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

Final report

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81 pages

Contract Reference: C2934

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Executive Summary

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

Preliminary work involved the assessment of the chemical and chromatographic behaviour of the PSP toxins dcNEO and dcGTX2,3. Development work indicated a need to improve the analytical sensitivity of the N-hydroxylated PSP toxins. New fractionation methodologies were subsequently investigated and resulted in the adoption of a new ion exchange method, enabling the collection of lower sample fraction volumes and resulting in the increased sensitivity of the analysis of the N-hydroxylated toxins. Validation results generally showed that the analysis of PSP toxins in the acetic acid extracts of mussels was selective enough to detect the presence of each toxin peak. Issues were encountered with the early eluting toxins (dcGTX2,3 and GTX1,4) where naturally fluorescing co-extracted components were present at the same chromatographic retention times, thus potentially contributing to false positive toxin assignment. The variable levels of these interferences in naturally contaminated mussels confirmed the need for running unoxidised samples alongside oxidised extracts as the AOAC 2005.06 method proposes, allowing the subtraction of non-toxin contributions from overall toxicity. Whilst subject to a degree of uncertainty, such calculations were shown to reduce the likelihood of false positives.

The linear relationship between the instrumental response and toxin concentration in mussel extract as well as solvent based solutions was shown to be acceptable, both visually and statistically, over the working range of 0 to 1.2 times the Action Limit (AL) for each toxin. Differences were observed between the calibration slopes of matrix-

matched and solvent spiked standards in only two of the toxins (dcNEO and GTX1,4) confirming that the use of matrix-matched calibration standards was not a requirement.

In relation to regulatory limits (0.8 µg STX eq./g shellfish tissue) and a target concentration level of 0.2 AL per toxin, a good level of instrumental sensitivity was illustrated for both the periodate screen (approximately 0.03 to 0.14 µg STX eq./g) and fully determined for the full quantitation method (~0.01 to 0.16 µg STX eq./g detection limits). The method has therefore been shown to detect all toxins in mussels at or below the 0.2 AL target level, with most toxins well below this concentration, showing a similar level of sensitivity to that described in the AOAC method. Limits of quantitation ranged from ~0.01 to 0.16 µg STX eq./g for all toxins except GTX1,4 (0.38 µg STX eq./g). Thus, with the exception of GTX1,4, the method can perform with an acceptable level of sensitivity at toxin concentrations less than or equal to 0.2 AL for each of these toxins. However, the acceptability of medium term precision data for GTX1,4 at 0.2 AL (16% RSD (n=6), HorRat < 2.0) argues that quantitation of this toxin may still be carried out at levels below the LOQ with a good degree of confidence and fitness for purpose.

Method recoveries at 0.2 AL and 0.5 AL showed recoveries to be similar to the levels described in the AOAC 2005.06 method, with results ranging from approximately 60% to 100% for all toxins, with noticeable differences between recoveries for each toxin and at different concentration levels. The extraction method was poor in terms of isolating dcNEO, but this toxin is not present within the AOAC 2005.06 so comparison and assessment was not possible. Extraction recoveries, as measured by exhaustive recovery experiments demonstrated an apparent extraction efficiency of 75-80%. Analysis of two different PSP positive mussel ‘candidate’ reference materials supplied by the NRCC illustrated a fair degree of accuracy associated with the AOAC method. A comparison of toxin profiles between pre- and post-column (NRCC) oxidation techniques showed an excellent level of agreement.

The precision of chromatographic retention times and peak area responses was shown to be excellent (RSD% <5%) and the level of method repeatability over the short term was found to be high at both 0.5 and 0.2 AL for each toxin (RSD < 10% for all toxins except dcGTX2,3 at 0.5 AL (18%)). Medium term repeatability showed acceptable levels of method precision for all toxins at both concentration levels (mean RSD% = 29% at 0.2 AL and 20% at 0.5 AL). Long term precision, assessed with the repeat analysis of a laboratory reference material (LRM) over two months, was also shown to demonstrate a good level of repeatability for the toxins detected (RSDs 11-25%). Further statistical analysis using HorRat values, provided further strong evidence of the acceptability of the repeatability of the method over short, medium and long term.

The ruggedness of the method was experimentally and statistically assessed, with results indicating that none of the method parameters monitored had any effect on method stability. Validation results obtained throughout the study were subsequently used to calculate preliminary standardised and expanded uncertainties for the analysis of PSP toxins in mussels. Combined standardised uncertainties ranged from 0.17 (for STX) to 0.52 (for NEO). Expanded uncertainties calculated using a coverage factor (k) of 2, ranged from 0.34 (STX) to 1.02(NEO).

The validation protocols were trialled with the parallel analysis of naturally contaminated mussels collected as part of the UK marine biotoxin monitoring programme. Results from the screening step of the AOAC method compared excellently with results

previously obtained using the current HCl screening method, and the agreement between HPLC screen positive and MBA positive results was 100%. The correlation between the MBA and HPLC total toxicity results was good, with visual and statistical results from the two methods (correlation coefficient, r = 0.93; t-test t = 0.74 (t-critical = 1.80); mean HPLC/MBA = 101%; RSD = 30%) indicating that there is no significant difference between the two methods, albeit on a relatively limited data set (n=40). An analysis of variability in toxicity equivalence factors demonstrated that different approaches may result in the variability of final toxicity results. However, results demonstrated that the approach taken by Cefas resulted in the highest correlation between HPLC and MBA results.

The method proved to be robust, but it was noted that the fully-quantitative HPLC method is challenging and costly to implement, with clear logistical differences existing between this method and the current use of HPLC-screen and MBA. A high level of training will be required and a significant increase in laboratory investment, equipment maintenance, quality control and sample turnaround times will be noticeable if this method is adopted. Assuming an absence of instrumental and/or QC failures, quantitation data interpretation would be completed on day three following sample receipt, resulting in an overall minimum turnaround time of 52 hours. Contingency measures are already enabled within the Cefas laboratory, but even so, future QC or instrument failures will result in further delays of an additional 24 hours to the already lengthy procedure. This method when exclusively applied to the routine monitoring of PSP toxins in mussels has the potential to provide significant reduction in the overall bioassay usage for PSP toxin determination in compliance with the 3Rs (Replacement, Refinement, Reduction) principles in both European Union (EU) and national legislation. Future work will continue with the validation of the AOAC 2005.06 for other shellfish species of importance to the UK marine biotoxin monitoring programme.

	Linearity	LOD	LOQ	Recovery %		Short term precision RSD%		Medium term precision RSD%		Long term precision	Ruggedness	Standardised Uncertainty
	(r^2)	ug/g STX equiv	ug/g STX equiv	0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL	RSD%	Stability	
GTX 1,4	0.970	0.16	0.38	112%	81%	4%	4%	17%	4%	25%	Yes	0.30
dcNEO	0.989	<0.16	0.16	29%	53%	4%	5%	20%	26%	nd	nd	0.36
NEO	0.963	0.068	0.14	136%	107%	2%	6%	51%	26%	nd	Yes	0.52
dcSTX	0.994	0.007	0.01	85%	68%	1%	8%	32%	23%	nd	nd	0.39
GTX 2,3	0.983	0.087	0.17	94%	66%	7%	9%	22%	10%	17%	Yes	0.26
GTX 5	0.993	0.003	0.006	82%	76%	1%	7%	26%	32%	nd	nd	0.39
STX	0.993	0.018	0.04	122%	68%	0.2%	5%	16%	5%	11%	Yes	0.17
dcGTX 2,3	0.986	0.053	0.11	82%	70%	1%	18%	9%	15%	nd	nd	0.32
C 1,2	0.970	0.018	0.04	79%	63%	6%	10%	41%	10%	nd	nd	0.34
Mean	0.982	0.067	0.123	91%	72%	3%	8%	26%	17%	18%	na	na

Nd = not determined; na = not applicable

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Glossary

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

1. **Introduction**

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

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

An HPLC method, commonly referred to as the "Lawrence method", has been developed and gone through single and inter-laboratory validation [6,7,8,9,10, 11]. In 2005, this method was adopted by the AOAC as an official, first action method (method AOAC 2005.06) [12] and has recently 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 2074/2005 as ammended) [2]. However, as a potential technique for routine Official Control monitoring of PSP toxins the method is still under investigation by a number of monitoring laboratories across Europe, and to our knowledge there are currently no laboratories using the 2005.06 method as the sole statutory monitoring technique for the analysis of PSP toxins in shellfish. Prior to in-house use, it is important that a validation scheme is adopted in order to assess the performance of the method thoroughly. It is also a requirement of EC regulation 882/2004 that official control methods should be validated and quality assured prior to adoption into EU monitoring programmes [13]. Annex III of this regulation sets out the following requirements: "the method of analysis should be characterised by the following criteria: a) accuracy, b) applicability (matrix and concentration range), c) limit of detection, d) limit of determination, e) precision, f) repeatability, g) reproducibility, h) recovery, I) selectivity, j) sensitivity, k) linearity, l) measurement of uncertainty, m) ruggedness n) other criteria that may be selected, as required".

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

present, the extract is fractionated using ion-exchange SPE cartridges, followed by periodate oxidation of the individual fractions.

The objective of the work presented here was to establish the performance characteristics of the method when applied to mussel matrix and validate the AOAC 2005.06 OM method for use in this matrix at the Cefas Laboratory. The validation was to be applied to those PSP toxins described in the OM, which were commercially available as certified reference standards. Two phases were undertaken and for Phase 1, an in-house validation of the method was performed following as closely as possible the guidelines laid down by the International Union of Pure and Applied Chemistry (IUPAC) [4], namely to obtain methodological information regarding selectivity, linearity, limits of detection, limits of determination, accuracy, recovery, instrumental precision, repeatability, reproducibility and ruggedness. For Phase 2, an assessment was made of the method through tandem testing of naturally contaminated mussel samples by the 2005.06 OM and by the reference method.

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2. Materials and methods

2.1 Overview of the AOAC 2005.06 Method

Mussel samples are shucked and homogenised and the shellfish homogenates extracted with acetic acid, before being cleaned up on a C18 Solid Phase Extraction (SPE) cartridge. After pH adjustment, aliquots of the extract are oxidised by periodate reagent in the presence of a matrix modifier, prior to high-performance liquid chromatography with fluorescence detection (HPLC-FLD) alongside periodate-oxidised standards of certified toxin standards. This provides a qualitative screen for the presence of the toxins GTX1,4, NEO/dcNEO/dcSTX, GTX2,3, dcGTX2,3, C1,2, GTX5 and STX. Samples are assigned positive if PSP toxin peaks are present, and positive samples are progressed to full-quantitation. This involves peroxide oxidation of the C18-cleaned extracts in order to calculate the amounts of 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 used during the course of this method as neither oxidant will alone successfully oxidise every toxin to give a suitable level of analytical sensitivity. Peroxide oxidation is utilised for the oxidation of all non-N-hydroxylated toxins (STX, dcSTX, GTX2,3, GTX5, dcGTX2,3 and C1,2) and is deemed to be a sensitive and reliable oxidation technique. N-hydroxylated toxins (GTX1,4, NEO and dcNEO), however, do not respond to peroxide oxidation, and must be oxidised using the periodate reagent. This oxidation method is inherently less reliable, due in part to the significant effect of small pH variations [15], and as such, the AOAC 2005.06 method describes the use of a matrix modifier to be used in all periodate oxidations for both standards and samples, which is believed to result in a more repeatable oxidation, particularly for the oxidation of NEO (Lawrence, personal communication). This modifier is the C18 SPE cleaned up, acetic acid extract of a Pacific oyster sample, which has been shown to be free from chromatographic peaks at the same retention times as any of the PSP toxin standards.

There is ambiguity in the AOAC 2005.06, particularly concerning whether toxins should be quantified pre or post fractionation. Whilst Lawrence indicates that the N-hydroxylated toxins (GTX1,4, NEO and dcNEO) should be analysed by periodate oxidation post fractionation, there is no specific guidance in the method regarding whether non N-hydroxylated toxins should be analysed by peroxide either pre or post fractionation. A decision was taken during this study, to define a set approach to quantitation of each toxin, and this approach would be applied to all phase I and phase II validation samples consistently (Figure 1; table 1). This approach enabled all toxins which have the same oxidation products to be separated, whilst reducing the overall number of analyses per sample to 4 (including the analysis of un-oxidised sample extract), essential in a high throughput environment. As such, the only post-fractionation oxidation undertaken is the periodate oxidation of fractions F2 and F3 (highlighted yellow in Figure 1), whilst the peroxide oxidation of F2 and F3, plus periodate and peroxide oxidation of F1 are omitted from the protocol (highlighted in grey, Figure 1).

