and the FSA. Criteria were selected given the need to ensure that the performance of the method for the most important (toxic and prevalent) toxins was acceptable in terms of accuracy, sensitivity and repeatability, but to conduct the tests using the minimum of resources, specifically the numbers of ampoules of certified reference material standards required for fortification studies. The chosen approach follows the guidance described by the AOAC Technical Division for Reference Materials (TDLM) and the Analytical Laboratory Accreditation Criteria Committee (ALACC) in the document, which describes the range of criteria required for the verification of methods to meet ISO 17025 depending on the nature of the test and the concentration of the analytes [21]. The requirements of method verification are described for six specific categories of chemical test methods, as summarised in Table 4.

Table 4. Categories of chemical test methods

Performance characteristic	Performance characteristics included in a validation					
	1	2	3	4	5	6
	Identification	Low concentration: quantitative	Low concentration: Limit test	High concentration: Quantitative	High concentration: Limit test	Qualitative
Accuracy	No	Yes	No	Yes	Yes	No
Precision	No	Yes	No	Yes	Yes	No
Specificity	Yes	Yes	Yes	Yes	Yes	Yes
LOD	No	Yes	Yes	Yes/No	No	No
LOQ	No	Yes	No	Yes/No	No	No
Ruggedness	No	Yes	No	Yes	No	No
Linearity	No	Yes	No	Yes	No	No

Table reproduced from [21]

The guidance describes that the activities required for method verification are a subset of those needed for validation, so the required performance checks will be taken from the list of specific performance characteristics tests generally applied during the validation process. The LC-FLD method for the quantitation of PSP toxins for regulatory testing involves the determination of total PSP toxicity and comparing them against a regulatory limit. Therefore the method is a category five, specifically a method which involves the determination of analyte concentrations in relation to a specified high concentration limit which is substantially above the method LOQ. As such, the guidance recommends the application of method verification checks to include the specific tests detailed in Table 5.

Table 5. Requirements for method performance checks for method involving the quantitation of analytes at high concentration either above or below a specified value (limit test).

Performance characteristic	Check	Activity	Reasons
Accuracy	Yes	If the concentration range of the validated method is narrow (≤1 order of magnitude), one reference material, standard or spike at one concentration. Otherwise demonstrate accuracy at each concentration level	Over a narrow concentration range, the accuracy and precision should not vary, demonstration at one level is therefore sufficient. Over a wider range, the accuracy and precision can vary, thereby resulting in the need to verify performance at different concentration levels
Precision	Yes	Perform the repeatability test once. If the method concentration range extends beyond 1 order ot magnitude, repeatability test must include more than one concentrations	Argument as above for use of more than one concentration level. Intermediate (inter-analyst) is handled by ensuring the analysts are trained and can adequately perform the method
Specificity	No/Yes	Checks dependent on whether the sample analysed are identical to those for which the method was validated. Samples with the “same matrix” do not need to be tested for specificity	For some methods, specificity can be instrument-related as with potential differences between intra-matrix variations (e.g. different sources/species of the same food product)

Following the above guidance, the aspects of method tested were the accuracy and precision through the use of spiked recovery tests. With the validated linear range close to 1, one concentration would be appropriate, but two concentration levels were still chosen for assessment. Due to the previously noted variability in method performance between different shellfish species, the specificity tests were included. In addition to the above, tests for LOD and LOQ were also included. This was incorporated due to the importance of determining method sensitivities and the potential for multiple toxins present at low concentrations to sum to produce toxicity levels close to the high concentration test limit (regulatory action level in this context). Table 6, summarises the actual method performance checks utilised in these studies and the specified target performance limits for each characteristic.

Table 6. Method performance checks employed for verification of the PSP LC-FLD method in minor clam species

Performance characteristic	Concentration levels	Number of repeats	FSA-specified target performance
Specificity	na	1 analysis for each oxidation method for each cleaned up extract	Absence of matrix interferences
Recovery	0.2 and 0.4 AL per toxin	Triplicate homogenates in two batches	70% to 110% for GTX1,4, GTX2,3 and STX, 60% to 120% for other toxins
Short term precision	0.2 and 0.4 AL per toxin	Triplicate homogenates in one batch	Repeatability ≤15%
Precision	0.2 and 0.4 AL per toxin	Six homogenates over period of time > 2 weeks	Repeatability ≤25% over medium term
LOD	0.2 AL per toxin	Six homogenates	Confirm presence of toxin peaks at s/n ratio ≥ 3:1.
LOQ	0.4 AL per toxin	Six homogenates	Confirm presence of toxin peaks at s/n ratio ≥ 10:1.

AL = regulatory action limit (80 µg STX eq/100g). na = not applicable

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

The scheme in 2.1 (Figure 1) details the steps involved in the normal method. Each of the clam species were shucked and homogenised prior to extraction by heating with 1% acetic acid solution. After centrifugation, the supernatants were collected. 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 [13,15].However, due to some inter-batch differences in the ion exchange cartridges used, the volume of 2M NaCl used for eluting fraction F3 has recently been increased to 4.5mL. 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 Assessment of the protocol and applicability to surf clams

Previous published work from Portugal [24] and the results from the LC-FLD analysis of PSP-positive surf clams received through the English official control monitoring programme have indicated the potential for the high presence of decarbamoyl toxins within surf clams. In addition, the Portuguese work described the potential conversion of both carbamate and N-sulfocarbamoyl PSP toxins into their decarbamoyl counterparts. Consequently some preliminary work was performed in addition to the agreed proposed methodology to assess whether there were likely to be any issues with recovery experiments involving the fortification of surf clam samples with the full range of PSP toxins. Specifically, samples were spiked with concentrations of each PSP toxin at a concentration equivalent to 0.5 AL (0.4 µg STX eq./g) per toxin, prior to extraction, clean-up, oxidation and analysis using the normal LC-FLD protocol. Subsequently, a second experiment was performed whereby carbamate and N-sulfocarbamoyl toxins were spiked into surf clam homogenates as follows:

- The N-hydroxylated PSTs (GTX1,4 and NEO) were spiked at 0.32 µg STX di-HCl eq./g per toxin into replicate (n=8) surf clam homogenates
- The non-N-hydroxylated PSTs excluding dcSTX and dcGTX2,3 (GTX2,3, GTX5, STX and C1,2) were spiked into separate replicate (n=8) homogenates of surf clam at the same concentrations.
- Each set of homogenates was spiked with toxins and once completed the time was noted. As quickly as possible, one homogenate of each spiked sample was extracted in 1% acetic acid using the standard AOAC 2005.06 extraction method.
- Each of the spiked homogenates was subsequently extracted at different times, specifically at 1, 2, 3, 4, 6, 24 and 28 hours post-fortification.
- After all samples had been extracted, all extracts were C18-cleaned, oxidised and analysed in the same analytical batch.

Results obtained were subsequently used to determine whether toxin conversion was likely to occur within the shellfish tissues, and used to determine the most effective protocol for conducting method performance checks in this species. Results from these tests are presented in section 3.1.

2.9 Method performance checks

Method performance checks were conducted as follows:

2.9.1 Method selectivity

Homogenised tissues of each of the four clam species 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 aliquots analysed using the LC-FLD method following periodate and peroxide oxidation. In addition, C18-cleaned extracts were further cleaned using ion exchange SPE, prior to periodate oxidation and analysis of the fractions. Results are presented in section 3.2.

2.9.2 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 method,

following periodate oxidation of the C18-cleaned extracts and the full quantitation method and 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 of the amount and results from the screening method and quantitation method are presented in sections 3.3.1 and 3.3.2 respectively.

2.9.3 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 3.3.3

2.9.4 Assessment of method recovery for N-hydroxylated toxins

Assessment of the recovery of PSP toxins from clam 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 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, before leaving the spiked homogenates for at least 1 hour. Tissues were extracted and analysed, with oxidation and analysis carried out in triplicate. Quantitation of toxin concentrations involved the comparison of toxin peak area responses obtained from oxidised spiked samples with those obtained from oxidised toxin mix calibration solutions. For surf clams, the work was conducted using only dcSTX and dcGTX2,3 toxins. One single homogenate of manila, otter and carpet clams were spiked at 0.4AL with dcGTX2,3 and analysed to highlight any potential issues with recovery of this toxin. Recovery results are presented in section 3.4.

2.9.5 Determination of method precision

Precision was assessed with the repeated analysis of shellfish extracts containing PSP toxins spiked at 0.16 μg STX eq./g (0.2 AL) and 0.32 μg STX eq./g (0.4 AL). The short term (intra-batch) repeatability was checked for each of 3 repeated sample analyses in the same run at both 0.2 AL and 0.4 AL per toxin. The medium term (inter-batch) repeatability was subsequently assessed for clams on 6 replicates (0.2 AL and 0.4 AL) analysed in 2 batches of 3 samples, more than two weeks apart. For surf clams, the work was conducted using only dcSTX and dcGTX2,3 toxins. The acceptability of the precision characteristics of the method was assessed in comparison to the method performance criteria stipulated. Precision results are presented in section 3.5.

2.9.6 Method uncertainty of measurement

Results from the method performance checks were used to calculate an overall value of uncertainty for the measurement of PSP toxins in each of the clam species investigated. Individual component uncertainties were calculated and propagated to calculate an overall

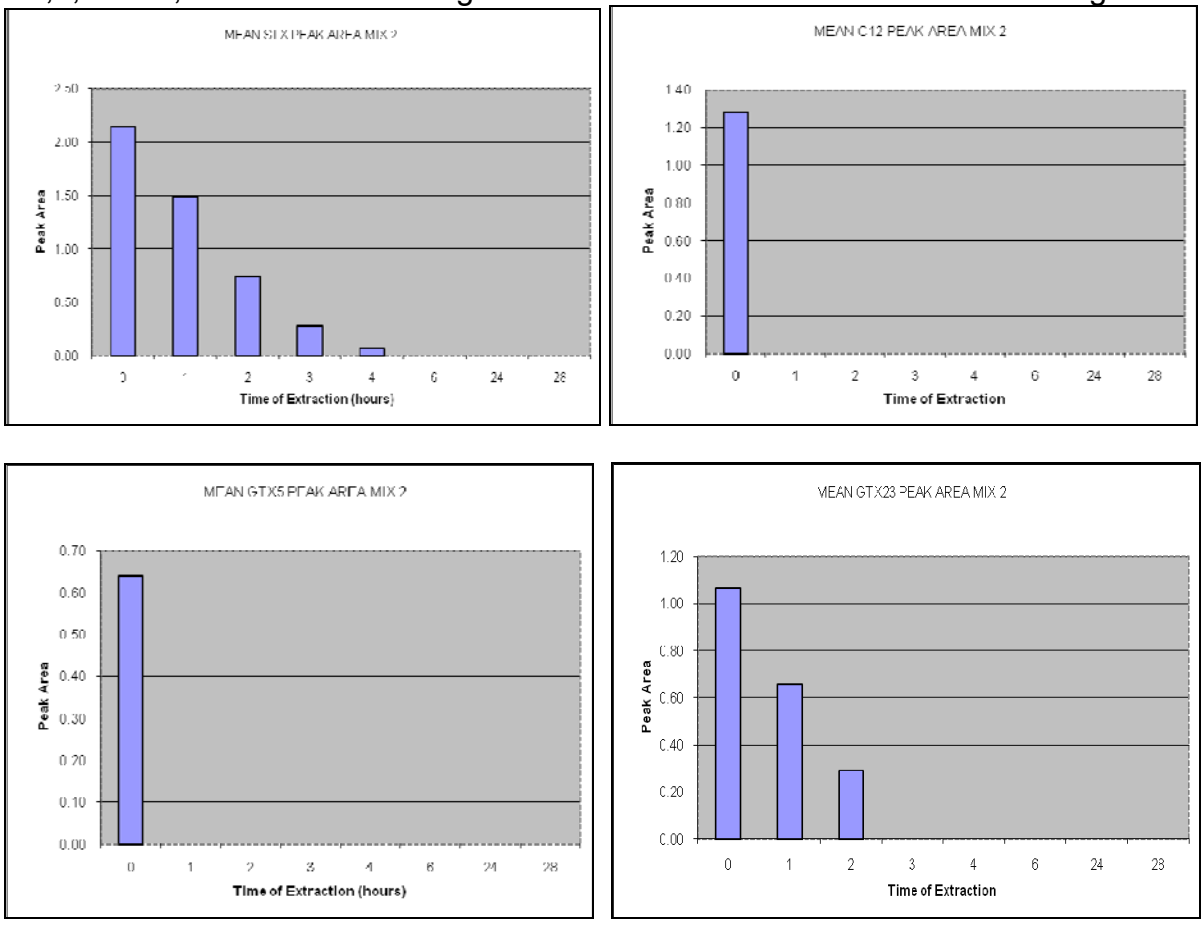
measurement uncertainty, as conducted previously in other major bivalve species [13-19]. Expanded uncertainties were calculated using an appropriate coverage factor (k), in order to provide “an interval expected to encompass a large fraction of the distribution of values that may be attributable to the measurand” [25,26]. Measurement uncertainty results are presented in section 3.6.