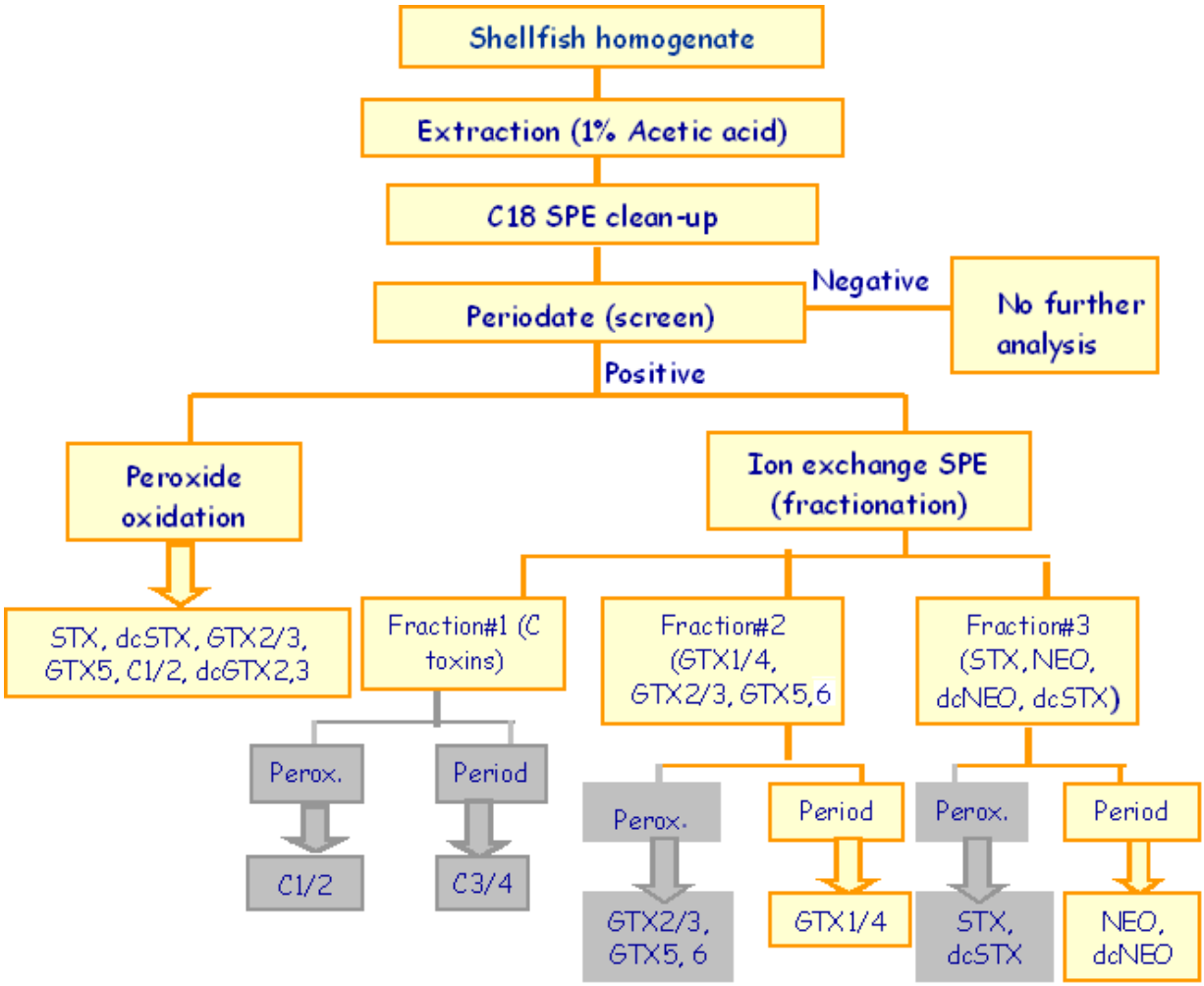


Figure 1. Scheme utilised for screening and quantitation of PSP toxins in mussel samples.

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

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

2.2 Laboratory equipment

The following general laboratory equipment was used throughout the validation scheme: hot water bath capable of holding boiling water, calibrated pH meters, 50 and 15 mL polypropylene centrifuge tubes, 5mL plastic graduated “DEC” tubes (with caps), vortex homogenisers, centrifuge, calibrated analytical balance (4 decimals), calibrated (10 to 1000 µL) pipettes, precision volumetric flasks (series A; 10, 100, 250 and 500 mL), nylon syringe filters (0.45 µm), precision graduated glass test tubes (7mL total

volume, graduated to 6mL), 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), large glass vessels suitable for test-tube deactivation, ice bath, 250mL beakers, 500mL solvent vessels, calibrated timer, Gilson automated SPE system, 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 are supplied prepared in acetic acid and/or hydrochloric acid.

Table 2. Concentrations of certified PSP calibration solutions.

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

Acetonitrile was of HPLC-grade (Rathburn Chemicals Ltd., Scotland) and water either HPLC-grade (Rathburn) or de-ionised water produced in-house. Analytical reagent grade acetic acid (99.9 % pure), ammonium formate (99 % pure), formic acid (>98 % pure), ammonium acetate (99 % pure), hydrogen peroxide, sodium hydrogen phosphate (99 % pure), periodic acid (99 % pure) were from Sigma-Aldrich (Poole, Dorset, UK), sodium chloride (99 % pure) and sodium hydroxide (99 % pure) were from BDH. Toxin standards were diluted in ~4.5g water to give concentrated stock standard solutions. These were subsequently diluted in appropriate volumes of 0.1 mM acetic acid to produce working analytical standards for instrument calibration purposes. The toxicity equivalence factors (TEF) quoted [20] for each toxin were incorporated into the calculations for preparation of calibration solutions for each toxin mix, so that the calibration range for each toxin equated to 0.2 to 1.0 AL in terms of STX equivalence. The exception was GTX5 where calibration solutions were prepared at 10% of the concentration of other toxins (0 to 0.12 AL) due to the very low relative toxicity of GTX5 to STX (Appendix 4). In the case of isomeric pairs (GTX1,4, GTX2,3, C1,2 and dcGTX2,3), the highest toxicity equivalence factor was used for each pair (Appendix 4). Individual toxin results obtained are therefore quoted in terms of µg STX eq./g, and the total PSP toxicity was calculated by summing the individual concentration contributions from all quantified toxins and is quoted in terms of µg STX eq./100 g.

2.4 Samples

Bulk shellfish samples for use in homogenate and extract spiking studies were obtained from M&J Seafood of Poole, Dorset. Approximately 0.5 kg each of both common mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*) (for matrix modifier) were shucked and homogenised on arrival and stored at –20 °C until use. 5.0 g (± 0.1 g) sub-samples were transferred to 50 mL polypropylene centrifuge tubes and extracted and analysed according to the AOAC 2005.06 method and results compared against PSP toxin standards to confirm that both mussel and oyster samples were free from all PSP toxins.

Archived shellfish samples were acquired from the UK Official Control monitoring programmes and had been stored at –20 °C since arrival and homogenisation.

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

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

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

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

An Agilent fluorescence detector (1200 model FLD) was used for the detection of the oxidation products of all PSP toxins. Fluorescence excitation was set to 340nm and emission to 395nm. The peak width was set to >0.2min and the detector gain (PMT) set to 11.

2.6 Optimisation of HPLC and FLD parameters

PSP toxin standards were prepared as working standards in 0.1 mM acetic acid to cover the analytical range equivalent to 0.2 to 1.0 times the action limit (AL) (0.8 µg STX equivalent /g) for each toxin. Individual toxins and toxin mixes were oxidised using both oxidation methods described in the method and 30µL – 50µL injected onto the LC-FLD instrument in order to establish retention times and ensure toxin peaks with a different expected retention time were well resolved. Chromatographic data was reviewed in order to ascertain the retention times and relative peak areas of the toxins oxidised under both oxidation conditions. The AOAC 2005.06 method describes the use of LC-FLD analysis for the separation and identification of the PSP toxins. In addition, two other toxin reference standards dcGTX2,3 and dcNEO recently became available commercially, and these toxins were included in the validation project. It should be noted, however, that these toxins were not validated in the original method and are not

part of the official control method AOAC2005.06, and as such represent a single laboratory extension to the official method. As defined by the FSA, the “target” detection limit in terms of concentration for all PSP toxins was set at 0.2 AL per toxin and thus it was one of the purposes of this work to demonstrate the performance of the method, in terms of sensitivity, at this concentration level.

2.7 Notes on matrix nomenclature and subsequent approaches to validation exercises

The Lawrence method utilises both a C18 clean-up of acetic acid extracts and a COOH-SPE fractionation step (Figure 1). The cleaned up extracts are expected to show variable levels of shellfish matrix co-extractives and therefore may be expected to show different effects on the analytical responses of toxins present within each matrix. As such, the C18 extracts and the fractionated extracts (or fractions) were assessed separately throughout the validation procedure.

For practical reasons, all validation work involving the spiking of mussels with toxins was carried out on homogenate aliquots taken from the same bulk sample. The potential variability between the matrix components present in mussels grown and harvested at different times of the season and in different locations is however noted, as highlighted in previous validation studies within our laboratory [14].

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

This method matches as closely as possible that of the original Lawrence method [7, 9, 10, 11]. Small deviations from the original procedure will be highlighted as such throughout the text of this report. The scheme in 2.1 (figure 1) details the steps involved in the method.

Mussel samples were shucked and homogenised and the shellfish homogenates extracted by heating with a 1% acetic acid solution and the supernatants collected post-centrifugation. A second extraction of the homogenate was performed with a further aliquot of 1% acetic acid at room temperature and the subsequent supernatant added to the first. Extracts were diluted to a known volume and cleaned-up using a solid phase extraction (SPE) SPE cartridges. After conditioning the cartridge with methanol and water, the extract was added to the top of the cartridge and the effluent collected into a suitable graduated collection tube. The cleaned-up extract was then pH-adjusted to pH 6.5 (± 0.5) before diluting the extract with water. Aliquots of this extract were then used for oxidation and subsequent LC-FLD analysis. SPE-COOH ion-exchange clean-up was used for all samples thought to contain N-hydroxylated PSP toxins (GTX1,4, NEO and dcNEO). A sample of cleaned-up extract was passed through an ion-exchange cartridge pre-conditioned with 0.01M ammonium acetate, and the effluent collected into a graduated tube labelled fraction 1 (F1). A further volume of water was added to the cartridge and the effluent collected also in F1. Further volumes of sodium chloride (NaCl) were passed through the cartridge; first a low molarity NaCl solution, then a higher molarity 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 are described below (section 2.9). F1 contains the N-sulfocarbamoyl C-toxins (C1,2 and C3,4), F2 contains the Gonyautoxins (GTX) group of toxins (GTX1,4, GTX2,3, GTX5 and dcGTX2,3) leaving the carbamates (STX, dcSTX and NEO) to elute in F3.

Sample extracts were analysed by first oxidising the relevant extracts and/or fractions to form fluorescent oxidation products. Oxidation methods used throughout the validation work were exactly those detailed in the AOAC 2005.06 method.

2.9 Optimisation of COOH fractionation

Initial experiments suggested that the periodate oxidation of the N-hydroxylated toxins (GTX1,4, NEO and dcNEO) at 0.2 AL would not achieve the level of sensitivity required as defined by the FSA (target detection limit of 0.2 AL per toxin). The AOAC 2005.06 method requires that these N-hydroxylated toxins are oxidised post fractionation. It is clear that such fractionation will introduce a further dilution to the sample extracts (by an additional factor of 2 to 3) and subsequently reduce the overall level of sensitivity for the N-hydroxylated toxins. If detector sensitivity problems are encountered, the AOAC method recommends concentrating each fraction by evaporating the solutions down to a smaller volume. This approach has implications on the application of this method to a routine monitoring programme. The extra time required for this evaporation, together with the additional sample transfer steps (and the additional potential sample losses associated with that transfer – CRL personal communication), would result in the overall sample preparation steps extending to an impractically high level in a routine monitoring environment.

As a result, investigations were conducted into the possibility of altering the fractionation methodology so as to achieve lower final dilution volumes and subsequent increased sensitivity of analysis for the N-hydroxylated toxins. Ion-exchange cartridges were investigated in-house, the most successful being the Phenomenex Strata-X-CW. This cartridge contains a polymer sorbent backbone, rather than the silica stipulated by Lawrence, and an additional reverse phase element. Due to the physical nature of the polymer, a higher degree of sample loading per mg of sorbent can be obtained as compared that exhibited by silica phases. This results in the potential to reduce the overall sorbent weight used in the cartridge and hence decrease the volume of solvent used to elute the fractions. However, the reverse-phase element to the cartridge results in selectivity differences between the Strata-X-CW and the standard silica-bound COOH sorbent. As such, the separation of fractions required in-house optimisation using toxin standards to ensure that the separation achieved by the Strata-X-CW cartridges was comparable to those stipulated in the original method. The results from this optimisation are shown in tables 4 and 5, and indicate that whilst different concentrations of sodium chloride solution must be used to elute fractions F2 and F3, the fractionation pattern observed was exactly the same as described in the AOAC 2005.06 method. Furthermore, low levels of PSP toxin carryover as reported verbally by other laboratories, was not observed using this new method. Hence, this approach represents an overall improvement to the Lawrence fractionation, both in terms of fractionation selectivity and subsequent analytical sensitivity. A third advantage is the lack of cartridge de-conditioning when air is pushed through. Polymer SPE cartridges are therefore ideally suited to automated SPE systems, where instrument optimisation will not require precisely checking that columns do not become dry each time they are used. The ability to push each solvent fully through the cartridges enables repeatable fraction volumes to be collected and ensures that the dilution of each fraction will always be consistent without the need for further manual dilution post fractionation.

Table 4. Fractionation results as defined in Lawrence method

Fraction	Toxins	Elution solvent	Fraction volume
F1	C1,2	Water	6.0mL
F2	GTX1,4, GTX2,3, dcGTX2,3, GTX5	0.05M NaCl	4.0mL
F3	STX, NEO, dcNEO, dcSTX	0.3M NaCl	5.0mL

Table 5. Fractionation results from in-house optimised fractionation using Strata-X-CW

Fraction	Toxins	Elution solvent	Fraction volume
F1	C1,2	Water	5.0mL
F2	GTX1,4, GTX2,3, dcGTX2,3, GTX5	0.3M NaCl	3.0mL
F3	STX, NEO, dcNEO, dcSTX	2.0M NaCl	3.0mL

As a result of this optimisation, the Strata-X-CW ion exchange SPE cartridges were employed throughout the validation project for use in the fractionation stages of the method.

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

2.10.1. Method selectivity

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

2.10.2 Linearity and linear range of the HPLC-FLD method

In order to determine the range of selected toxin concentrations over which the quantitation method can be applied, PSP toxins were spiked into mussel extracts and also shellfish extract-free solvent (0.1mM acetic acid) to give a range of toxin concentrations, before subsequent oxidation and HPLC-FLD analysis. To prepare each calibration level, specific volumes of each PSP toxin mix (toxin mixes defined in section 3.1.2; table 9) were spiked into known volumes of C18-cleaned mussel extract or solvent to produce the following concentrations (expressed in terms of fraction of the action level for STX i.e. 80 μg STX eq./100g): 0.0, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, 1.2, 1.5, 2.0 and 2.5 AL. The exception was GTX5, which was spiked at 10% of the concentrations described above. The linearity of the calibrations was assessed over the standard working range (0 to 1.2 AL; GTX5 = 0 to 0.12 AL) and over an extended range (0 to 2.5 AL; GTX5 = 0 to 0.25 AL). Three aliquots of each calibrant solution were then oxidised and analysed. Linear regression equations were generated and no weighting was placed on the calibration plot. The linearity of the analytical method was initially evaluated graphically, with visual inspection of calibration plots generated for individual toxins. Linear calibration graphs are presented (section 3.3) along with the associated correlation coefficients, gradients and intercepts. Correlation coefficients were generated using all triplicate data points rather than the mean value at each concentration level, and calibration graphs are also plotted as such. Results are displayed together with calibrations generated by the analysis of toxins spiked into a shellfish extract-free solvent (0.1 mM acetic acid). This enables a visual comparison of gradients between solvent and mussel-spiked standards and indicates whether the use of matrix-matched standards is a possible requirement in future sample analysis. Linear plots for fraction spikes are plotted using a wider concentration range for fraction results (0 to > 1.5 AL) than for extract and solvent spikes.

A further examination of the extended data set involved the examination of the residuals after linear regression, graphs for which are also displayed (Appendix 1). Additionally an

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

2.10.3 Determination of limits of detection

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

Predicted LOD = 3C/S

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

Once these predicted LOD values were established, matrix extract spiking was performed at the predicted LOD concentration and triplicate oxidations and injections of the spiked solutions were carried out to confirm the s/n and establish the actual LOD and the variability of the screening step. Results are presented in section 3.4.1.
Full quantitation method LODs were estimated using s/n ratios measured in repeat analyses of mussel tissue homogenates spiked at 0.2 AL for each toxin. Predicted method LODs were calculated using the same calculation as above and further triplicate homogenate spiking was performed at the predicted LOD concentration in order to confirm the measured s/n ratios and LOD values for each toxin. Triplicate oxidations for each triplicate spike were used to assess variability of the amount. Results are presented in section 3.4.2.

2.10.4 Determination of limits of quantitation

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. Mussel tissue homogenate was spiked in triplicate at 0.2 AL for each toxin and the samples extracted, cleaned, fractionated and oxidised according to the method. Using the same approach as above, signal-to-noise ratios for each LC-FLD peak were measured to calculate the predicted concentration which would result in a signal to noise ratio of 10:1. This concentration of each toxin was subsequently spiked in triplicate into mussel homogenates, extracted and analysed to experimentally confirm the predicted LOQs. Results are presented in section 3.4.3.

2.10.5 Determination of the accuracy of the extraction and analytical method for the quantitation of PSP toxins in mussels

The accuracy is defined as the extent of the agreement between the analytical data generated and the ‘true’ concentration value of the analyte [4]. In order to undertake such accuracy studies, it is preferable that a reference material containing a certified quantity of analyte is extracted and analysed. Currently no such material of

PSP toxins is available. In this work, the accuracy determinations were undertaken using two different candidate and non-certified but well characterised mussel homogenate reference materials provided by the NRCC. Extraction of three samples of each reference material was carried out, with each extract subsequently being cleaned-up, fractionated. Triplicate aliquots of each fraction were then oxidised prior to analysis by LC-FLD. The PSP toxins identified were quantified against toxin mix standards prepared in 0.1mM acetic acid and results calculated following the method described by AOAC 2005.06. Samples were analysed blind before final results were sent back to the NRCC for comparative analysis. PSP toxin concentrations in both candidate reference materials were calculated as the mean values obtained from homogeneity studies using NRCC post-column LC-FLD analysis and are thought to accurately represent the toxin profile but not necessarily give highly accurate concentrations (Quilliam, personal communication). However, the use of this material for accuracy studies should at least give further insight into the potential accuracy of the AOAC method and any future release of certified toxin concentrations should subsequently allow a more thorough assessment of method accuracy. Results are presented in section 3.5.

2.10.6 Determination of the method recovery

2.10.6.1. Homogenate spiking

Assessment of the recovery of PSP toxins from mussel tissue involved the spiking of homogenates with known amounts (addition by volume) of each toxin. Each 5 g sample of mussel tissue homogenate was spiked with a PSP toxin mix to provide expected concentrations relating to 0.2 and 0.5 AL for each toxin (with the exception of GTX5 which was spiked at 0.02 and 0.05 AL), assuming 100 % method recovery. For each concentration, three separate 5g aliquots of homogenates were spiked, the sample tube was capped and vortex mixed for 1 min. Tissues were extracted and analysed according to the method described above, with oxidation and analysis carried out in triplicate. Analysis was carried out as soon as possible post-clean-up and oxidation, but in some instances extracts had to be stored overnight (4^{o} C) prior to oxidation. This was due to the high number of analyses required. Quantitation involved the comparison of toxin peak area responses obtained from oxidised spiked samples with those obtained from oxidised toxin mix calibration solutions as described by AOAC 2005.06. Due to issues with spiking high numbers of toxins into samples (data not shown), toxins were spiked as part of a toxin mix (mixes defined section 3.1.2). The exception was dcNEO, where recovery was only assessed as part of a total toxin homogenate spike. Unfortunately, with the current lack of availability of dcNEO standard, it is not possible to repeat these experiments any further for this toxin. Recovery results are presented in section 3.6.

2.10.6.2. Exhaustive extraction

Eleven different naturally contaminated mussel samples were first extracted according to AOAC 2005.06 and the PSP toxin content quantified. The samples ranged in total toxin content between 0.25 and 1.16 µg STX eq./g and all contained varying proportions of GTX1,4, GTX2,3 and STX. Subsequently, the remainder of the tissue material was extracted three more times, each with a further 3 mL of 1% acetic acid, before the combination of all three extract supernatants were diluted to 10 mL, cleaned-up and analysed. Additionally, four spiked mussel tissue samples (spiked with GTX2,3 and STX each at 0.5 AL), and a further mussel tissue spiked with all toxins each at 0.2 AL, were analysed first following the standard AOAC method and secondly following a subsequent triple extraction as described above. Results are presented in section 3.6.2.

2.10.7 Determination of method precision

2.10.7.1 Estimation of instrumental precision

Instrumental precision was assessed in two ways. Firstly, the precision of toxin peak retention times was assessed. Toxin peak retention times were measured on seven separate days over a two month period. Toxin standard mixes prepared in 0.1mM acetic acid were oxidised and analysed by HPLC-FLD and the apex of each chromatographic peak assigned as the toxin retention time. Secondly, in order to assess the instrumental precision inherent in peak area response, one sample of a 0.5 AL Mix IV-spiked Mussel homogenate (GTX5 spiked at 0.05 AL) was extracted, cleaned-up, fractionated and oxidised using both periodate and peroxide oxidants. Each extract and fraction were then analysed six times by LC-FLD. The relevant quantitation peaks were measured and recorded. Peak area data was used to assess the level of instrumental precision inherent in the method.

2.10.7.2 Estimation of method repeatability

Method repeatability was assessed with the repeated extraction, clean-up and analysis of spiked Mussel tissue. Repeatability assessment was also performed involving the repeat clean-up and analysis of naturally contaminated Mussel extracts, although it should be noted that these do not contain the full suite of PSP toxins available as certified standards.

Due to noted instability of toxin oxidation products, instrumental analysis run time and the subsequent limitation on the number of samples that can be analysed in a single batch, also noting that short term (intra-batch) precision studies must be by definition carried out within a single batch of samples, the experiment was designed as follows:

- short term (intra-batch) repeatability was assessed on 3 repeat sample analyses in the same run at both 0.2 AL and 0.5 AL;
- medium term (inter-batch) repeatability was assessed on 6 replicates (0.2 AL and 0.5 AL) analysed in 2 batches of 3 samples, more than two weeks apart;
-long term precision was assessed using the repeated extraction, clean-up and analysis of a naturally contaminated laboratory reference material (LRM) over a period longer than one month, although it is noted that such material does not contain all the individual toxins studied in this project. A sample was selected which contained significant concentrations of PSP toxins and enough sample was collected to produce an LRM which would last several months. On receipt of the sample, the sample was homogenised according to laboratory protocol and 6-7 g aliquots measured into plastic vials, before being labeled with a unique LRM number, capped and stored at –20^{o}C. The homogeneity of the LRM was established previously with the repeat HPLC-FLD analysis (n=12) of C18 cleaned extracts following extraction with hydrochloric acid [14]. Each LRM was removed from the freezer prior to use, allowed to thaw and the required amount of homogenate weighed into a 50 mL centrifuge tube and extracted according to the AOAC 2005.06 method. LRMs were cleaned up by C18 SPE, pH-adjusted, fractionated, oxidized and analysed according to the exact same method applied to samples.

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

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

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

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

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

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

HorRat values will be calculated with values ≤ 2.0 inferring satisfactory levels of precision.

Additionally, an extended precision experiment was devised, involving six different naturally contaminated mussel samples. Extracts remaining from the Mussels extracted as part of the parallel testing (section 4.1) were combined to create five different sample extracts each with a total volume > 15 ml. This allowed the repeated C18 clean-up of each sample ten times. In addition, the extract of a single sample (sample code 811), was cleaned-up a further 6 times (maximum number achievable with available volume of extract). The cleaned-up extracts were all pH-adjusted and fractionated according to the AOAC method. Inter-batch long-term repeatability was investigated by analysing each of the ten cleaned-up extracts in different batches and using different calibrations. This enabled data to be generated which would identify any problems with C18-clean-up repeatability and/or calibration batch quantitation. Intra-batch repeatability was studied by analysing all ten cleaned-up extracts for each sample in the same sequence. HorRat values were again calculated in order to assess the acceptability of intra-batch (short term) and inter-batch (medium term) method precision.

It is recognised that these approaches do not conform exactly to those definitions described in the IUPAC guidelines for single laboratory validation, but this departure represents a necessary approach when dealing with the practical nature of this complex and lengthy analytical method. Precision results are presented in section 3.7.

2.10.8 Method ruggedness

Ruggedness is assessed with the deliberate introduction of small variable changes into the method and the subsequent statistical analysis of any variations in analytical data emerging from these changes. Experimental protocol involves the use of Plackett-Burman design, allowing information to be obtained on the effects of minor changes in an efficient and systematic manner, avoiding the more lengthy approach where each parameter is investigated separately.
Other approaches to determining method ruggedness included the assessment of the effects of changing users for the entire procedure and the use of consumables sourced from different suppliers. Such variables are included in an overall assessment of method long term repeatability and will be reported in the section 3.7.2.
As part of each of the ruggedness experiments, 7 key method parameters were identified and chosen. Due to the complexity of the method and the associated ruggedness testing, and the need to run all ruggedness experiments in the same batch, two separate ruggedness tests were conducted, the first for the non-N-hydroxylated toxins (GTX2,3 and STX) and the second for the N-hydroxylated toxins (GTX1,4 and NEO). This allowed the removal of the fractionation step from the first test, whilst

focussing primarily on the fractionation, periodate oxidation and matrix modifier in the second. Two toxins were chosen for each test due to the high level of toxin standard required for spiking eight replicate homogenate samples. The toxins chosen were those most commonly quantified in the naturally contaminated mussel samples analysed during phase II (section 4.1) and thus potentially of most relevance to the future use of the AOAC method as part of routine PSP monitoring in the UK. The following method parameters were chosen as illustrated by table 6:

Table 6. Experimental parameters chosen for two ruggedness experiments

Parameter	Test 1 (GTX2,3 and STX)	Test 2 (GTX1,4 and NEO)
A	Extraction temperature (A= 100°C, a = 95°C)	C18 extract pH (A= pH6, a = pH7)
B	Vortex time for extraction (B = 30sec, b =90 sec)	Fractionation flow rate (B = 2ml/min, b =3ml/min)
C	C18 clean-up flow rate (C=2ml/min, c =3ml/min)	Periodate oxidant pH (C=8.15, c = 8.25)
D	pH of cleaned-up extract (D=pH6, d=pH7)	Vortex mixing time (D=3s, d=6s)
E	Ambient temperature during oxidation (E=22°C, e=25°C)	Ambient temperature during oxidation (E=22°C, e=25°C)
F	Oxidation time (F= 1min 55sec, f = 2min 5 sec)	Oxidation time (F= 55sec, f = 65 sec)
G	Chromatography flow rate (G=1.9ml/min, g=2.1ml/min)	Matrix modifier (G=Modifier 1, g=Modifier 2)

Extractions were conducted on eight 5g sub-samples of blank Mussel tissue homogenate for each test, each spiked with the appropriate toxins at concentrations of 0.5 AL per toxin. Each sample was subsequently extracted, cleaned, pH adjusted, oxidised and analysed according to the experimental design described below in table 7. For the investigation of matrix modifier, samples were oxidised using two different matrix modifiers prepared using two different sources of Pacific oysters. Matrix modifier 1 was the modifier used day to day in the validation work, being derived from locally sourced Pacific oysters (M&J Seafood of Poole, Dorset) in March 2007. Pacific Oyster homogenate used for Matrix modifier 2 was supplied by the CRL and is believed to be sourced from Spanish waters in August 2007. Both modifiers were kept frozen until use. Samples oxidised using matrix modifier 1 were quantified against calibration standards also oxidised using matrix modifier 1 and those oxidised using matrix modifier 2 were quantified against calibration standards also oxidised using matrix modifier 2.

Table 7. Experimental design for ruggedness testing.

	Experiment number							
Parameter	1	2	3	4	5	6	7	8
A or a	A	A	A	A	a	a	a	a
B or b	B	B	b	b	B	B	b	b
C or c	C	c	C	c	C	c	C	C
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	e	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g
Observed result	s	t	u	v	w	x	y	z

Post analysis, results were statistically analysed (t-test) to assess whether or not the parameters investigated had any significant effect on method stability. Results are presented in section 3.8.