3. Results

3.1 Behaviour of PSP toxins in surf clams

Results obtained from the LC-FLD analysis of the spiked surf clam samples were found to show strong evidence for the conversion of all carbamate and N-sulfocarbamoyl PSTs into their decarbamoyl counterparts. The conversion of the N-sulfocarbamoyl toxins (C1,2 and GTX5) was found to occur rapidly, within the first hour of spiking, whereas conversion of carbamates (STX, GTX2,3, GTX1,4 and NEO) was seen to occur more slowly but still resulted in the conversion of the toxins to decarbamoyls. It was noted that the presence of the primary GTX1,4 quantitation peak at 24 and 28 hours relates to the presence of higher proportions of the dcGTX2,3 secondary peak appearing due to the toxin conversion. Although no decarbamoyl toxins were spiked into the surf clams, concentrations of both dcSTX and dcGTX2,3 were found to rise. Furthermore, there is some evidence for the conversion into dcNEO, with the increasing presence of the dcSTX/dcNEO toxin oxidation product peak in the Mix 1 spiked homogenates, resulting from the transformation from NEO. The results obtained from these experiments are given in Figures 2 and 3.

Figure 2. Reductions in the quantified concentrations over 28 hours of GTX2,3, STX, GTX5, C1,2, GTX1,4 and NEO following fortification of each toxin in surf clam homogenates



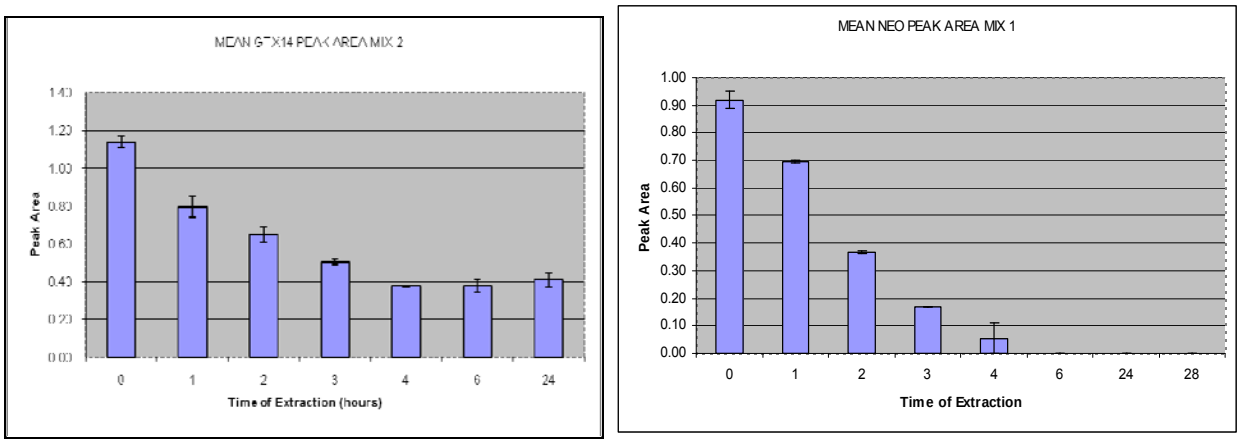
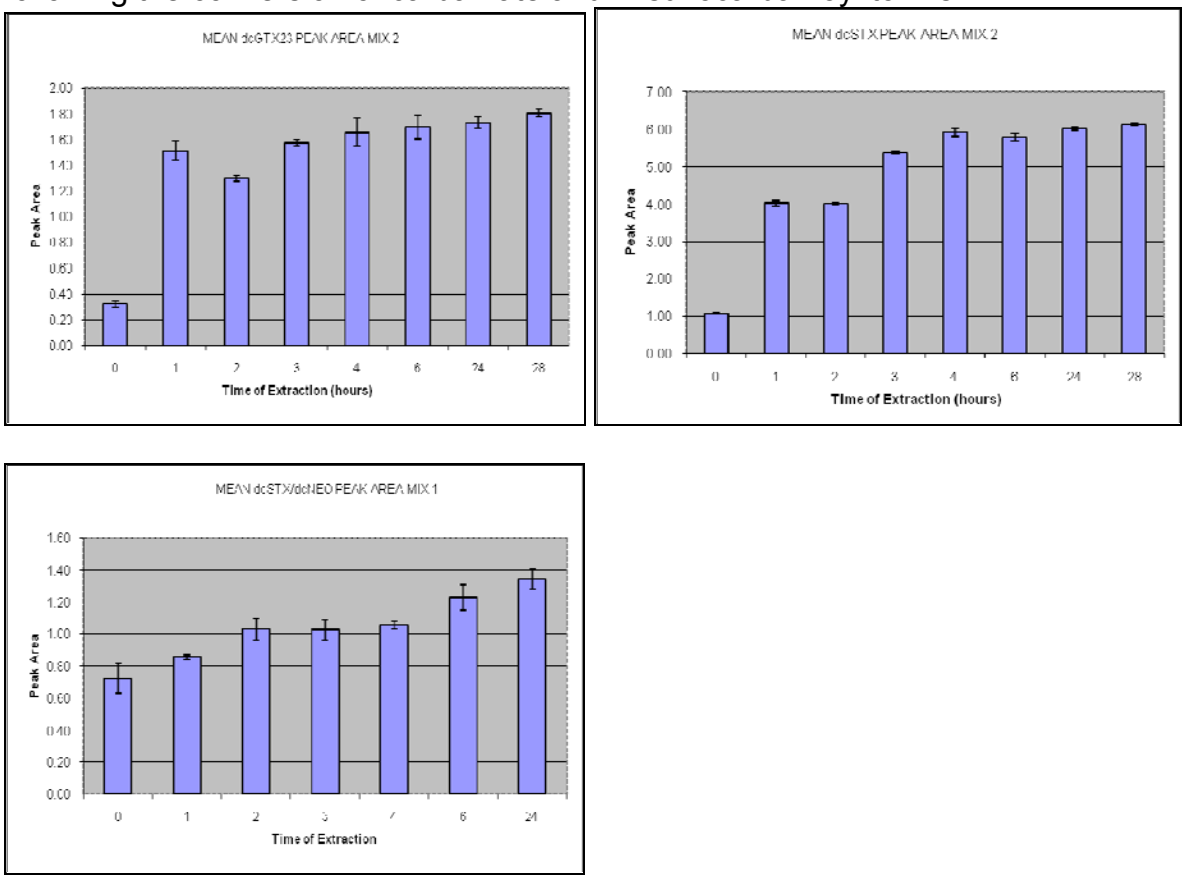


Figure 3. Increases in concentrations of dcSTX, dcGTX2,3 and dcNEO over 28 hours following the conversion of carbamate and N-sulfocarbamoyl toxins.



This confirms the results presented by [24] and also the toxin profiles detected previously from PSP-positive UK surf clam samples. Figure 4 illustrates the chemical structures of the saxitoxin analogues, showing the various sub-groups and subsequent analogue names. With the transformation of both carbamate and N-sulfocarbamoyl toxins into their decarbamoyl equivalents, specific toxin transformations would be expected, as summarised in Table 7. These transformations occur through the actions of carbamoylase enzymes which catalyse the hydrolysis of the larger R_4 groups into their hydroxyl-substituted equivalents [24]. Given the predominance of the transformations converting the major toxins into either dcSTX or dcGTX2,3, these are the transformation products most likely to be observed in our samples. However, the table also shows the potential transformation of the N-hydroxylated toxins GTX1,4 and NEO into dcGTX1,4 and dcNEO respectively and the subsequent presence of these toxins in some shellfish. However, with the current non-availability of both dcNEO and

dcGTX1,4, no further work was possible with either of these toxins. Nevertheless, the performance checks conducted on the non-N-hydroxylated toxins dcGTX2,3 and dcSTX should still give a good indication of the performance of the method for the determination of PSTs in surf clams.

Figure 4. Chemical structures of saxitoxin analogues (reproduced from Ref 23)

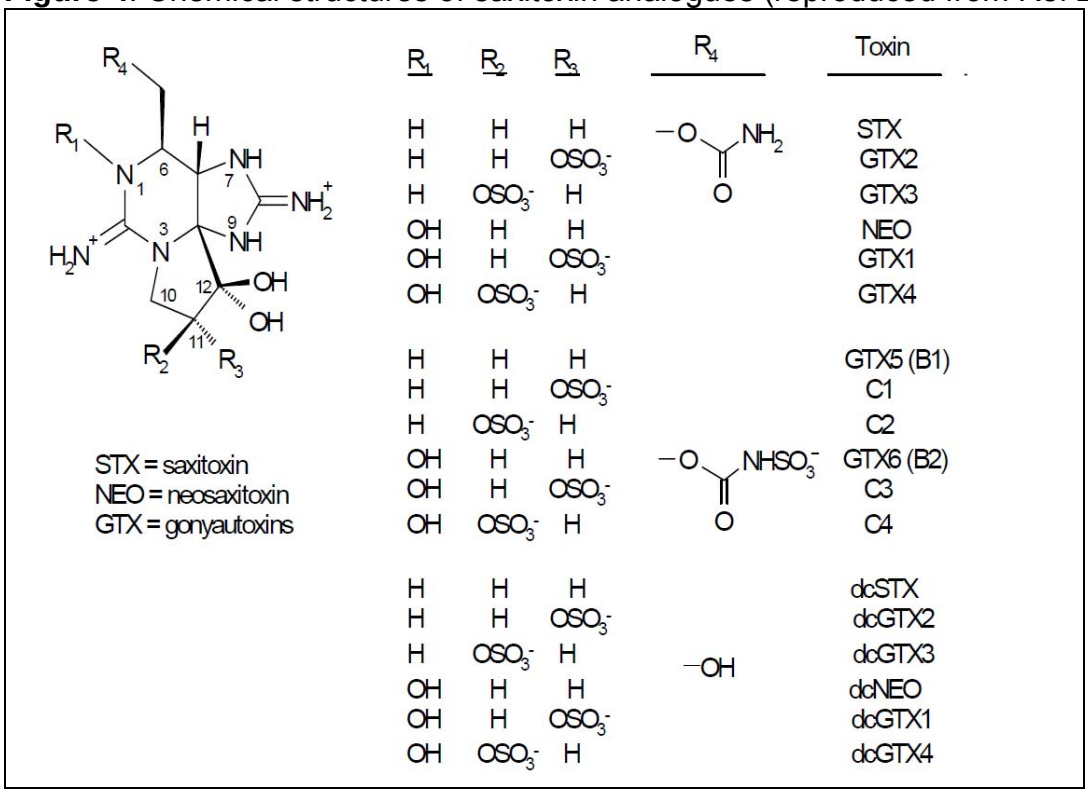
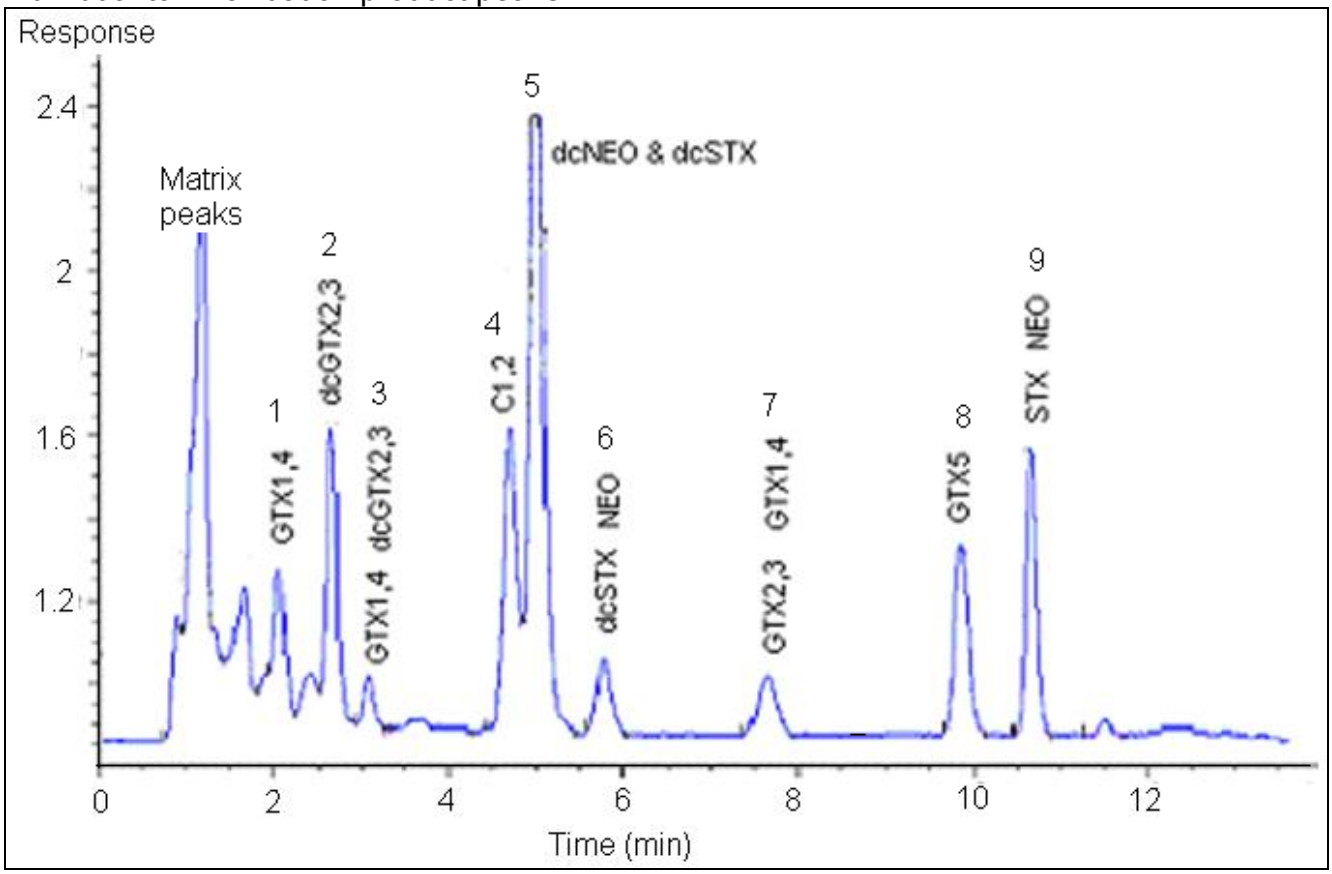


Table 7. Summary of the expected transformations and corresponding chromatographic elution patterns

Toxin	Product after transformation	Chromatographic elution of product peaks	
		Peak number (Fig. 5)	Toxins at same retention time
STX	dcSTX	5,6	dcSTX/NEO/dcNEO
GTX2,3	dcGTX2,3	1,2	dcGTX2,3/GTX1,4
C1,2	dcGTX2,3	1,2	dcGTX2,3/GTX1,4
GTX5	dcSTX	5,6	dcSTX/NEO/dcNEO
NEO	dcNEO	5	dcSTX/NEO/dcNEO
GTX1,4	dcGTX1,4	1,2	dcGTX2,3/GTX1,4

Figure 5. Chromatograms of PSTs showing retention times and peak numbers (1 to 9) of individual toxin oxidation product peaks.



As a result, no meaningful data could be obtained for the recovery of the non-decarbamoyl toxins from surf clams. The recommendation from this preliminary work was therefore that the performance checks for surf clams should be limited to only the decarbamoyl toxins dcSTX and dcGTX2,3 currently available as certified reference standards.

3.2 Selectivity of the method

In order to assess whether components of the clam matrices may have an effect on the quantitation of PSP toxins following periodate and peroxide oxidation, clam tissue extracts were cleaned-up using C18 SPE, fractionated by ion exchange SPE and the appropriate aliquots analysed by periodate and peroxide oxidation. Specifically, periodate oxidation was conducted on the C18 SPE-cleaned extracts and fractions F2 and F3 from all species, and peroxide oxidation was applied only to the C18-cleaned extracts. 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 [27,19].

3.2.1 Selectivity in periodate oxidised C18-cleaned extracts and post ion-exchange fractions

Matrix components were observed (Figures 6 to 9) eluting up to 2.0 minutes in the chromatograms for the periodate oxidation of C18-cleaned extracts in all four species, as is typically encountered for all other shellfish species [12-19]. A low number of small peaks corresponding in retention to the toxin oxidation products of GTX1,4 and dcSTX were observed in the C18-cleaned extracts of surf clams and manila clams, but were present at levels giving rise to peaks well below a signal to noise ratio of 3. As such these would not interfere with the screening analysis of these species. No such peaks were observed in any of the fractions for any of the four species (Figures 10 to 17), therefore indicating the

selectivity of the quantitation method for the N-hydroxylated toxins (GTX1,4 and NEO) each species.