2.10.9 Method uncertainty

Results were used from the phase 1 validation studies to calculate an overall value of uncertainty for the measurement of PSP toxins in Mussel tissue. In order to assess the overall method uncertainty, it was first necessary to identify each of the method parameters that contribute to measurement uncertainty. Once sources were described, the individual component uncertainties were calculated and propagated to estimate an overall measurement uncertainty. Expanded uncertainties were calculated using an appropriate coverage factor (k), in order to provide "an interval expected to encompass a large fraction of the distribution of values that may be attributable to the measurand" [16]. The contribution and effects of matrix modifier and sampling uncertainty are not discussed within this study. Measurement uncertainty results are presented in section 3.9.

2.11 Phase II. Analysis of routine Mussel samples.

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

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

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

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

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

Ordinarily, only positive samples would be progressed to full quantitation using the full AOAC 2005.06 method for further evaluation. However, for the purposes of this study, all samples were fully quantified to ensure none of the samples screened negative exhibited any positive toxin concentrations in subsequent quantitation. PSP toxin concentration data derived from LC-FLD analysis were compared with MBA results and the correlation between the two data sets analysed statistically to determine whether there is any significant difference between the two methods. Results from the phase II testing are given in section 4.

<u>Internal quality controls (IQC):</u>
Draft SOPs have been written following completion of the phase 1 and phase 2 validation studies described in this report which detail the application of IQC to the

future routine monitoring of mussels by the AOAC 2005.06 method. Parallel testing of naturally contaminated mussels in particular allowed an assessment on the numbers of samples which could be run in an analytical batch and highlighted the need for a good level of QC throughout the lengthy and complex analytical batches.
The full quantitation sequence begins with a number of instrumental blanks and subsequently consists of initial calibrations of the three toxin standard mixes at five different concentration levels, evenly spaced over the 0 to 1.0 AL working range. Correlation coefficients calculated from these calibrations are checked to ensure toxin oxidation of standards is successful and the calibration curve fit for purpose for toxin quantitation. The sensitivity of analysis is checked by ensuring the signal to noise ratios of all quantitation peaks are $\geq$ 3.0. The most significant secondary peak must also be present for the toxins with more than one oxidation product. Retention times of initial calibration toxin standards are used to assign retention time windows enabling the integration and quantitation of toxins in samples. The retention time drift of toxin peaks within an analytical batch must be $\leq \pm 2.5\%$. A system suitability test is performed to assess the chromatographic efficiency of the instrumental system, with values generated for peak resolution, peak tailing and column efficiency (plates per metre). Acceptable tolerances have been defined and will be applied to the method. Within each batch, a procedural blank is run alongside the samples throughout the entire procedure (using 5 g 1% acetic acid or water instead of Mussel tissue) and oxidised with periodate and peroxide oxidants prior to LC analysis. Additionally, an un-oxidised aliquot of C18 cleaned extract of each sample is analysed alongside the oxidised samples, so as to determine whether any of the peaks were due to the presence of naturally-fluorescent co-extractive interferences. Any components identified in either of these controls, are subtracted from the peak responses of any PSP toxins with the same retention times. A laboratory reference material is extracted, cleaned, fractionated (if applicable) and analysed alongside each batch of samples. The toxin areas (for screening analysis) and concentrations (for quantitation analysis) calculated for the peaks of interest are recorded on a Shewhart quality control chart and must fall within ± 3 standard deviations (SD) for each toxin analysed. The low level (0.2 AL) calibration standard for each toxin mix is run through the sequence after every 5 samples, each sample consisting of 4 separate analyses as described previously. The results from the LRM are used to ensure signal to noise ratios of each quantitation peak are $\geq$3.0 and that peak area responses for each quantitation peak do not drop more than 20% during the analytical sequence. The analytical batch may continue for up to 24 hours.

Toxin quantitation

Each toxin was quantified by direct comparison of peak areas to external, certified analytical standards prepared at 0.2, 0.4, 0.6, 0.8 and 1.0 AL in 0.1 mM acetic acid for each toxin (GTX5 prepared at 10% concentration of other toxins). Calibration solutions were prepared and oxidised in suitable mixes of toxins as advised by the AOAC 2005.06 method. Individual toxin peak areas were included in the toxicity calculations if the diagnostic peak exhibited a signal to noise ratio of $\geq$3.
With the potential for issues with the use of specific TEFs and the current use of conservative values for isomeric pair TEFs, the effects of variability of these factors were examined in relation to final toxicity calculations. The former was undertaken to assess the effects on possible inaccuracy with the Oshima [20] TEFs by comparing the results obtained with those calculated using the TEFs quoted by Genenah and Shimizu [21]. The latter was tested to model the potential for the lowest toxicity toxin isomer to be present and thus examine the degree to which over-estimation of PSP toxicity is possible (table of TEFs given in Appendix 4).

3. Results and discussion

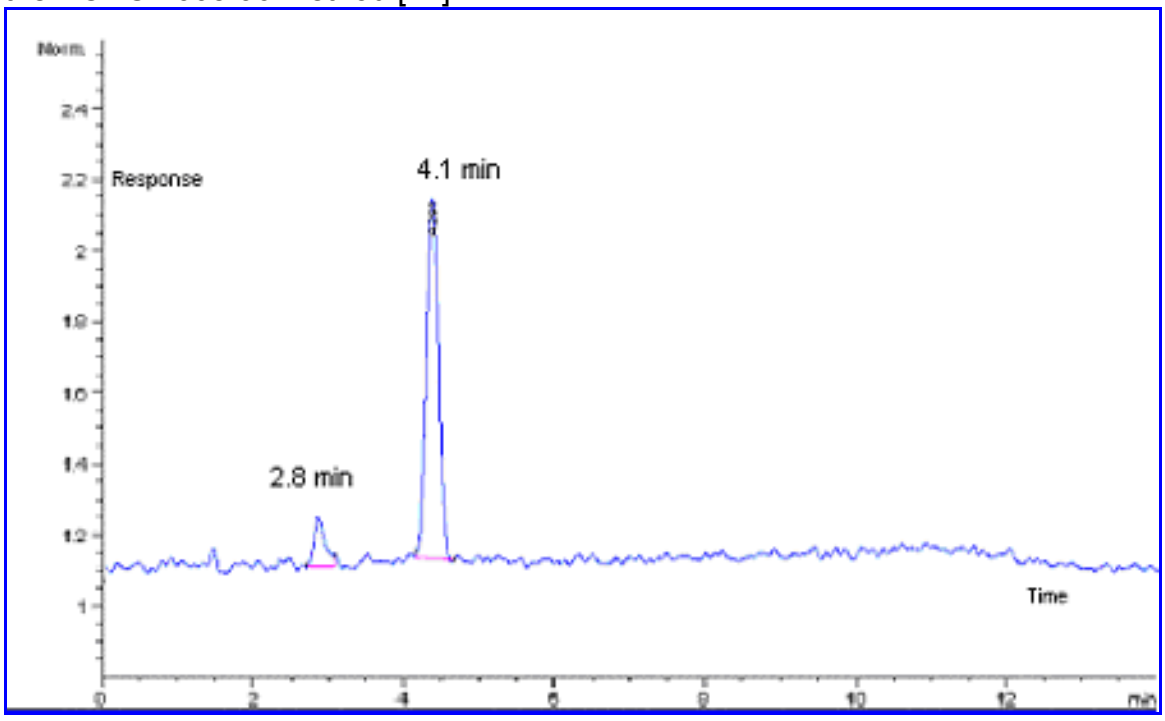
3.1. Method refinement

3.1.1. Individual toxins in 0.1mM acetic acid.

The chromatographic characteristics of GTX1,4, NEO, GTX2,3 dcSTX, GTX5 and STX has been described previously by Cefas and shown to be comparable to results obtained by Lawrence [14]. Results reported in this section focus on the three new toxins used in this validation programme(C1,2, dcNEO and dcGTX2,3). Chromatographic results enabled a comparison between the success of each oxidation method to these toxins and enabled decisions to be made regarding the use of toxin mixes for standard calibrations (table 8).

C1,2

Periodate oxidation of C1,2 generated one major fluorescent product with an analytical retention time of approx. 4.2 minutes (Figure 2a). Peroxide oxidation generated a product at the same retention time (Figure 2b), although it was noted that the peak area size of the peroxide peak was ~10 times larger than that observed in the periodate, indicating that peroxide would be the more suitable oxidant for C1,2. Additionally, a small impurity peak eluting at 2.8 minutes (with GTX1,4 and dcGTX2,3 – see below) was found to exhibit a peak area size 2-3% of the main diagnostic peak in both periodate and peroxide. Whilst it may be the case that this peak represents a small GTX1,4 or dcGTX2,3 impurity in the C1,2 toxin, these toxins are not shown to be present in the NRCC standard documentation so would not be expected to be seen in the chromatograms. It is therefore possible that this small impurity represents a smaller secondary oxidation product of C1,2. Evidence for this comes from the fractionation of a pure C1,2 standard showing the 2.8 minute peak eluting in fraction 1 (F1) along with the main C1,2 peak, whereas a GTX1,4 or dcGTX2,3 impurity peak would be expected to elute in fraction 2 (F2). These results agree with chromatographic results described in the AOAC 2005.06 method [12].



(a) periodate oxidation

Figure 2. LC-FLD chromatograms of C1,2 in 0.1 mM acetic acid.

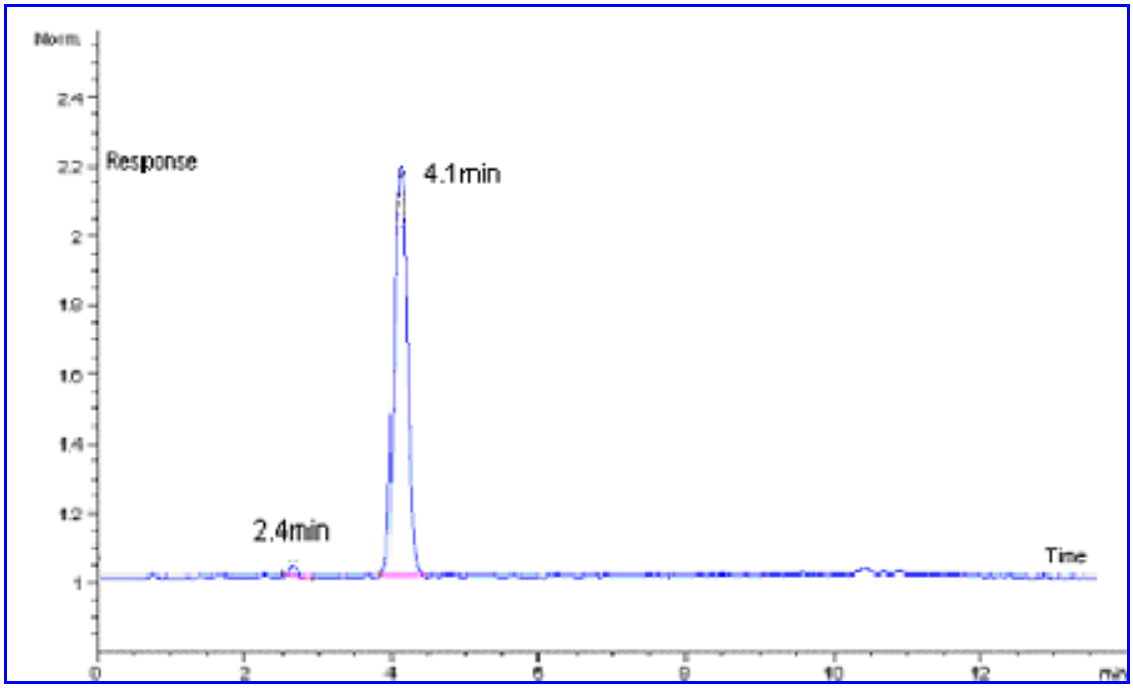
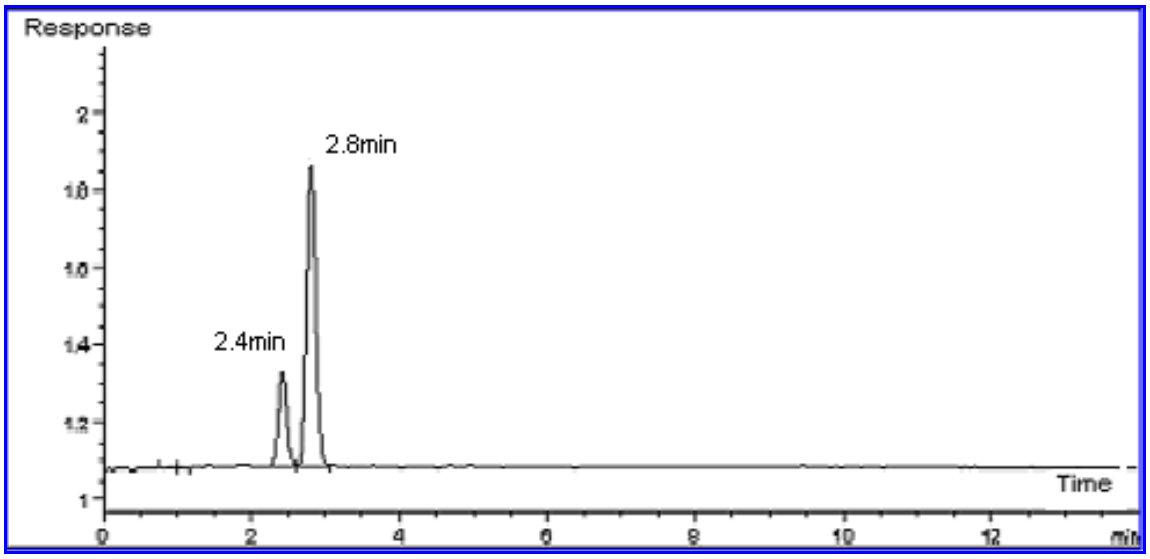


Fig 2(b) peroxide oxidation

dcGTX2,3

This toxin was not included in the Lawrence study [1,6,7,8] and there are no current guidelines for establishing the diagnostic peak. Periodate and peroxide oxidation of dcGTX2,3 each generated two fluorescent products, with retention times of approx. 2.4 and 2.8 minutes (figure 3a,b). The 2.8 min peak co-elutes with the quantitation peak of GTX1,4, but the 2.4 min peak is unique amongst the PSP toxin suite, if the unknown C1,2 impurity peak is disregarded. It was additionally observed that the relative intensities of the two peaks are inversed when comparing the two oxidation methods, with the largest peak in periodate being 2.8 min and the largest in peroxide being 2.4 min. Therefore the unique 2.4 min peroxide peak is the peak of choice for quantitation purposes. However, it should be noted that the presence of dcGTX2,3 in any sample will compromise the quantitation of GTX1,4 unless the contribution of dcGTX2,3 to the GTX1,4 peak can be estimated and removed (see 3.1.2.1 for discussion).



(a) periodate oxidation
Figure 3. LC-FLD chromatograms of dcGTX2,3 in 0.1 mM acetic acid.

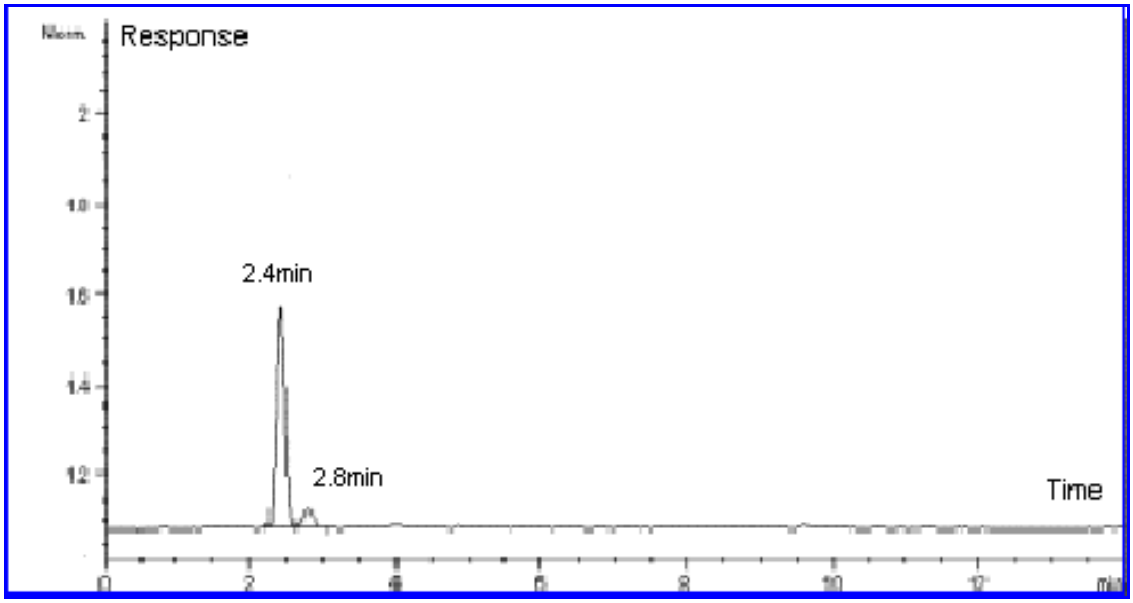
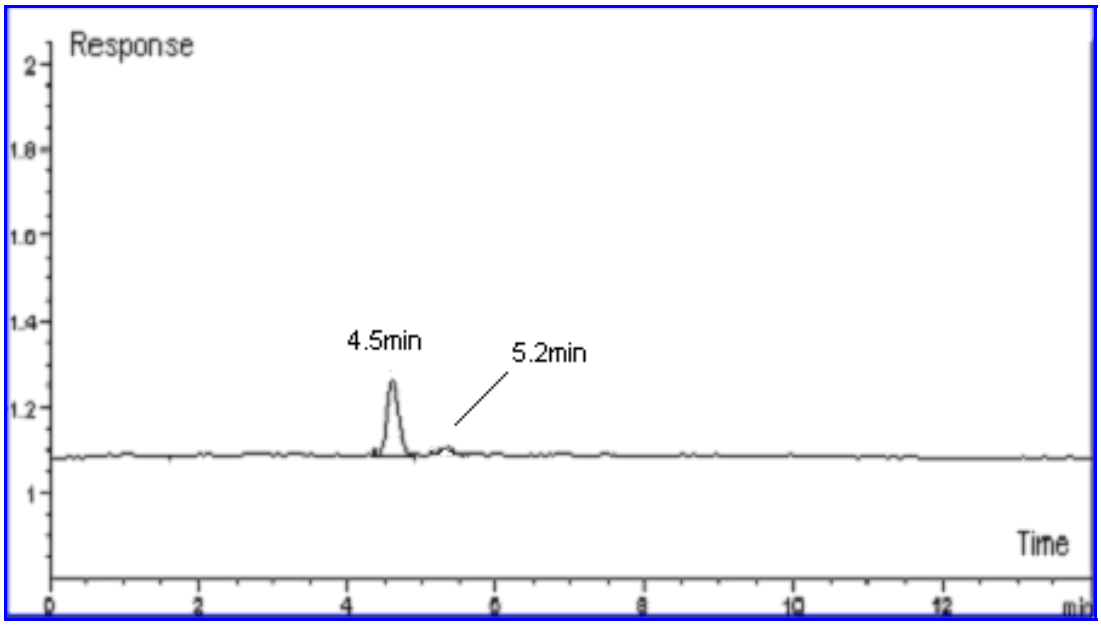


Fig 3(b) peroxide oxidation

dcNEO

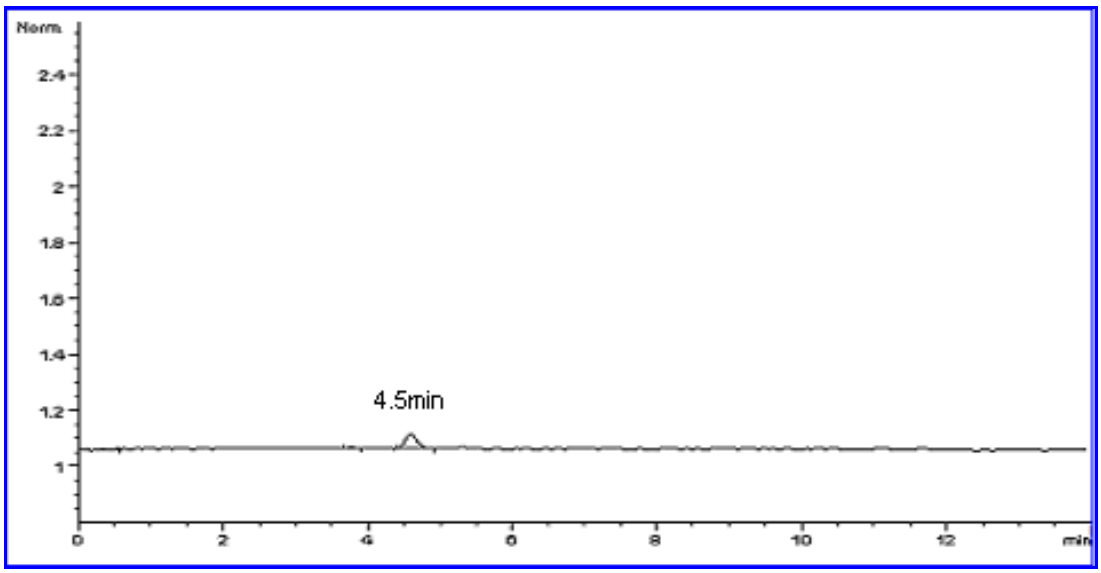
As an N-hydroxylated toxin, dcNEO, is only expected to exhibit fluorescent products after periodate oxidation. As shown in figure 4a, a prominent peak is observed in the chromatogram of dcNEO after periodate at approx. 4.5 minutes. This peak therefore co-elutes with the smaller of the two major dcSTX peaks after periodate oxidation. An additional peak is seen at ~5.2 minutes, with the same retention time as the other major dcSTX fluorescent periodate product.

Peroxide oxidation shows the presence of one small fluorescent product (figure 4b), also with a retention time of ~4.5 min. This is thought to be an oxidation product of dcSTX, as the certificate of analysis for the NRCC dcNEO standard shows the presence of a minor impurity of dcSTX within the dcNEO standard. Due to the relative size of the possible dcSTX impurity to the dcNEO, it is thought that the effects of this impurity on dcNEO quantitation will be negligible.



(a) periodate oxidation

Figure 4. LC-FLD chromatograms of dcNEO in 0.1mM acetic acid



(b) peroxide oxidation

3.1.2. Toxin mixes

Table 8 shows the full range of toxin peak retention times and the individual toxins and co-eluting found within the chromatogram. Lawrence previously recommended the use of two toxin mixes for convenience of working standard preparation and subsequent analysis. However, with the inclusion in this study of three more toxins (C1,2, dcNEO and dcGTX2,3), two of which have found to contain co-eluting fluorescent products with other toxin peaks, three toxin mixtures were designed and used for toxin standard analysis, with a fourth utilised containing a mixture of all available toxins (Table 9).

Table 8. Chromatographic PSP toxin peaks exhibited after periodate or peroxide oxidations

Approx. Retention time (min)	1.9	2.4	2.8	3.3	4.1	4.5	5.2	6.9	8.8	9.5
Toxin peaks after periodate oxidation [a]	GTX1,4	dcGTX2,3	GTX1,4 dcGTX2,3	NEO dcSTX[b]	C1,2	dcNEO dcSTX	NEO dcSTX	GTX1,4 GTX2,3	GTX5	STX NEO
Toxin peaks after peroxide oxidation [a]		dcGTX2,3	dcGTX2,3	dcSTX[b]	C1,2	dcSTX	dcSTX	GTX2,3	GTX5	STX

[a] Peak considered as diagnostic quantitation peak is underlined
[b] Not clear whether early eluting dcSTX peak is real oxidation product or not, as other laboratories do not see this peak [14]

Table 9. Toxin mix nomenclature utilised during this study.

Toxin Mixture	Toxins
I	GTX1,4, NEO, dcNEO
II	STX, dcSTX, GTX2,3, GTX5
III	C1,2, dcGTX2,3
IV	All the above toxins

The first part of the AOAC2005.06 method involves the periodate oxidation of C18-cleaned extracts to obtain qualitative information on the presence of PSP toxins within the shellfish sample. Hence, it is important to assess the chromatographic behaviour of the three toxin mixes after periodate oxidation. Chromatographic results are shown in Figures 5, 6 and 7.

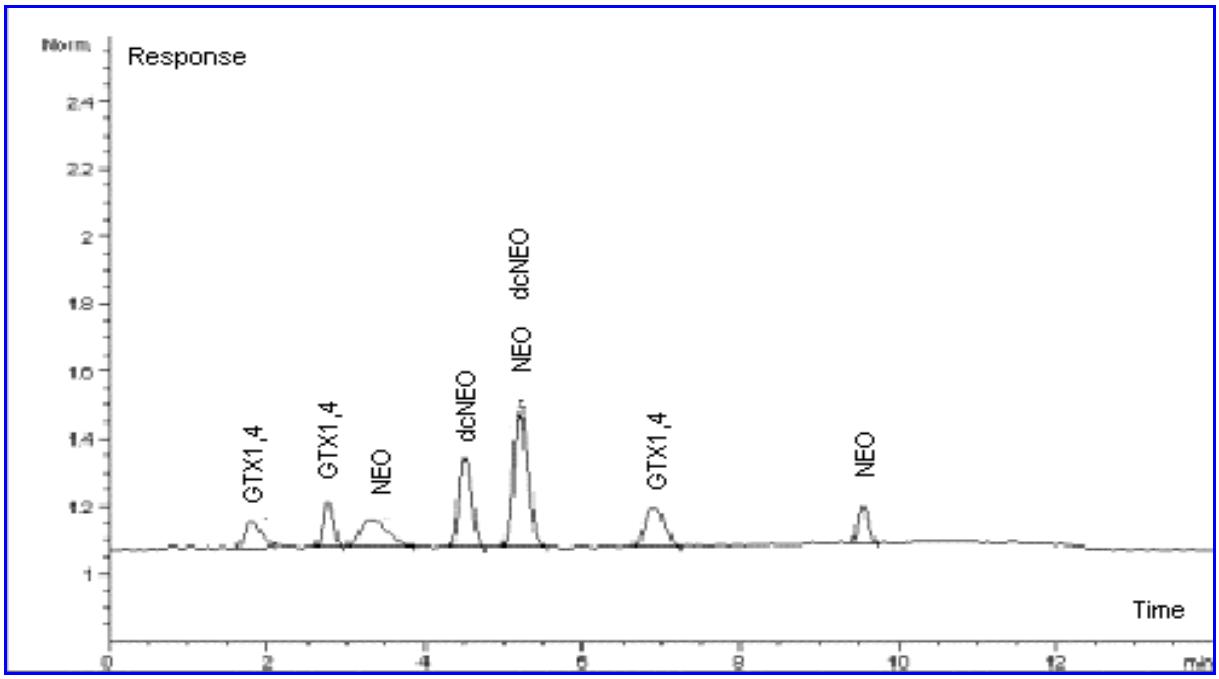


Figure 5. Chromatograms showing periodate oxidation products of Mix I standard (0.5 AL) in 0.1mM acetic acid.