Figure 6. Periodate oxidation of C18 cleaned surf clam extract

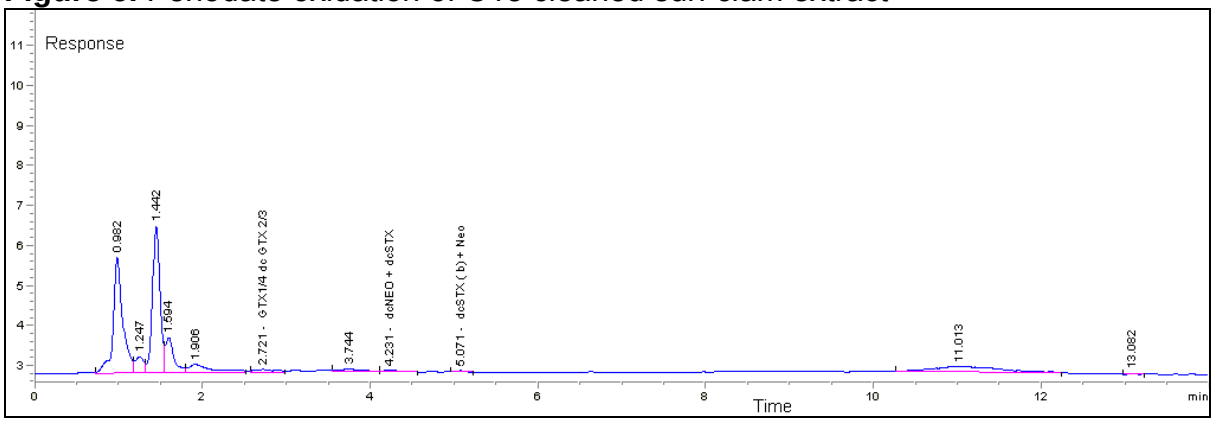


Figure 7. Periodate oxidation of C18 cleaned otter clam extract

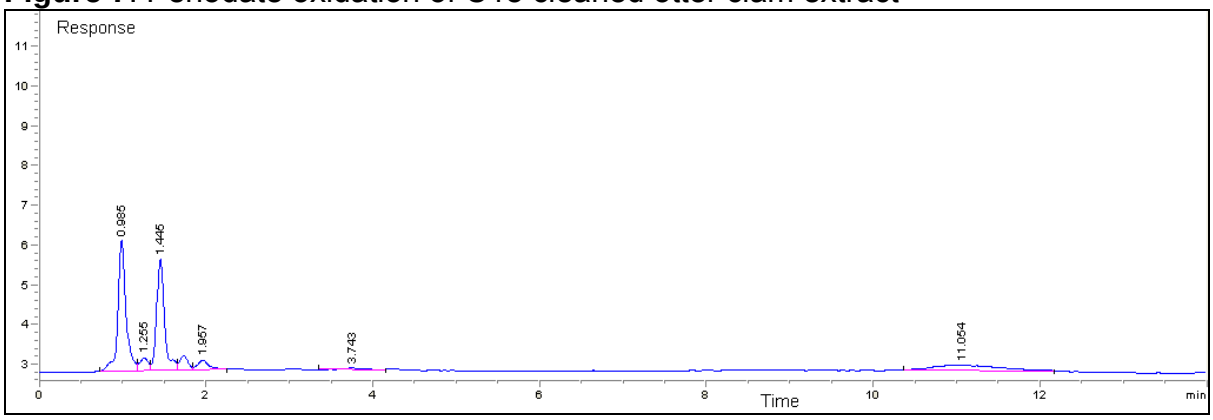


Figure 8. Periodate oxidation of C18 cleaned manila clam extract

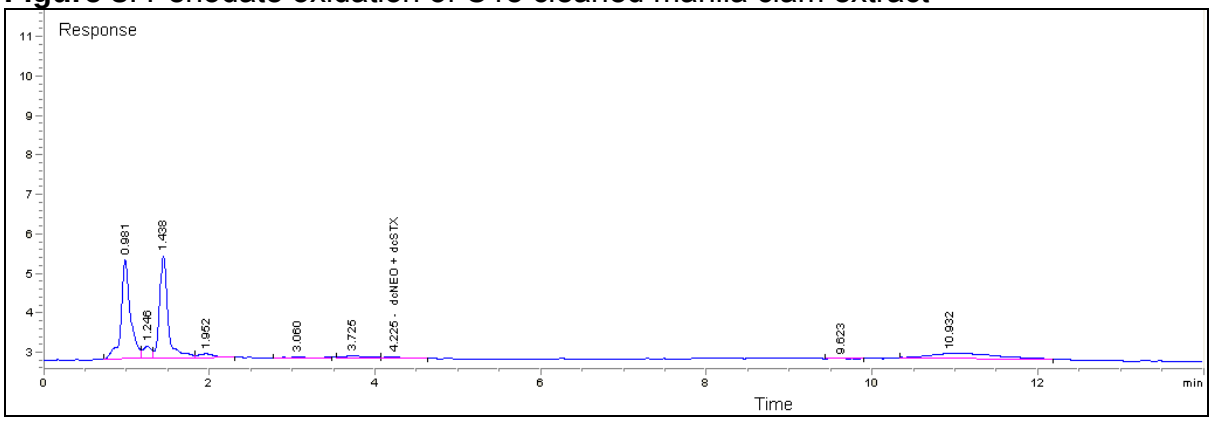


Figure 9. Periodate oxidation of C18 cleaned carpet shell clam extract

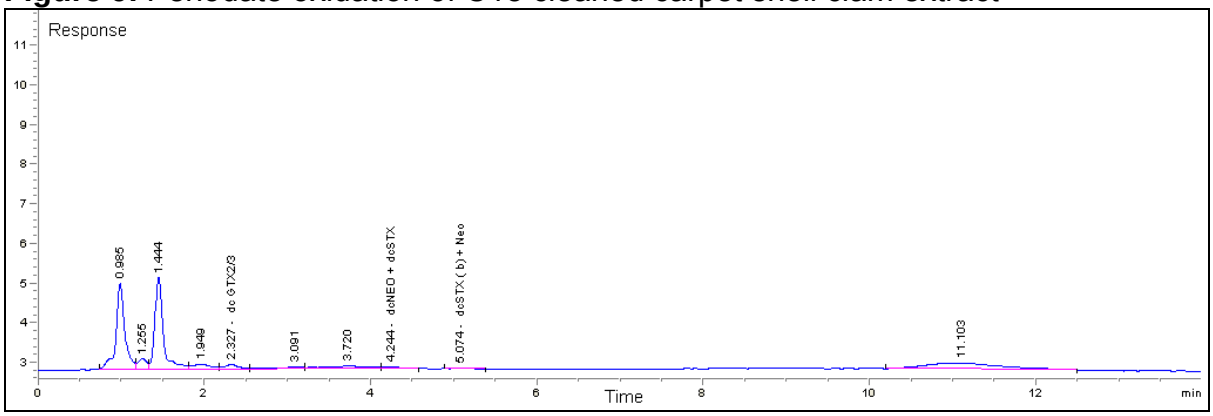


Figure 10. Periodate oxidation of fraction F2 of surf clam extract

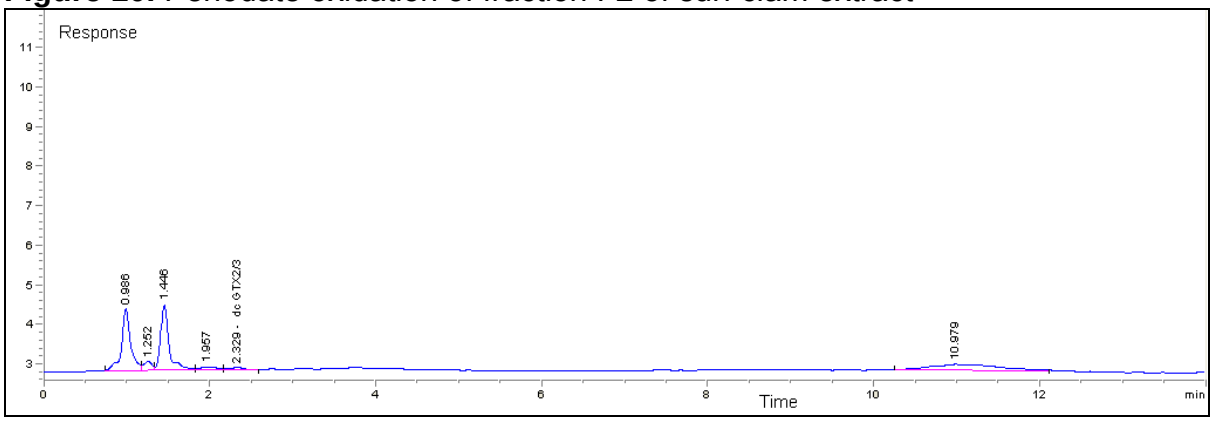


Figure 11. Periodate oxidation of fraction F2 of otter clam extract

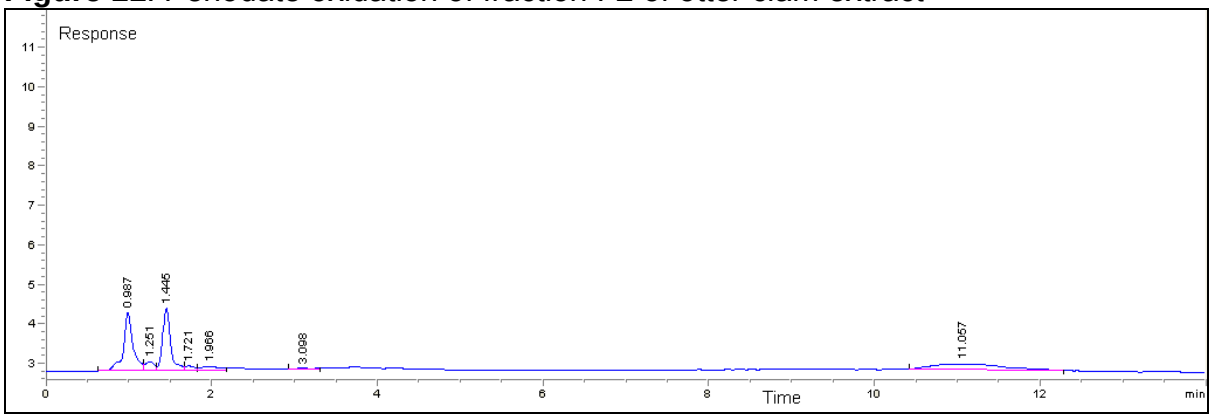


Figure 12. Periodate oxidation of fraction F2 of manila clam extract

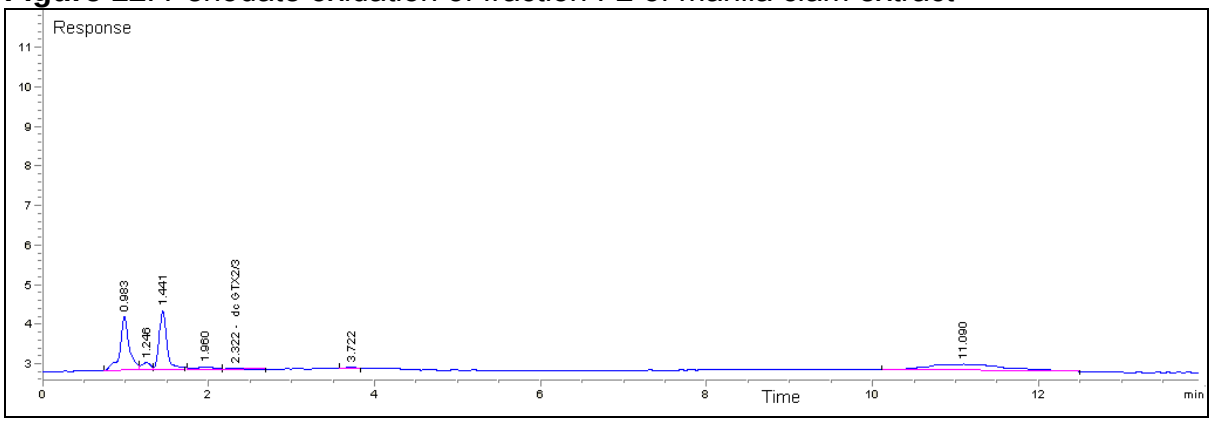


Figure 13 Periodate oxidation of fraction F2 of carpet shell clam extract

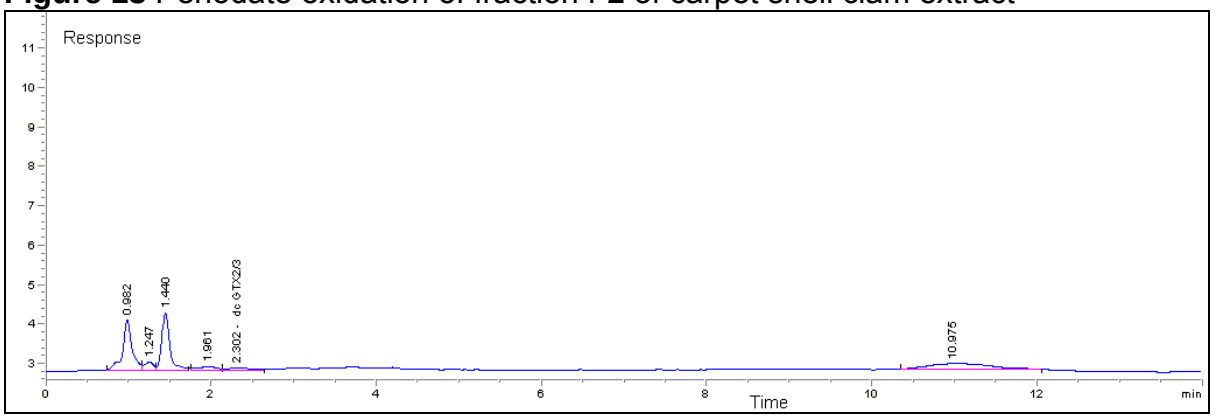


Figure 14. Periodate oxidation of fraction F3 of surf clam extract

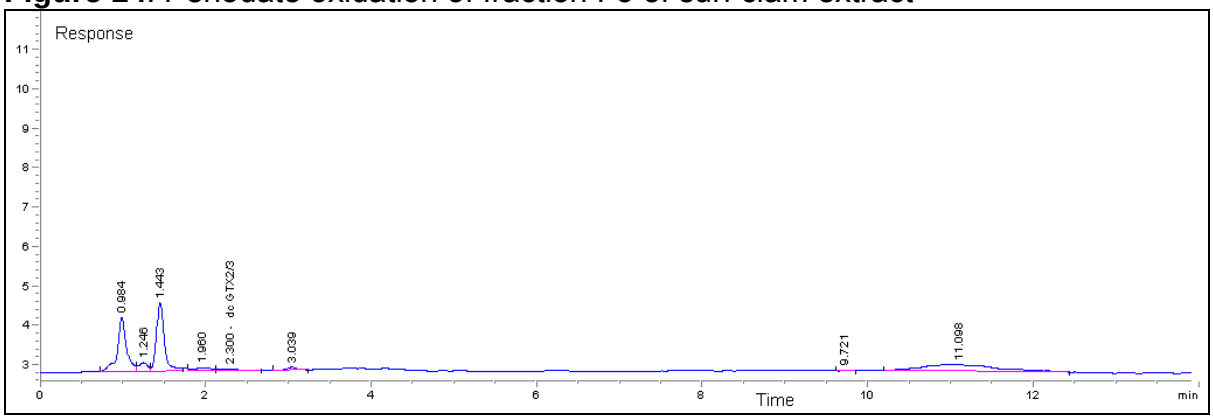


Figure 15. Periodate oxidation of fraction F3 of otter clam extract

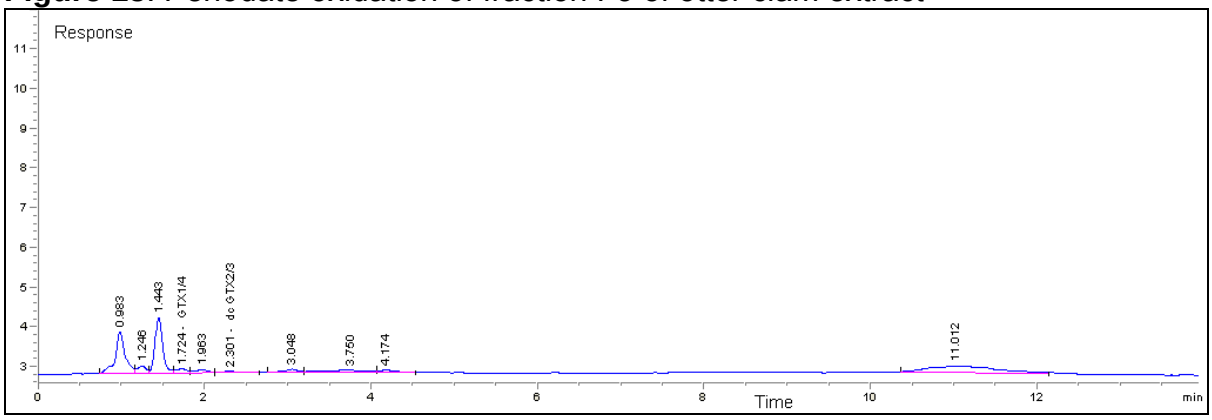


Figure 16. Periodate oxidation of fraction F3 of manila clam extract

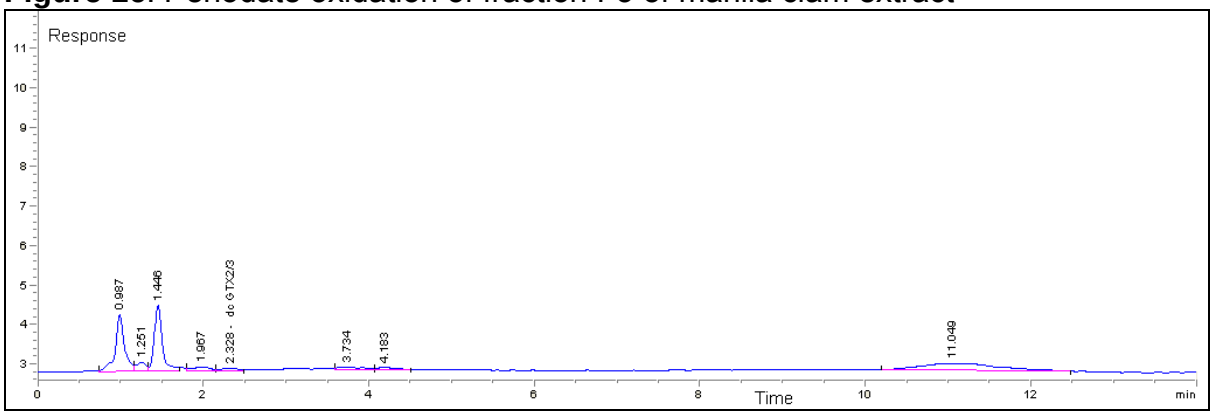
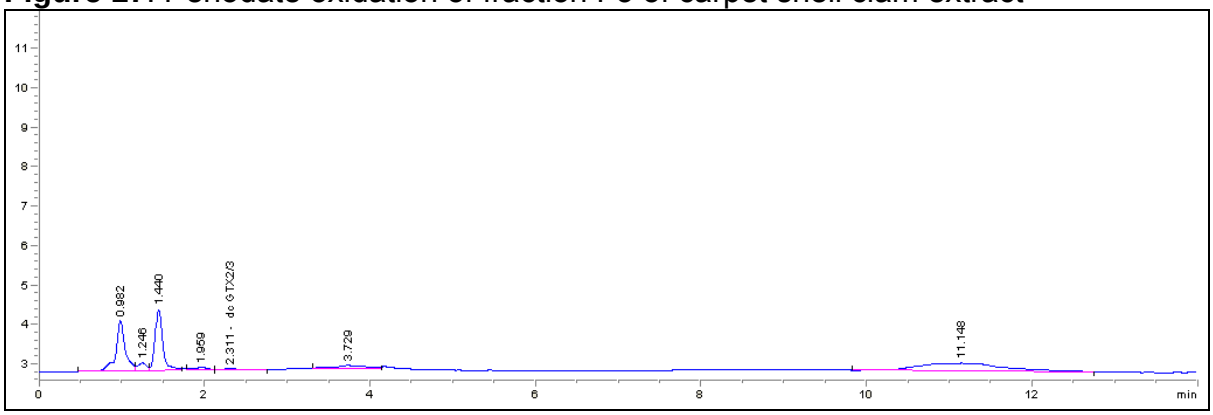