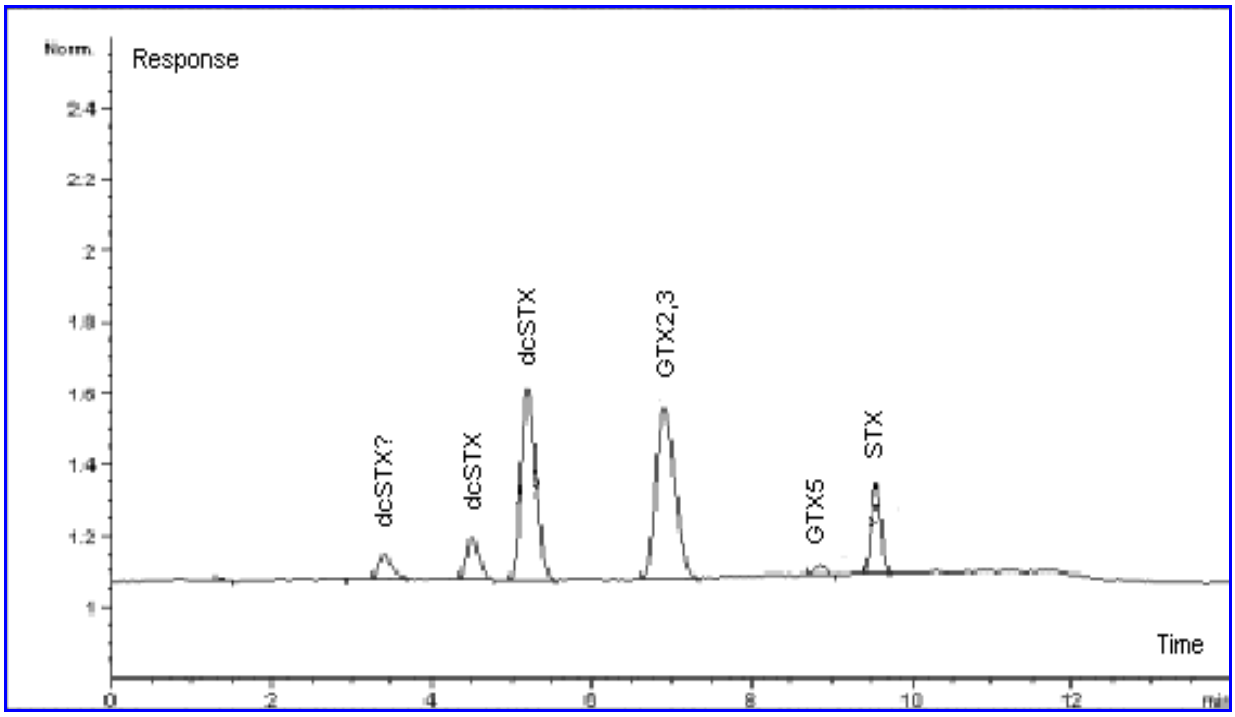


Figure 6. Chromatograms showing periodate oxidation products of Mix II standard (0.5 AL) in 0.1mM acetic acid.

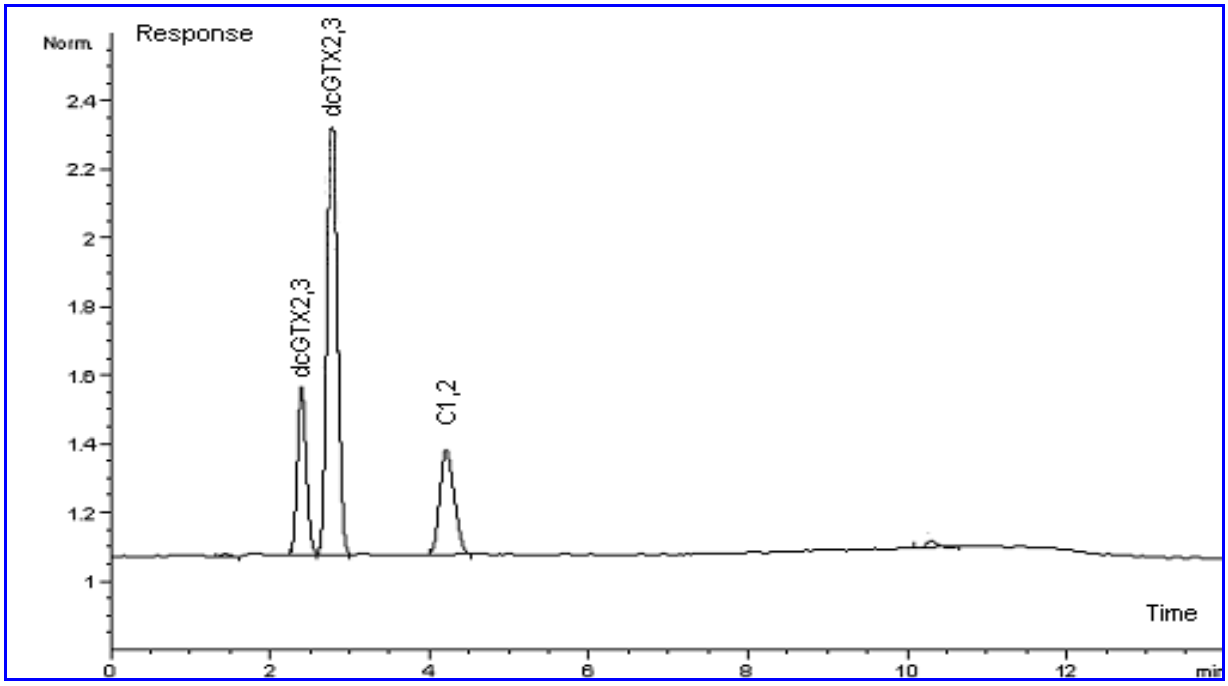


Figure 7. Chromatograms showing periodate oxidation products of Mix III standard (0.5 AL) in 0.1mM acetic acid.

Visual inspection of the chromatograms of the three toxin mixes indicated that all chromatographic peak shapes were symmetrical, with the exception of the non-quantitation secondary NEO peak at ~3.3min. No peak tailing was observed even at the highest concentration of standard (1.0 AL per toxin; data not shown). Peaks were well resolved within individual toxin mixes, indicating that the choice of chromatography column was good and the toxin mixes chosen would be suitable for the application of this LC-FLD method.

The chromatographic profile of the total toxin mix (Mix IV, with matrix modifier) was also established using periodate oxidation. Figure 8 shows the peaks of Mix IV at 0.6 AL eluting at the expected retention times (table 8), and shows that most of the toxin peaks are resolved. The exception is the C1,2 toxin (4.2 min) which partially co-elutes with dcSTX and dcNEO at 4.5 min. This indicates the potential for quantitation inaccuracies to occur during analysis of naturally contaminated samples containing elevated levels of C1,2 with either dcSTX or dcNEO. A further point to note was the presence of early eluting interferences present due to the matrix modifier which co-elute with the 2.4 min dcGTX2,3 peak. Further selectivity studies (see section 3.2) and linearity calculations should help determine whether or not such interferences will significantly affect the quantitation of dcGTX2,3.

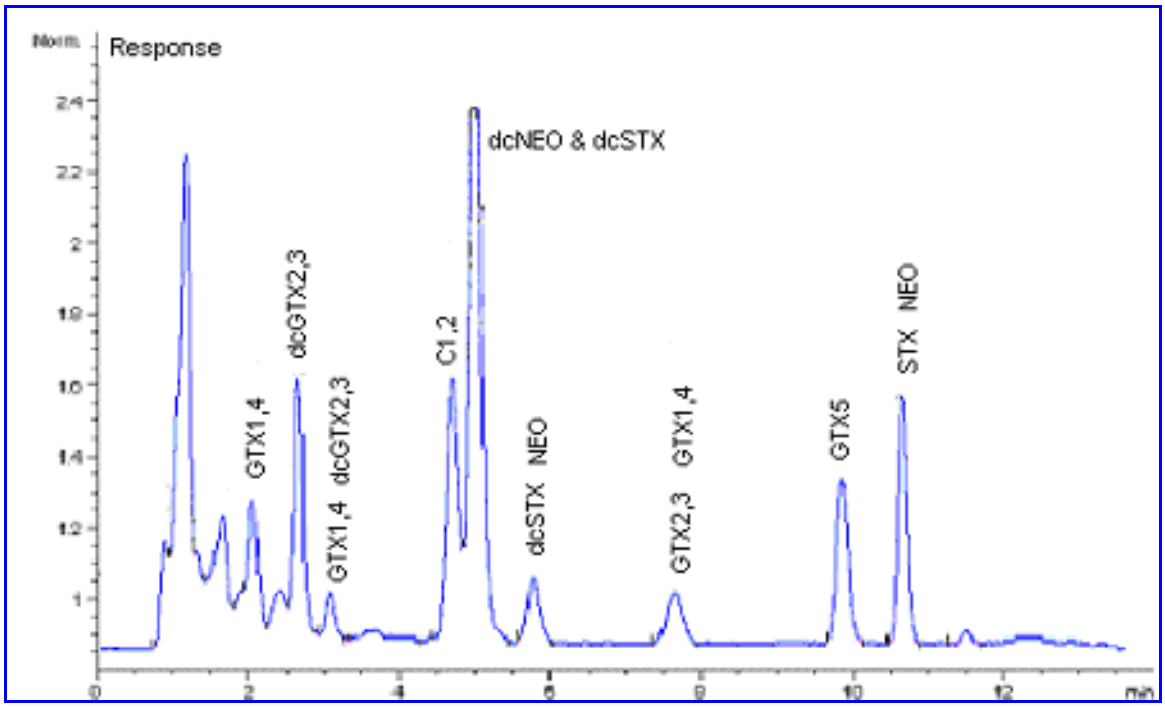


Figure 8. Mix IV (0.6 AL) analysed by LC-FLD post periodate oxidation with matrix modifier

As mentioned in section 2, there are co-elution issues which may only be resolved if the contribution of interfering toxin components are removed. The co-elution of GTX1,4 and GTX2,3 at 6.9 min and STX and NEO and 9 min is resolved with the separate use of periodate and peroxide oxidations, as described in the AOAC method [12]. Lawrence additionally recommended the use of peak ratios between periodate and peroxide to assess and remove the estimated contribution of dcSTX to NEO at 5.3 minutes [12] but this is no longer possible with the presence of dcNEO in standards and potentially in samples. Similarly, the dcGTX2,3 contribution to the GTX1,4 diagnostic peak at 2.8 minutes will need to be removed as will the dcSTX contribution to dcNEO at 4.5 minutes. The following techniques will therefore be used to assess and remove such effects:

GTX1,4 (2.8 min): The contribution of dcGTX2,3 to the GTX1,4 chromatographic peak at 2.8 min is removed by estimating the peak area of dcGTX2,3 which would be expected at 2.8 min. This calculation is performed in the following way. Firstly, the ratio of peak areas is measured between the 2.4 min and 2.8 min dcGTX2,3 peaks in the

periodate oxidised Mix III standards. Secondly, the concentration of any dcGTX2,3 toxin quantified in a sample by peroxide oxidation is calculated. Thirdly, this concentration is multiplied by the ratio obtained above to estimate the peak area of dcGTX2,3 expected at 2.8 min after periodate oxidation of the sample. Finally, this value is subtracted from the total peak area measured at 2.8 min in the sample, the result giving the total GTX1,4 peak area, minus the contribution of dcGTX2,3. To reduce the number of oxidations and analyses required, the dcGTX2,3 peak present in fraction F2 will be used rather than periodate-oxidising a second aliquot of the C18-cleaned extract.

NEO (5.3 min): A complication with the method relates to the presence of dcNEO in the toxin suite and the effect this has on the quantitation of NEO in the presence of dcSTX. As the AOAC 2005.06 method suggests, due to the co-elution of the NEO and dcSTX, the relative peak areas of dcSTX in both periodate and peroxide are to be used in order to estimate the amount of dcSTX which is contributing the NEO quantitation peak. However, dcNEO, a toxin not studied in the original 2005.06 method, co-elutes with the major NEO peak and as such, NEO cannot be directly quantified as suggested in the AOAC 2005.06 method. The amount of dcSTX present in the sample is therefore first quantified using the peroxide oxidation of both the sample and the dcSTX calibration standard. The calculated concentration of dcSTX is then used against a calibration graph for dcSTX generated using periodate oxidation to calculate the expected peak area of dcSTX at 5.3 min in periodate. This value is then subtracted from the overall peak area at 5.3 min to give the estimated peak area for NEO alone and subsequent concentration of NEO in the sample.

dcNEO (4.5 min): Similarly to the removal of dcSTX from NEO, the dcSTX contribution to the 4.5min dcNEO must be removed by subtracting the expected periodate peak area of dcSTX calculated from the calibration curve of dcSTX at 4.5 min in periodate. In all three cases, it is noted that such calculations involve a high degree of estimation and as such will contribute significantly to the overall uncertainty of the method for these three toxins. However, these approaches have been recommended by the CRLMB [17].

3.2. Selectivity of the method

In order to assess whether components of the mussel matrix may have an effect on the quantitation of PSP toxins, the C18 cleaned PSP-free mussel tissue extract was analysed un-oxidised and after both periodate and peroxide oxidations. The results indicate an example of the interferences observed in chromatograms, but it is noted that variability of co-extractive interferences is expected to vary from sample to sample, as highlighted by Cefas by previous work [14].

Un-oxidised extracts

A complex profile of co-extractive matrix components was observed (Figure 9), in particular showing substantial early interference peaks, not clearing the column until 3.8 – 4.0 min. A substantial peak, was identified coinciding with the secondary peak of NEO, (Rt 3.4 min), although other NEO peaks were not observed. A large peak is identified at ~3.3 min and could compromise identification of the secondary NEO peak. However, this component does not interfere with the quantitation peaks of any of the PSP toxins. A peak of > 3.0 signal to noise ratio is also seen at 4.7 mins. This has the potential to be inadequately resolved from dcSTX/ dc NEO peak at 4.5 min, and could introduce additional inaccuracies to the quantitation of dcSTX. This in turn will have an effect on the estimation of the dcSTX contribution to the NEO and dcNEO quantitation peaks.