Figure 17. Periodate oxidation of fraction F3 of carpet shell clam extract



3.2.2 Selectivity in peroxide oxidised C18-cleaned extracts

All the chromatograms obtained following analysis of the peroxide-oxidised C18 extracts of the four clam species show typically clean profiles (Figures 18-21). Again, matrix peaks typical of all shellfish species are observed eluting up to 2 minutes. The chromatogram for surf clams shows a small peak eluting at the same retention time as the quantitation peak for dcSTX. However, the peak was found to have a signal to noise ratio of <3, thereby not interfering with the quantitation of the toxin. All other chromatograms were found to be devoid of any other matrix interference peaks. As such, there is good evidence for the selectivity of the method for the non-N-hydroxylated toxins in the four clam species following the peroxide oxidation of the C18-cleaned extracts.

Figure 18. Peroxide oxidation of C18-cleaned surf clam extract

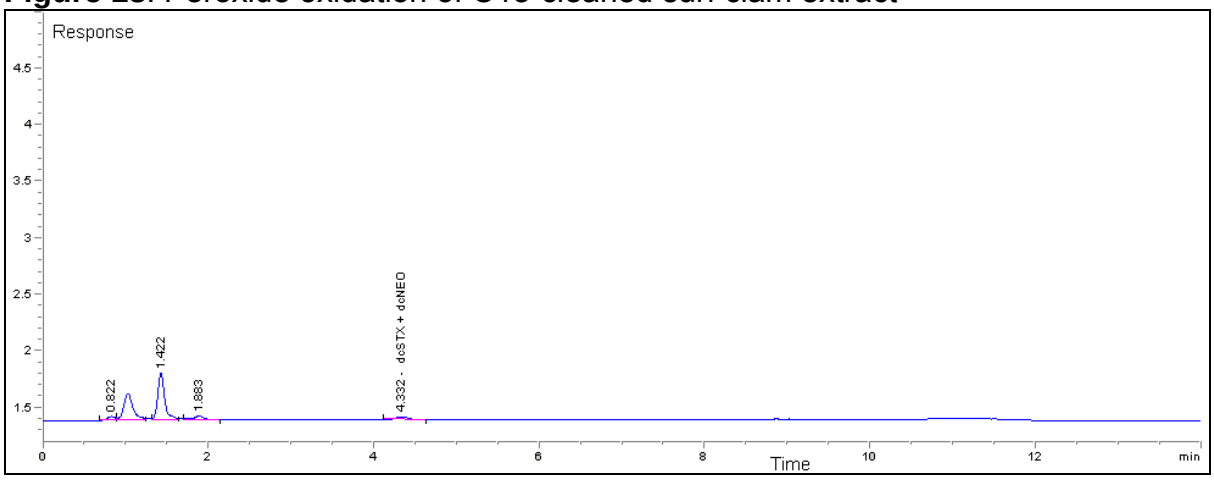


Figure 19. Peroxide oxidation of C18-cleaned otter clam extract

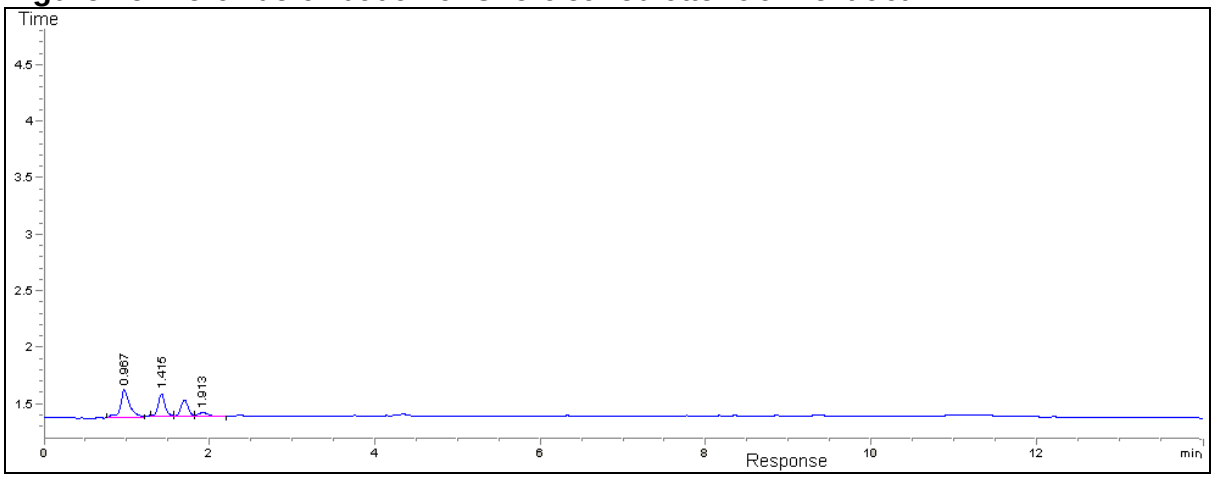


Figure 20. Peroxide oxidation of C18-cleaned manila clam extract

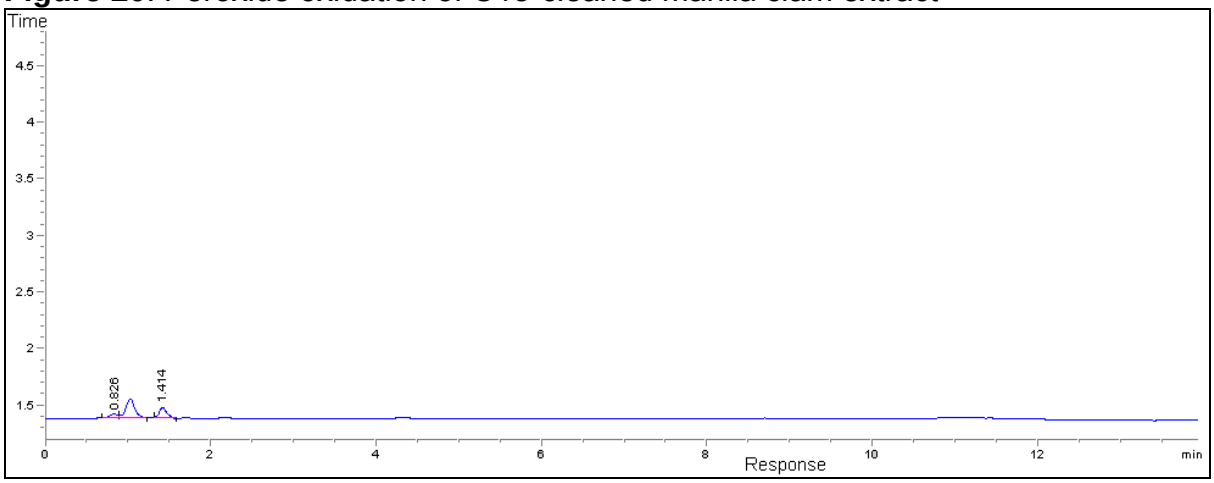
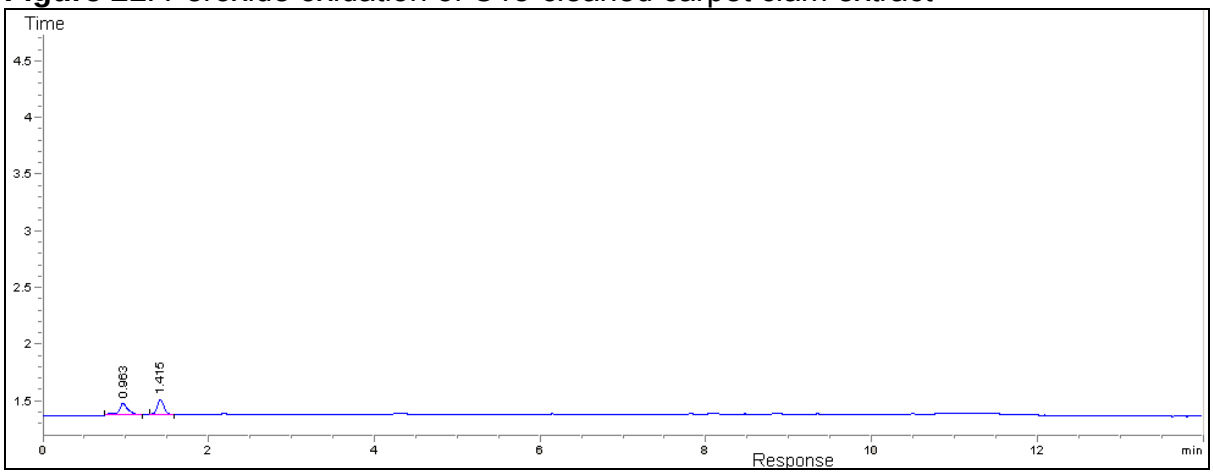


Figure 21. Peroxide oxidation of C18-cleaned carpet clam extract



3.3. Limits of detection and quantitation for PSP toxins in minor clam species

3.3.1 Limits of detection for screening method

LODs were calculated for the periodate oxidation of all toxins in cleaned-up clam extracts 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 8) in terms of mean ± one standard deviation (sd, n=3) where the standard deviation is calculated from the signal to noise values measured for each of the triplicate results for each individual toxin. Values were calculated for the primary (diagnostic) toxin peak only.

For the range of toxins investigated here, predicted LODs of ~0.02 to 0.15 µg STX eq./g (0.03 to 0.19 AL) were determined for the periodate screening method of C18-cleaned extracts of clam tissues. Whilst these values were not experimentally confirmed with homogenate spiking experiments at the actual LOD concentrations, they give a good indication of the sensitivity of the screening method. Results clearly shoiw the acceptable sensitivity of the periodate screen for the range of toxins studied, with the majority of toxins exhibiting LODs <0.1 µg STX eq./g. Given the results summarised here, periodate oxidation of the C18-cleaned extracts of all clam species is considered as a suitable qualitative screening step prior to the full quantitation of any positive samples.

Table 8. Predicted limits of detection (LOD; µg STX eq./g ± 1 sd) of the LC-FLD screening method for the primary toxin peaks of PSP toxins following periodate oxidation of C18-cleaned clams (no target specified for screening method).

Toxin	Carpet	Manila	Otter	Surf
GTX 1,4	0.052 ± 0.014	0.064 ± 0.007	0.056 ± 0.006	*na*
NEO	0.021 ± 0.004	0.026 ± 0.003	0.022 ± 0.003	*na*
C 1,2	0.066 ± 0.018	0.081 ± 0.029	0.048 ± 0.017	*na*
dcSTX	0.096 ± 0.021	0.112 ± 0.032	0.073 ± 0.041	0.096 ± 0.011
GTX 2,3	0.037 ± 0.008	0.044 ± 0.009	0.031 ± 0.009	*na*
STX	0.138 ± 0.04	0.154 ± 0.051	0.097 ± 0.068	*na*
dcGTX 2,3	*na*	*na*	*na*	0.022 ± 0.022

na = not analysed

3.3.2 Limits of detection for quantitation method

Table 9 tabulates the predicted LODs for the non-N-hydroxylated toxins following peroxide oxidation at 0.16 µg STX eq./g (0.2 AL) of the C18-cleaned extracts. Results illustrate that the sensitivity of the quantitation method is sufficient to quantify these toxins at this target concentration of ≤0.2AL per toxin, with predicted LODs for each of the peroxide-

quantified toxins well below this target. Initial data generated (not shown) indicated the close proximity of the estimated LOD to the 0.2 AL target for the quantitation of the N-hydroxylated toxins GTX1,4 and NEO. In addition, the values calculated for predicted LOQ were close to or higher than the target concentration of 0.4AL per toxin. Consequently, the analysis of the F2 and F3 fractions for GTX1,4 and NEO was modified, by using a higher analytical injection volume, specifically doubling the volume from 50µL to 100µL. The predicted LODs tabulated in Table 9 were subsequently calculated from the analysis of periodate-oxidised fractions after injection at this higher volume. Results for both GTX1,4 and NEO showed an acceptable level of sensitivity for the quantitative analysis, with predicted LODs well below the target 0.2 AL per toxin, in each of the three species. Overall therefore, the analysis has demonstrated the suitability of the quantitative LC-FLD method for the sensitive quantitation of all PSTs investigated, with the target LOD being achieved without any exception.

Table 9. Predicted limits of detection (LOD; µg STX eq./g ± 1 sd.) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions with 100µL injection volume and peroxide oxidation of C18-cleaned clam extracts (target LOD = 0.16 µg STX eq./g).

Toxin	Carpet	Manila	Otter	Surf
GTX 1,4	0.074 ± 0.005	0.076 ± 0.016	0.075 ± 0.025	*na*
NEO	0.082 ± 0.009	0.098 ± 0.025	0.084 ± 0.004	*na*
C 1,2	0.011 ± 0.002	0.010 ± 0.003	0.010 ± 0.002	*na*
dcSTX	0.009 ± 0.003	0.007 ± 0.003	0.008 ± 0.003	0.006 ± 0.002
GTX 2,3	0.038 ± 0.009	0.032 ± 0.011	0.033 ± 0.009	*na*
GTX5	0.032 ± 0.006	0.025 ± 0.010	0.029 ± 0.007	*na*
STX	0.014 ± 0.003	0.011 ± 0.004	0.012 ± 0.003	*na*
dcGTX 2,3	*na*	*na*	*na*	0.024 ± 0.003

na = not analysed

3.3.3 Determination of the limit of quantitation of the method

LOQs were experimentally confirmed at ≤0.16 μg STX eq./g for non-N-hydroxylated toxins. Predicted LOQs for the non-N-hydroxylated toxins were calculated as ranging from 0.033 to 0.17 μg STX eq./g for all species (Table 10). LOQs were again higher for the less sensitive analysis of the N-hydroxylated toxins after periodate oxidation, but with the higher injection volume are still confirmed at <0.32 μg STX eq./g, the target LOQ. Predicted LOQs for the N-hydroxylated toxins were found to range from 0.19 to 0.30 μg STX eq./g (Table 10). As such, the LOQs for all toxins are lower than the target concentration level of 0.32 μg STX eq./g (0.4 AL), and in the vast majority are well below this target level. Consequently, it is clear from these results that the method performance characteristics are acceptable in terms of LOQ for the detection and quantitation of PSTs in each of the clam species investigated.

Table 10. Predicted limits of quantitation (LOQ; μg STX eq./g ± 1 sd.) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions with 100μL injection volume and peroxide oxidation of C18-cleaned clam extracts (target LOQ = 0.32 μg STX eq./g).

Toxin	Carpet	Manila	Otter	Surf
GTX 1,4	0.188 ± 0.016	0.239 ± 0.040	0.294 ± 0.054	*na*
NEO	0.241 ± 0.035	0.259 ± 0.025	0.227 ± 0.012	*na*
C 1,2	0.045 ± 0.010	0.046 ± 0.010	0.050 ± 0.009	*na*
dcSTX	0.033 ± 0.011	0.035 ± 0.007	0.037 ± 0.008	0.024 ± 0.008
GTX 2,3	0.156 ± 0.038	0.156 ± 0.034	0.169 ± 0.031	*na*
GTX5	0.125 ± 0.035	0.124 ± 0.024	0.136 ± 0.027	*na*
STX	0.055 ± 0.015	0.054 ± 0.011	0.059 ± 0.011	*na*
dcGTX 2,3	*na*	*na*	*na*	0.097 ± 0.012

na = not analysed

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

Recoveries were calculated in terms of the expected mean recovery for each toxin in each of the three spiked, extracted, cleaned and derivatised samples in each of two batches. Table 11 presents the mean recovery percentages of PSP toxins from the four spiked clam species spiked at 0.2 AL and 0.4 AL with RSDs calculated from the mean recovery of each triplicate oxidation and analysis. Results show the mean recoveries of all PSP toxins spiked at 0.4 AL of C18-cleaned shellfish extracts falling in the range of 68% to 106% for carpet clams, 61% to 91% for manila clams, 64% to 87% for otter clams and 63% to 83% for surf clams. The analysis of the manila, otter and carpet clams spiked once at 0.4AL with dcGTX2,3 indicate no obvious problems with recovery of this toxin in each of these three species, although no precision was associated with this determination. RSDs associated with the recoveries of most other toxins indicated a fair degree of repeatability associated with such measurements. It was therefore shown that in all clam species at 0.4 AL, the recoveries for all PSP toxins are acceptable, being within the target range of recoveries (Table 11) and being similar to results reported previously in other species [13]. Recoveries determined at the lower concentration spiking level (0.2 AL) were similar for each of the studied toxins, with a slight overall increase in variability noted at the lower concentration, as expected. As such, the recoveries are deemed acceptable in comparison with the performance characteristics limits defined.

Table 11. Mean percentage recoveries (and RSDs of triplicate spikes, n=6) of PSP toxins from batch 1 and 2 of the four clam species homogenate spiked at expected concentrations of 0.2 AL and 0.4 AL* (GTX5 1/10 concentration).

Toxin	Target Recovery (%)	Carpet clams		Manila clams		Otter clams		Surf clams	
		0.2 AL	0.4 AL	0.2 AL	0.4 AL	0.2 AL	0.4 AL	0.2 AL	0.4 AL
GTX 1,4	70-110	98% (14%)	106% (10%)	89% (16%)	87% (14%)	89% (13%)	84% (3%)	*na*	*na*
NEO*	60-120	72% (15%)	68% (22%)	64% (12%)	61% (7%)	70% (15%)	64% (13%)	*na*	*na*
dcGTX 2,3	60-120	*na*	*81%***	*na*	*68%***	*na*	*82%***	71% (25%)	63% (23%)
C 1,2	60-120	90% (15%)	80% (3%)	82% (13%)	73% (5%)	89% (13%)	75% (9%)	*na*	*na*
dcSTX	60-120	91% (21%)	83% (6%)	85% (16%)	76% (6%)	93% (18%)	81% (10%)	88% (22%)	83% (25%)
GTX 2,3	70-110	91% (23%)	78% (3%)	89% (17%)	74% (5%)	96% (19%)	78% (10%)	*na*	*na*
GTX 5	60-120	96% (7%)	88% (4%)	91% (5%)	85% (3%)	97% (6%)	87% (5%)	*na*	*na*
STX	70-110	91% (12%)	83% (4%)	86% (8%)	79% (4%)	95% (10%)	83% (8%)	*na*	*na*

**Batch 2 carpet, manila and otter clams were spiked with NEO at 0.3 and 0.54 AL due to spiking error. na = not analysed. **One homogenate was spiked at 0.4AL with dcGTX2,3 and analysed in duplicate*

3.5 Determination of the precision of the method

3.5.1 Estimation of short-term repeatability

Tables 12 to 15 show the concentrations calculated for triplicate homogenate spikes of all four clam species at 0.2 AL and 0.4 AL for each PSP toxin following single batch analysis. Standard deviations calculated from the resulting concentrations illustrate an acceptable level of short-term method repeatability for each of the toxins. RSD% values are less than or equal to 12% at both 0.2 AL and 0.4 AL for all non N-hydroxylated toxins and are ≤15% for the N-hydroxylated toxins subjected to the additional ion exchange clean up steps. The results therefore show that at individual PSP toxin concentrations equivalent to 0.2 and 0.4 AL, the short term repeatability for the LC-FLD analysis of each toxin is within the specified limits to short term repeatability of 15%. In addition, it is noted that the values compare well with those generated previously for other species [13-18] with equivalent or improved levels of precision.

Tables 12-15. Calculated mean concentrations (µg STX eq./g ±1 sd) of triplicate spiked clam homogenates at 0.2 AL and 0.4 AL per toxin (GTX5 at 0.02 and 0.04 AL), showing estimations of short-term method repeatability in terms of percentage relative standard deviation (n=3; same batch).

Table 12. Carpet shell clams

	Concentration at 0.2 AL ± sd	RSD%	Concentration at 0.4 AL ± sd	RSD%
GTX 1,4	0.17 ± 0.019	11%	0.36 ± 0.028	8%
NEO	0.10 ± 0.004	4%	0.17 ± 0.002	1%
C 1,2	0.12 ± 0.006	5%	0.26 ± 0.006	3%
dcSTX	0.12 ± 0.005	4%	0.25 ± 0.005	2%
GTX 2,3	0.12 ± 0.006	5%	0.25 ± 0.005	2%
GTX 5	0.01 ± 0.001	4%	0.03 ± 0.001	2%
STX	0.13 ± 0.006	5%	0.27 ± 0.005	2%

Table 13. Manila clams

	Concentration at 0.2 AL ± sd	RSD%	Concentration at 0.4 AL ± sd	RSD%
GTX 1,4	0.16 ± 0.013	8%	0.31 ± 0.017	6%
NEO	0.10 ± 0.007	7%	0.18 ± 0.012	7%
C 1,2	0.13 ± 0.007	6%	0.23 ± 0.004	2%
dcSTX	0.12 ± 0.004	4%	0.24 ± 0.021	9%
GTX 2,3	0.13 ± 0.005	4%	0.23 ± 0.027	12%
GTX 5	0.01 ± 0.000	2%	0.03 ± 0.002	6%
STX	0.14 ± 0.004	3%	0.25 ± 0.018	7%

Table 14. Otter clams

	Concentration at 0.2 AL ± sd	RSD%	Concentration at 0.4 AL ± sd	RSD%
GTX 1,4	0.16 ± 0.008	5%	0.27 ± 0.002	1%
NEO	0.100.015	15%	0.18 ± 0.017	9%
C 1,2	0.13 ± 0.006	5%	0.23 ±0.026	11%
dcSTX	0.12 ± 0.004	4%	0.24 ± 0.021	9%
GTX 2,3	0.13 ± 0.005	4%	0.23 ± 0.027	12%
GTX 5	0.01 ± 0.000	2%	0.03 ± 0.002	6%
STX	0.14 ± 0.004	3%	0.25 ± 0.018	7%

Table 15. Surf clams

	Concentration at 0.2 AL ± sd	RSD%	Concentration at 0.4 AL ± sd	RSD%
dcGTX2,3	0.09 ± 0.005	5%	0.16 ± 0.009	6%
dcSTX	0.11 ± 0.004	3%	0.20 ± 0.006	3%

3.5.2 Estimation of medium-term repeatability

Tables 16-19 show the precision for the four clam species following the analysis of six replicate spiked homogenates (both 0.2 and 0.4 AL) performed over a longer period of time (> 2 weeks). For carpet clams RSD percentages range from 7% to 23% at 0.2 AL (mean = 15%) and 3% to 22% at 0.4 AL (mean = 7%) for all toxins. For manila clams RSD percentages range from 5% to 17% at 0.2 AL (mean = 11%) and 3% to 14% at 0.4 AL (mean = 6%) for all toxins. For otter clams RSD percentages range from 6% to 19% at 0.2 AL (mean = 13%) and 3% to 13% at 0.4 AL (mean = 8%) for all toxins and surf clams RSD percentages were 25% and 22% at 0.2 AL (mean = 24%) and 23% and 25% at 0.4 AL (mean = 24%) for dcGTX2,3 and dcSTX toxins. Results therefore show similarities to values generated previously for other species [13-18] and that at each concentration, the medium term repeatability is within the specified limits of 25% for each species. Further evidence for an acceptable level of precision is provided by the HorRat values, which are <2.0 for all toxins at both concentration levels, with only dcSTX in surf clams > 1.3.