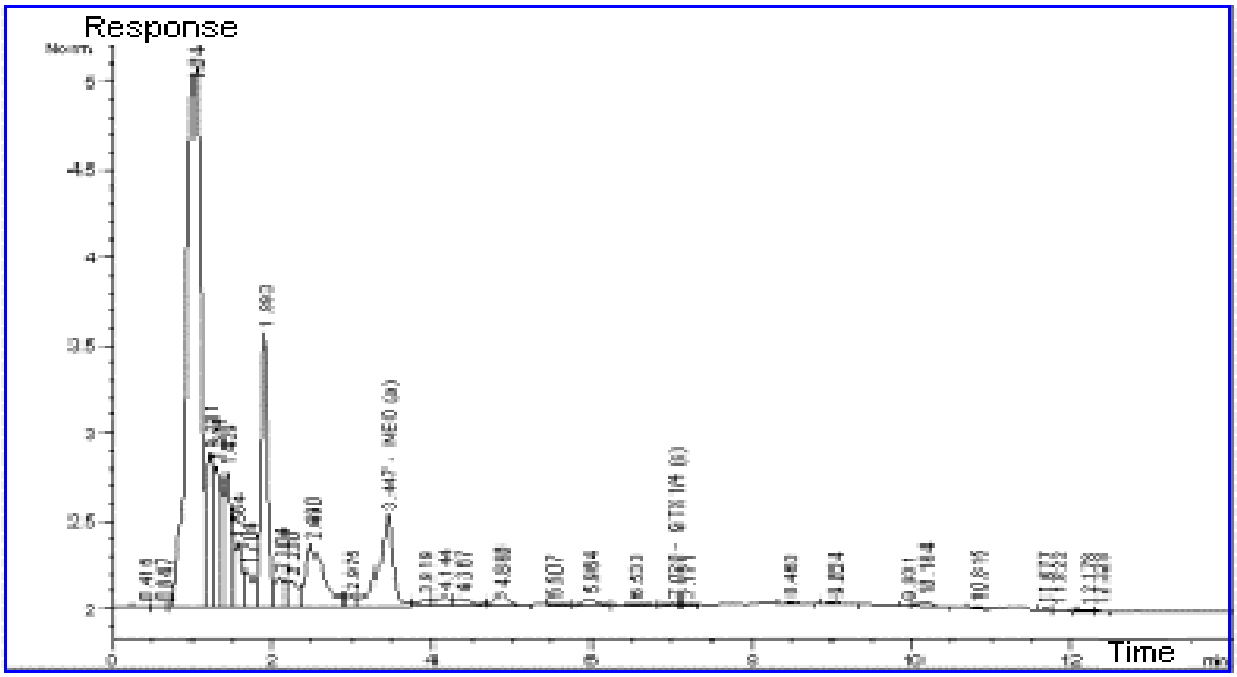


Figure 9. LC-FLD chromatogram of un-oxidised C18-cleaned, mussel extract

Periodate oxidation

A similar profile is observed (figure 10), although relatively large amounts of naturally-fluorescing compounds are again present and elute up to 3.5 min. These components, as described above, may cause problems in the correct identification and the accurate quantitation of GTX1,4 and dcGTX2,3. Definite early eluting peaks similar to those retention times of GTX1,4 and dcGTX2,3, (Rt's 1.8, and 2.8), were not identified in these samples, but the location of a general background of interference could potentially cause problems with identification and quantitation of these toxins.

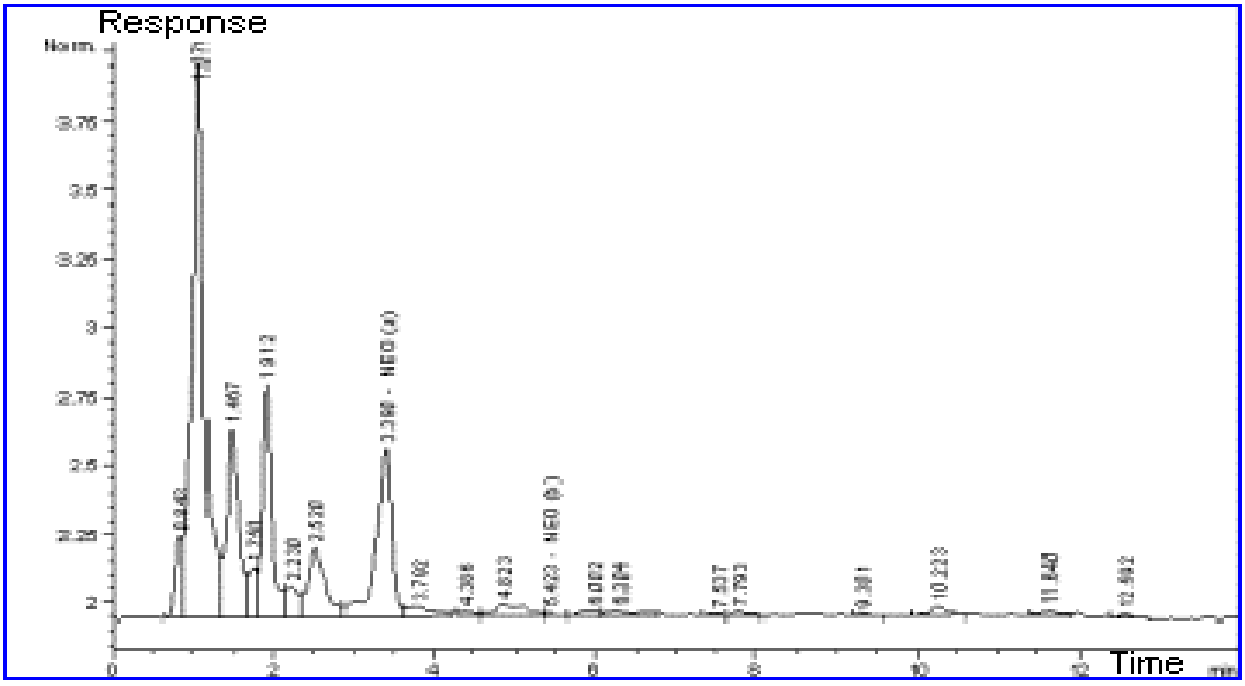


Figure 10. Chromatogram of C18-cleaned, PSP toxin-free mussel extract after periodate oxidation

Peroxide oxidation

Analysis of the mussel extract following peroxide oxidation illustrated another complex profile (figure 11), but arguably 'cleaner' relative to the periodate profile shown in figure 8. Large amounts of material are noted eluting from column up to 4.0 min, with smaller, 'shoulder', type peaks up to 5 min. There is a notable peak at Rt.3.4min, in all aliquots, and a further small peak at around 4.7 min, which could interfere with quantitation of the dcSTX peak at 4.5 mins. With large amounts of interfering material eluting up to 2.4 min, the dcGTX2,3 peak may be compromised and the level of methodological uncertainty increased during quantitation. A 'noisy', baseline at the retention times of GTX 5 and STX, (8.8 – 9.6 min), could influence identification and quantitation of these toxins, although the signal to noise ratio was measured as < 3.0.

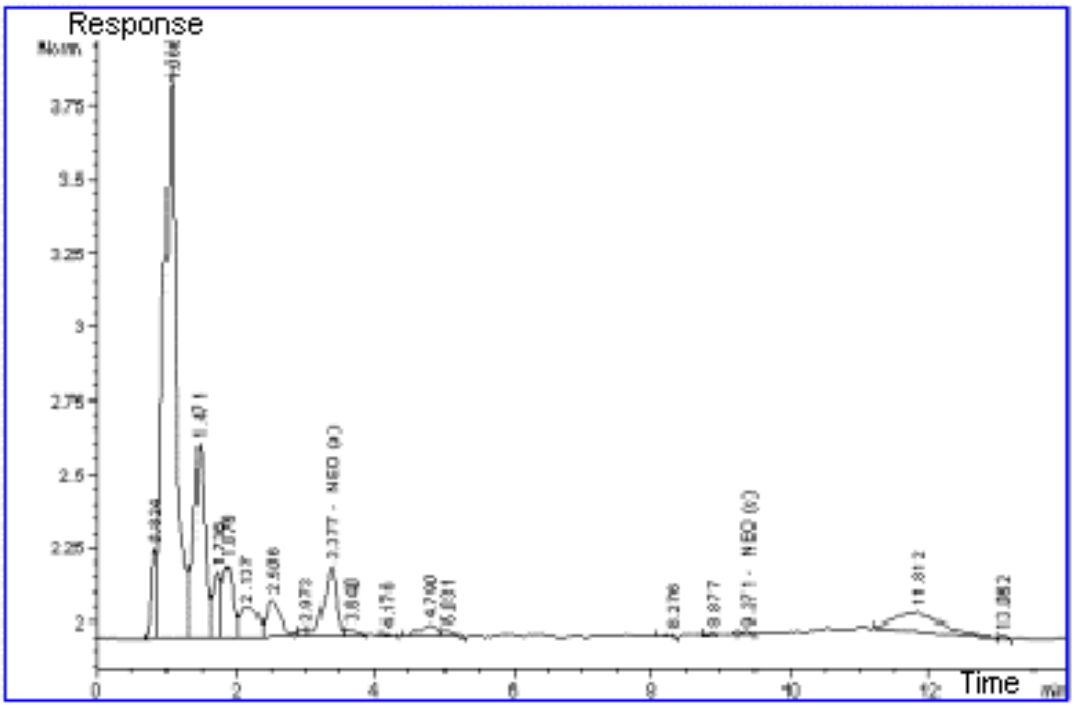


Figure 11. Chromatogram of C18-cleaned Mussel extract after peroxide oxidation

3.3. Linearity of the analytical method

The LC-fluorescence detector was calibrated with individual PSP toxins prepared in either acetic acid or mussel extracts (after C18 and fractionation by COOH SPE). The linearity was tested by fitting a sequence of statistical models to the signal responses of each level of calibrant solution to find the best fit. R-squared correlation coefficient (r^2) measures the proportion of variance explained, so is usually interpreted as indicating the usefulness of the model. In all cases, results showed that a linear-fit model is the preferred model, with separate slopes for each matrix (solvent and extract/fraction). A summary of all the results is shown in table 10 and the graphical results for each toxin are presented individually as follows.

3.3.1. Linearity for GTX1,4

Quantitation of GTX1,4 is performed by analysing the presence of this toxin in fraction F2, generated post ion exchange fractionation. Linearity observed in the fraction calibration graph (figure 12) appears acceptable (r^2 = 0.970) and the random errors measured in the response factors were in the order of 14 %. The values calculated for

the linearity of GTX1,4 in fraction matrix result in a calibration gradient with a gradient noticeably lower than the gradient of the solvent graph, indicating possible peak suppression associated with the fraction extract. As such, toxin concentrations in real samples may be underestimated using a solvent calibration.
The F-test goodness of fit results and F-critical value for the data set and visual examination of the residual plots (Appendix 1) for the fraction plot indicates a linear relationship exists between toxin concentration and detector response over the tested range.

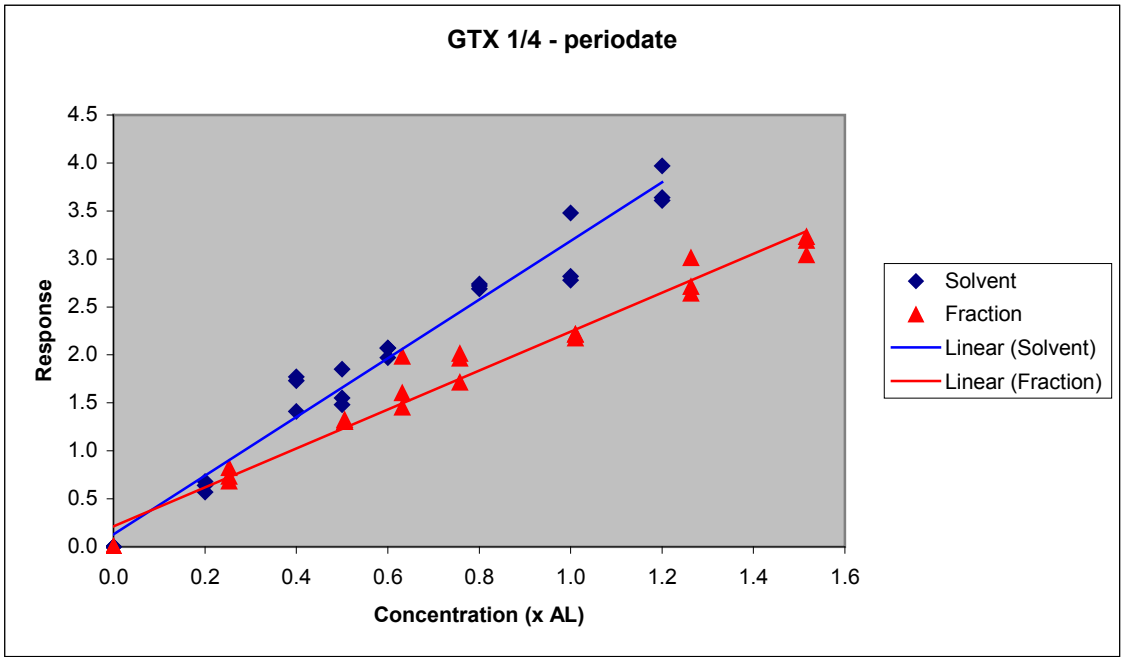


Figure 12. Calibration plots of GTX1,4 concentration against detector response for standard prepared in mussel extract fraction (F2) and solvent over the working calibration range of 0 to 1.2 AL.

3.3.2. Linearity for dcNEO

Figure 13 shows an apparent visual linearity for both F3 fraction and solvent matrices-based dcNEO calibrants. The intercept value is small and the F-test goodness of fit value is significantly lower than F-critical, indicating a linear relationship exists. Visual examination of the residual plots (Appendix 1) does not indicate anything other than a random distribution for all calibrations associated with the random errors of the triplicate oxidations. The gradient of the fraction plot is lower than the solvent calibration curve, indicating a possible dcNEO peak suppression effect associated with the fraction extract, and toxin concentrations in real samples may be underestimated using a solvent calibration.