Tables 16-19. Calculated mean concentrations (µg STX eq./g ±1 sd) of triplicate spiked clam homogenates at 0.2 AL and 0.4 AL per toxin (GTX5 at 0.02 and 0.04 AL), showing estimations of medium-term method repeatability in terms of percentage relative standard deviation (n=6; inter-batch).

Table 16. Carpet shell clams

	Concentration at 0.2 AL ± sd	RSD%	HorRat	Concentration at 0.4 AL ± sd	RSD%	HorRat
GTX 1,4	0.16 ± 0.023	14%	0.68	0.34 ± 0.035	10%	0.55
NEO	0.11 ± 0.018	15%	0.73	0.22 ± 0.049	22%	1.17
C 1,2	0.14 ± 0.022	15%	1.03	0.26 ± 0.007	3%	0.20
dcSTX	0.14 ± 0.030	21%	0.99	0.26 ± 0.015	6%	0.29
GTX 2,3	0.15 ± 0.034	23%	1.18	0.25 ± 0.008	3%	0.17
GTX 5	1.15 ± 0.011	7%	0.50	0.28 ± 0.012	4%	0.31
STX	0.15 ± 0.018	12%	0.59	0.27 ± 0.011	4%	0.22

Table 17. Manila clams

	Concentration at 0.2 AL ± sd	RSD%	HorRat	Concentration at 0.4 AL ± sd	RSD%	HorRat
GTX 1,4	0.14 ± 0.023	16%	0.77	0.28 ± 0.038	14%	0.72
NEO	0.10 ± 0.012	12%	0.57	0.20 ± 0.014	7%	0.37
C 1,2	0.13 ± 0.017	13%	0.34	0.23 ± 0.011	5%	0.37
dcSTX	0.14 ± 0.022	16%	0.76	0.24 ± 0.016	6%	0.32
GTX 2,3	0.14 ± 0.025	17%	0.87	0.24 ± 0.012	5%	0.28
GTX 5	0.15 ± 0.007	5%	0.34	0.27 ± 0.008	3%	0.22
STX	0.14 ± 0.011	8%	0.38	0.25 ± 0.011	4%	0.21

Table 18. Otter clams

	Concentration at 0.2 AL ± sd	RSD%	HorRat	Concentration at 0.4 AL ± sd	RSD%	HorRat
GTX 1,4	0.14 ± 0.018	13%	0.61	0.27 ± 0.008	3%	0.15
NEO	0.11 ± 0.017	15%	0.73	0.20 ± 0.026	13%	0.68
C 1,2	0.14 ± 0.019	13%	0.87	0.24 ± 0.021	9%	0.67
dcSTX	0.15 ± 0.027	18%	0.85	0.26 ± 0.027	10%	0.53
GTX 2,3	0.15 ± 0.029	19%	0.97	0.25 ± 0.024	10%	0.57
GTX 5	0.16 ± 0.009	6%	0.40	0.28 ± 0.015	5%	0.37
STX	0.15 ± 0.016	10%	0.47	0.26 ± 0.020	8%	0.42

Table 19. Surf clams

	Concentration at 0.2 AL ± sd	RSD%	HorRat	Concentration at 0.4 AL ± sd	RSD%	HorRat
dcGTX2,3	0.11 ± 0.03	25%	1.36	0.20 ± 0.05	23%	1.39
dcSTX	0.14 ± 0.03	22%	1.04	0.26 ± 0.07	25%	1.32

3.6 Uncertainty of measurement

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 [13-19]. Whilst this study does not comprise of a full set of method validation exercises, the data generated from the method performance checks, including the short-term, medium-term and long-term precision can be used to estimate the various contributions of the components which can be summed to provide an overall estimate of measurement uncertainty.

3.6.1 Precision – Repeatability

The measurement uncertainty inherent in the precision component was evaluated here from the statistical distribution of the results of a series of measurements and can be characterised by standard deviations [23]. Uncertainties were calculated at two concentration levels (0.2 AL and 0.4 AL) for medium term precision and RSDs were pooled to give total standardised precision uncertainties in each of the four clam species (Table 20):

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

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 20. Pooled standard uncertainties calculated for PSP toxins in minor clams associated with the medium-term precision of the method.

	Precision			
Toxin	Carpet	Manila	Otter	Surf
GTX 1,4	0.13	0.15	0.09	*na*
NEO	0.19	0.10	0.14	*na*
C 1,2	0.11	0.10	0.11	*na*
dcSTX	0.15	0.12	0.15	0.24
GTX 2,3	0.16	0.13	0.15	*na*
GTX 5	0.06	0.04	0.06	*na*
STX	0.09	0.06	0.09	*na*
dcGTX2,3	na	*na*	*na*	0.24

3.6.2 Within-lab reproducibility or long-term repeatability

The uncertainties associated with long term precision (Table 21) were estimated from the precision data generated by the repeated extraction, clean-up, fractionation, oxidation and analysis of the spiked homogenates over >2 batches.

Table 21. Within-lab reproducibility uncertainties calculated from repeat analysis (>2 batches) of spiked clam homogenates

	Reproducibility			
Toxin	Carpet	Manila	Otter	Surf
GTX 1,4	0.12	0.15	0.11	*na*
NEO	0.18	0.09	0.13	*na*
C 1,2	0.11	0.09	0.11	*na*
dcSTX	0.14	0.11	0.13	0.24
GTX 2,3	0.16	0.12	0.14	*na*
GTX 5	0.06	0.07	0.06	*na*
STX	0.10	0.06	0.10	*na*
dcGTX2,3	na	*na*	*na*	0.24

3.6.3 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 0.2 AL and 0.4 AL in Table 22 below in each of the four species. Pooled uncertainties are calculated for each toxin using the same formula as in section 3.7.1 (above) and are shown to be of relatively small magnitude as expected.

Table 22. RSDs and pooled uncertainties associated with determination of recovery in spiked clam homogenates.

	Recovery			
Toxin	Carpet	Manila	Otter	Surf
GTX 1,4	0.10	0.07	0.04	*na*
NEO	0.03	0.10	0.13	*na*
C 1,2	0.04	0.03	0.09	*na*
dcSTX	0.03	0.04	0.07	0.03
GTX 2,3	0.04	0.03	0.09	*na*
GTX 5	0.04	0.01	0.05	*na*
STX	0.04	0.02	0.06	*na*
dcGTX2,3	na	*na*	*na*	0.05

3.6.4 Calculation of combined standard uncertainty

Preliminary combined standardised uncertainties for each PSP toxin in each of the four clam species (Table 23) 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 23. Combined uncertainties calculated from performance data for the four clam species showing uncertainties as (a) standardised uncertainty and (b) expanded uncertainty (k=2).

	Standardised uncertainty				Expanded uncertainty			
Toxin	Carpet	Manila	Otter	Surf	Carpet	Manila	Otter	Surf
GTX 1,4	0.20	0.22	0.15	*na*	0.39	0.45	0.30	*na*
NEO	0.26	0.17	0.23	*na*	0.53	0.33	0.46	*na*
C 1,2	0.16	0.14	0.18	*na*	0.32	0.28	0.35	*na*
dcSTX	0.21	0.17	0.21	0.33	0.42	0.34	0.42	0.67
GTX 2,3	0.23	0.18	0.22	*na*	0.46	0.36	0.45	*na*
GTX 5	0.09	0.08	0.10	*na*	0.18	0.16	0.19	*na*
STX	0.14	0.09	0.14	*na*	0.28	0.18	0.29	*na*
dcGTX2,3	*na*	*na*	*na*	0.34	*na*	*na*	*na*	0.69

The values for uncertainty of measurement reported in Table 23 are preliminary as further work on the method, potential generation of 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 the clam species. The results above show the combined standardised uncertainties for individual toxins ranging from 0.08 to 0.26 for all toxins in the carpet, manila and otter clams. In surf clams, the method performance checks on the decarbamoyl toxins dcSTX and dcGTX2,3 show higher levels of measurement uncertainty, primarily due to the variability observed in the medium term repeatability. Expanded uncertainties, calculated using a coverage factor (k) of 2, result in a range of values from 0.16 to 0.53 for carpet, manila and otter clams, with expanded uncertainties in surf clams of 0.67 and 0.69 for dcSTX and dcGTX2,3 respectively. The coverage factor was taken to be 2 in order to provide a 95% confidence in the distribution of values, assuming a normal distribution [26] and as assumed previously for other bivalve species. Results show a similar range of values for the toxin suite compared with values reported previously for the major bivalve species [13-19].

3.7 Notes on analysis to date of naturally contaminated clam samples

The number and availability of clam samples with measurable PSP toxicity is generally found to be low in samples received as part of the UK official control monitoring programme. Specifically, only one surf clam sample has been received in recent years which was found to contain levels of PSP above the MBA detection limit. The sample (BTX/2008/1563) was found to exhibit a toxicity of 92 µg STX eq./100g following MBA and 66 µg STX eq./100g following LC-FLD, when using the Oshima TEFs for toxicity calculations. This value would be revised to 81 µg STX eq./100g with use of the EFSA TEFs [23], as now used for calculation of routine results since June 2010 at the request of the CRL and UK NRL. As such, for the very limited amount of information obtained to date from naturally contaminated surf clams, there is no evidence for under or over-estimation of PSP toxicity if using the LC-FLD in replacement of the official MBA. Other than the one surf clam received, no PSP-positive manila, otter or carpet shell clams have been received through the UK monitoring programme.

4. Conclusions

The AOAC 2005.06 LC-FLD "Lawrence" method was subjected to an in-house programme of method verification exercises for the determination of performance characteristics of the method in a range of minor clam species. The species investigated were manila clams (*Ruditapes philippinarum*), European otter clams (*Lutraria lutraria*), grooved carpet shell clams (*Ruditapes decussatus*) and surf clams (*Spisula solida*). The performance characteristics were agreed before-hand between Cefas and the FSA and consisted of a series of investigations to check the performance in terms of method selectivity, sensitivity (LOD and LOQ), toxin recovery and method precision. In order to keep the number of expensive toxin standards to a minimum, required for recovery and precision assessment, the specific toxins to be investigated were also agreed in advance. The full suite of toxins to be investigated included all those of highest toxicity and commonly found in UK naturally contaminated bivalve shellfish samples over recent years. The toxins utilised for the study were 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)). Additionally, the work was extended to the non N-hydroxylated decarbamoylgonyautoxin-2 and 3 (dcGTX2,3) for the assessment of method performance in surf clams, toxins not covered by the AOAC method, but previously incorporated into the validation at Cefas for the major bivalve species and known to occur naturally in surf clams due to toxin interconversion within the surf clam flesh. The overall aim of this work was to verify the performance of the AOAC 2005.06 method for the determination and quantitation of PSTs in all four clam species, thus enabling the implementation of the method into the UK routine monitoring programme for these species. Quantitative results from the study are summarised for each species in Tables 24-27.

Initial studies focussed on the potential issues of the testing regime as applied to surf clams. Analysis of homogenates spiked with known concentrations of PSTs, revealed significant levels of inter-conversion of toxins, seemingly with the carbamate and N-sulfo-carbamoyl toxins being transformed into their decarbamoyl counterparts. A focussed investigation ensued, examining the transformation of individual PSP toxins over a 28 hour period, after each toxin was spiked into surf clam homogenates. Results indicated a very rapid transformation of the N-sulfocarbamoyl toxins (GTX5 and C1,2) into the decarbamoyl toxins, with transformation occurring within a few minutes of matrix spiking and near total conversion of the toxins within the first hour. Carbamate toxins were found to convert more slowly, but still showed near total conversion within twelve hours of toxin spiking. Potentially, the toxins dcSTX, dcGTX2,3, dcNEO and dcGTX1,4 could be formed from the transformation of toxins within the surf clam homogenate. Given the current non-availability of dcNEO and dcGTX1,4 toxins as reference standards, method performance checks for surf clams were limited to just dcSTX and dcGTX2,3, as agreed with the FSA.

Following the chromatographic analysis of PSP-negative clams in order to demonstrate the selectivity of the method, some matrix components were observed eluting within the first two minutes of most of the periodate and peroxide chromatograms. These peaks relate solely to the presence of matrix components as is typically encountered for all other shellfish species. A low number of small peaks corresponding in retention to the toxin oxidation products of GTX1,4 and dcSTX were observed in the C18-cleaned extracts of surf clams and manila clams, but were present at levels giving rise to peaks well below a signal to noise ratio of 3. As such these would not interfere with the screening analysis of these species. No such peaks were observed in any of the fractions for any of the four species,

therefore indicating the selectivity of the quantitation method for the N-hydroxylated toxins in each species. Chromatograms obtained following analysis of the peroxide-oxidised C18 extracts of the four clam species also showed relatively clean profiles. The results for surf clams showed a small peak eluting at the same retention time as the quantitation peak for dcSTX, but was found to be present at a signal to noise ratio of <3, thereby not interfering with the quantitation of the toxin. All other chromatograms were found to be devoid of any other matrix interference peaks. Overall therefore, there was good evidence for the selectivity of the method for all PSP toxins in the four clam species.

Instrumental sensitivity was determined for toxins following the periodate screen in terms of the regulatory action limit, specifically determining whether the screening method was capable of detecting toxins present in each of the clam matrices at concentrations equivalent to 0.16 µg STX eq./g (0.2 AL) per toxin. Predicted limits of detection (LODs) ranged from ~0.02 to 0.15 µg STX eq./g (0.03 to 0.19 AL) for the four species under investigation, thus indicating the suitability of the screening method. It would therefore be suitable to use the periodate oxidation of the C18-cleaned extracts of all clam species as a qualitative screening step prior to the full quantitation of any positive samples.

Method LODs for the full quantitation method were experimentally confirmed at 0.16 µg STX eq./g (0.2 AL) for each toxin in each of the four species. Predicted LODs were calculated as ranging from 0.007 to 0.1 µg STX eq./g per toxin, following implementation of a larger injection volume (100µL) for the analysis of periodate oxidised fractionated extracts for the N-hydroxylated toxins. Consequently, the results indicated the successful verification of the method in terms of its ability to detect toxins at concentrations ≤0.16 µg STX eq./g (0.2 AL).

LOQs were experimentally confirmed at ≤0.16 µg STX eq./g for non-N-hydroxylated toxins and predicted LOQs were found to tange from 0.033 to 0.17 µg STX eq./g in all species. LOQs were found to be higher for the analysis of the N-hydroxylated toxins after periodate oxidation, but with the higher injection volume were confirmed at <0.32 µg STX eq./g, with predicted LOQs ranging from 0.19 to 0.30 µg STX eq./g. As such, the LOQs for all toxins are lower than the target concentration level of 0.32 µg STX eq./g (0.4 AL). Consequently, the results indicate the acceptable performance characteristics of the method in terms of its ability to quantify toxins at concentrations ≤0.32 µg STX eq./g in each of the four clam species.

Recoveries calculated for each toxin present in homogenate tissues at 0.2 AL and 0.4 AL showed values falling in the range of 68% to 106% for carpet clams, 61% to 91% for manila clams, 64% to 87% for otter clams and 63% to 83% for surf clams. Method recovery was therefore shown to be acceptable, being within the target range of recoveries specified (70%-110% for GTX1,4, GTX2,3 and STX and 60%-120% for other toxins) and being similar to results reported previously in other species [13].

Analysis of the short-term (within-batch) precision of the method showed RSD% values ≤12% at both 0.2 AL and 0.4 AL for all non N-hydroxylated toxins and ≤15% for the N-hydroxylated toxins subjected to the additional ion exchange clean up steps. The results therefore show that, at individual PSP toxin concentrations equivalent to 0.2 and 0.4 AL, the short term repeatability for the LC-FLD analysis of each toxin is within the specified limits to short term repeatability of 15%. In addition, it was noted that the values compare well with those generated previously for other species [13-18] with equivalent or improved levels of precision. Results also showed the medium-term precision (inter-batch) for the four clam

species to be acceptable over a longer period of time (> 2 weeks). RSD percentages ranged from 3% to 23% at both concentrations (manila clams), 3% to 17% (carpet shell clams), 3% to 19% (otter clams) and 22% to 25% (for two decarbamoyl toxins in surf clams). Results therefore show similarities to values generated previously for other species [13-18] and that at each concentration, the medium term repeatability is within the specified limits of 25% for each species. Further evidence for an acceptable level of precision was provided by the HorRat values, which are <2.0 for all toxins at both concentration levels, with only dcSTX in surf clams > 1.3.

Results obtained during these studies were used to calculate standardised and expanded uncertainties for the analysis of PSP toxins in each of the four clam species. Uncertainty contributions were assessed and included contributions from the uncertainty inherent in the precision, reproducibility and recovery of the method. The combined standardised uncertainties for individual toxins were found to range from 0.08 to 0.26 for all toxins in the carpet, manila and otter clams. In surf clams higher levels of measurement uncertainty were calculated, ranging from 0.33 to 0.34 for the two decarbamoyl toxins, dcSTX and dcGTX2,3. Expanded uncertainties, calculated using a coverage factor (k) of 2, result in a range of values from 0.16 to 0.53 for carpet, manila and otter clams, with expanded uncertainties in surf clams of 0.67 and 0.69 for dcSTX and dcGTX2,3 respectively. Results therefore show a similar range of values for the suite of toxins studied in comparison with the values reported previously for the major bivalve species.

5. Final recommendations

Work presented here has been conducted to determine method performance characteristics for the LC-FLD analysis of PSP toxins in four species of clams: otter clams, manila clams, carpet shell clams and surf clams. The results from these studies have demonstrated the acceptable performance characteristics of the method in relation to pre-specified limits for each of the four species. Consequently, the recommendation is for the implementation of the method for the determination of PSP toxins in these species into the routine UK official control monitoring programme.

Table 24. Summary of method verification data for the LC-FLD analysis of carpet shell clams following AOAC 2005.06.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
GTX 1,4	y	0.074		0.188		98%	106%	70-110	11%	8%		14%	10%		10%		0.20
NEO	y	0.082		0.241		72%	68%	60-120	4%	1%		15%	22%		19%		0.26
C 1,2	y	0.011		0.045		90%	80%	60-120	5%	3%		15%	3%		6%		0.16
dcSTX	y	0.009		0.033		91%	83%	60-120	4%	2%		21%	6%		7%		0.21
GTX 2,3	y	0.038	0.16	0.156	0.32	91%	78%	70-110	5%	2%	15%	23%	3%	25%	6%	None	0.23
GTX 5	y	0.032		0.125		96%	88%	60-120	4%	2%		7%	4%		4%		0.09
STX	y	0.014		0.055		91%	83%	70-110	5%	2%		12%	4%		8%		0.14
dcGTX 2,3	y	na		na		na	81%	None	na	na		na	na		na		na
Mean	-	0.04		0.12		90%	84%		5%	3%		16%	7%		8%		0.18

na = not analysed.

Table 25. Summary of method verification data for the LC-FLD analysis of manila clams following AOAC 2005.06.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
GTX 1,4	y	0.076		0.239		89%	87%	70-110	8%	6%		16%	14%		15%		0.22
NEO	y	0.098		0.259		64%	61%	60-120	7%	7%		12%	7%		7%		0.17
C 1,2	y	0.010		0.046		82%	73%	60-120	6%	2%		13%	5%		5%		0.14
dcSTX	y	0.007		0.035		85%	76%	60-120	4%	9%		16%	6%		6%		0.17
GTX 2,3	y	0.032	0.16	0.156	0.32	89%	74%	70-110	4%	12%	15%	17%	5%	25%	6%	None	0.18
GTX 5	y	0.025		0.124		91%	85%	60-120	2%	6%		5%	3%		8%		0.08
STX	y	0.011		0.054		86%	79%	70-110	3%	7%		8%	4%		4%		0.09
dcGTX 2,3	y	na		na		na	68%	None	na	na		na	na		na		na
Mean	-	0.04		0.13		84%	77%		5%	7%		13%	6%		7%		0.09

na = not analysed.

Table 26. Summary of method verification data for the LC-FLD analysis of otter clams following AOAC 2005.06.

	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
GTX 1,4	y	0.075	0.16	0.294	0.32	89%	84%	70-110	5%	1%	15%	13%	3%	25%	10%	None	0.15
NEO	y	0.084		0.227		70%	64%	60-120	15%	9%		15%	13%		12%		0.23
C 1,2	y	0.010		0.050		89%	75%	60-120	5%	11%		13%	9%		9%		0.18
dcSTX	y	0.008		0.037		93%	81%	60-120	4%	9%		18%	10%		9%		0.21
GTX 2,3	y	0.033		0.169		96%	78%	70-110	4%	12%		19%	10%		10%		0.22
GTX 5	y	0.029		0.136		97%	87%	60-120	2%	6%		6%	5%		7%		0.10
STX	y	0.012		0.059		95%	83%	70-110	3%	7%		10%	8%		9%		0.14
dcGTX 2,3	y	na		na		na	82%	None	na	na		na	na		na		na
Mean	-	0.04		0.14		90%	79%		5%	8%		14%	8%		9%		0.14

na = not analysed.

Table 27. Summary of method verification data for the LC-FLD analysis of surf clams following AOAC 2005.06.

Surf clams	Selectivity	LOD µg/g STX equiv		LOQ µg/g STX equiv		Recovery %			Short term precision RSD%			Medium term precision RSD%			Long term		Standardised uncertainty
		Actual	Limit	Actual	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.2 AL	0.4 AL	Limit	0.4 AL	Limit	
dcSTX	y	0.024	0.16	0.097	0.32	71%	63%	60-120	3%	3%	15%	22%	25%	25%	na	None	0.33
dcGTX 2,3	y	0.006		0.024		88%	83%	60-120	5%	6%		25%	23%		na		0.34
Mean	-	0.015		0.060		80%	73%		4%	4%		24%	24%		na		0.34

na = not analysed.

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Appendix 1. Protocol for method performance checks for PSP LC method on additional shellfish species

The agreed protocol covers the performance checks on LOD, LOQ, recovery and precision of testing. The experimental could all be incorporated into the analysis of shellfish homogenates spiked in triplicate at 0.2 and 0.4 AL (0.16 and 0.32 μg STX eq./g respectively). Analysis of each homogenate in two separate batches over a period of time > 2 weeks, would enable an assessment of medium term repeatability, toxin recovery and would confirm LOD and LOQ at 0.2 and 0.4 AL respectively.

Specificity

- Analysis of MBA-negative shellfish material to determine the presence or absence of matrix interferences present.

LOD

- Analysis of homogenate spiked at 0.2 AL (0.16 μg STX eq./g) per toxin, to confirm presence of toxin chromatographic peaks at s/n ratio ≥ 3:1.

LOQ

- Analysis of homogenate spiked at 0.4 AL (0.16 μg STX eq./g) per toxin, to confirm presence of toxin chromatographic peaks at s/n ratio ≥ 10:1.

Recovery

- Analysis of triplicate homogenates spiked at 0.2 and 0.4 AL for the assessment of toxin recovery at both concentrations. Experiments to be conducted two times over a period of time > 2 weeks, with mean recoveries calculated.
- Recovery of 70% to 110% to be demonstrated for GTX1,4, GTX2,3 and STX and 60% to 120% for other toxins (less prevalent in UK samples).

Precision

- Analysis of triplicate homogenate spikes used for recovery assessment to calculate repeatability in a single batch.
- A performance criterion of ≤15% is to be demonstrated for new shellfish species.
- Analysis of triplicate homogenate spikes used for recovery assessment to calculate repeatability over medium term of the method (> 2 weeks).
- A performance criterion of ≤25% is to be demonstrated for new shellfish species.

Continuous Quality Control during routine analysis

- Unoxidised samples, blanks and standards to be analysed in each analytical batch.
- To be open to active participation in proficiency tests for these species if any are made available.
- Running of control samples of new species – pending further discussion.

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***Appendix 2**: Relative toxicity factors used 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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