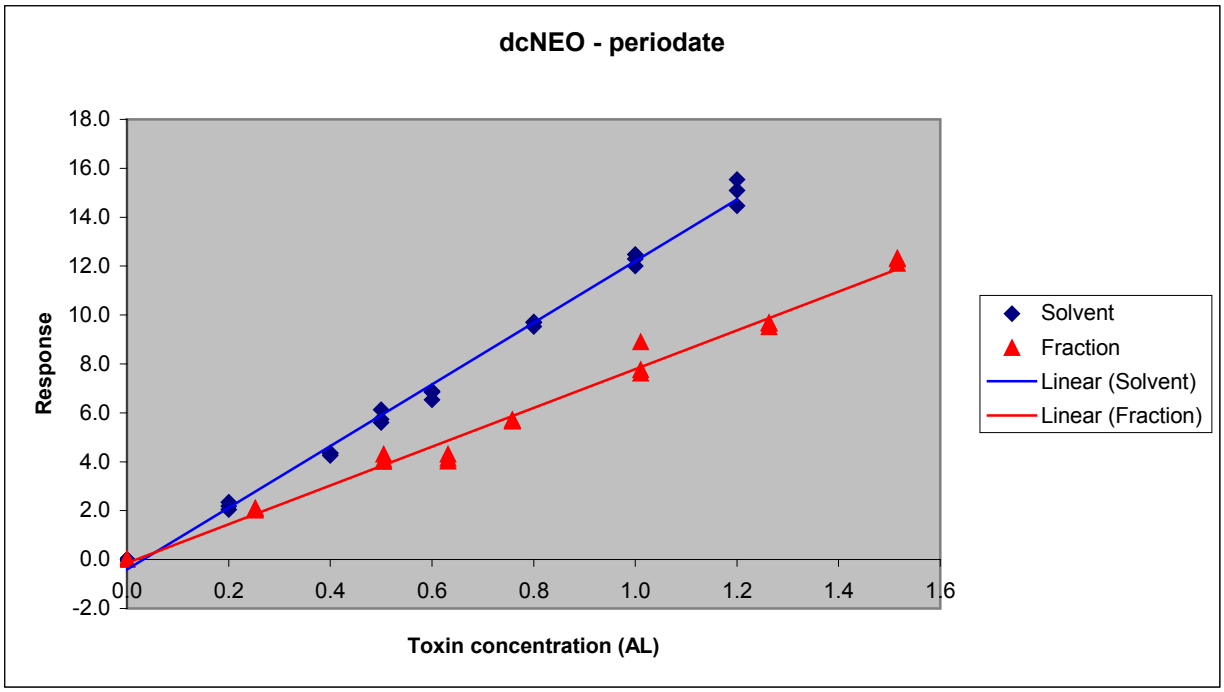


Figure 13. Calibration plots of dcNEO concentration against detector response for standard prepared in fraction extract (F3) and solvent over the working calibration range of 0 to 1.2 AL.

3.3.3. Linearity for NEO

Figure 14 displays the calibration graphs in fraction (F3) and solvent matrix for the periodate oxidation of NEO toxin. Visual inspection and correlation coefficients (table 10) indicate acceptable linearity for both fraction and solvent calibrations. F-test goodness of fitness tests for both matrices indicated a linear relationship existed for both calibrations, with values all significantly lower than F-critical. Visual examination of residual plots (Appendix 1) confirms that there is no apparent systematic deviation of the residuals from zero and the highly negative residual values at one concentration (0.6 AL) relate almost certainly to a preparation error rather than any inherent deviation from linearity. Interestingly, the relative gradient values are different for dcNEO, GTX1,4 and NEO.

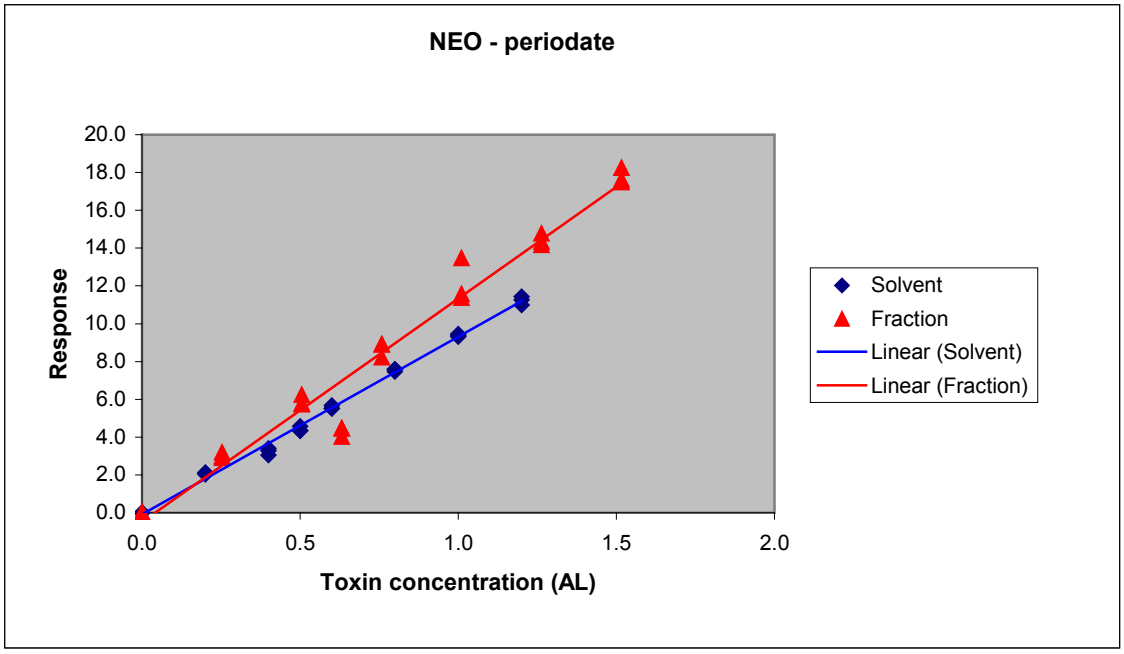


Figure 14. Calibration plots of NEO concentration against detector response for standard prepared in cleaned-up tissue extract, fraction extract and solvent over the working calibration range of 0 to 1.2 AL.

3.3.4. Linearity for dcSTX

Peroxide oxidation of both solvent and matrix dcSTX-spiked solutions reveals two calibrations very close in gradient (figure 15). The random errors associated with the calibrations appear smaller than those observed in the periodate calibrations, with an RSD of 5% for the response factors of the dcSTX calibration in mussel extract. The F-test furthermore indicates a linear relationship (table 10), and an insignificant intercept value confirms the qualitative observation that there are no significant interferences associated with the quantitation of this toxin.

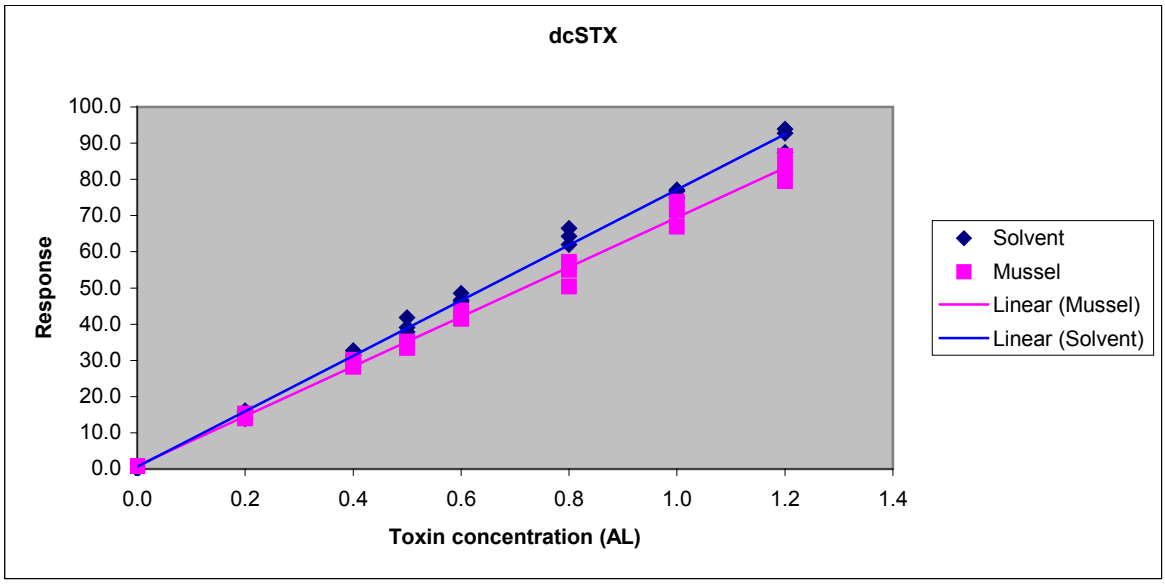


Figure 15. Calibration plots of dcSTX concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.3.5. Linearity for GTX2,3

Visual inspection (figure 16) indicates a fair linear fit for both solvent and matrix, although a degree of scatter of the solvent calibration is noticeable at the 0.8 and 1.0 AL concentration levels. F-test goodness of fit also indicates a linear relationship between concentration and detector response and the closeness of agreement between mussel extract and solvent standards shows there are no matrix effects present in the mussel extract for this particular toxin.

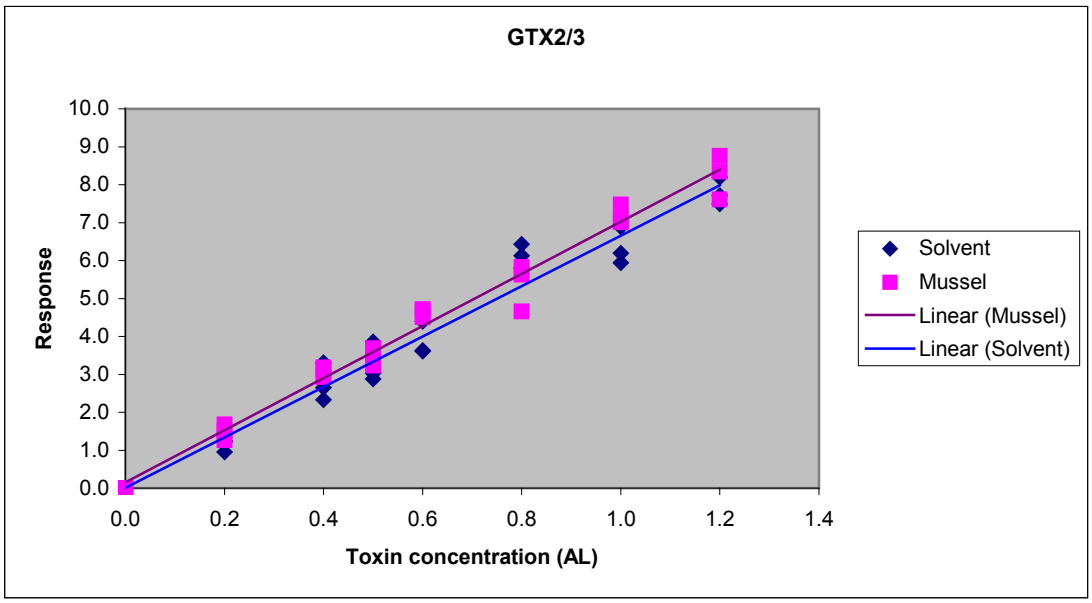


Figure 16. Calibration plots of GTX2,3 concentration against detector response for standard prepared in C18 cleaned tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.3.6. Linearity for GTX5 (1/10 concentration of other toxins)

Figure 17 shows that the gradient of the C18 cleaned mussel extract appears linear between 0 and 0.12 AL and is similar to the slope of the solvent calibration. However, deviation from linearity is noted in the solvent curve and from the lowered correlation coefficient (table 10), originating mainly from high residuals at 0.08 AL. The F-test results indicates a linear relationship between concentration and response in the mussel extract and this is further evidenced by the random nature of the residual scatter plot (Appendix 1).

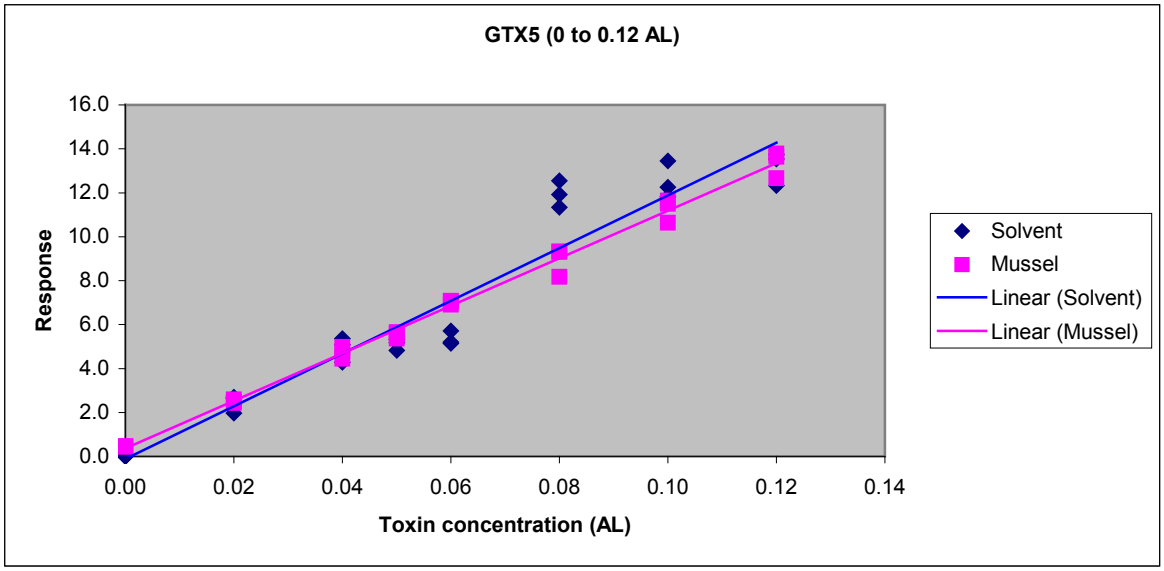


Figure 17. Calibration plots of GTX5 concentration against detector response for standard prepared in C18 cleaned tissue extract and solvent over the working calibration range of 0 to 0.12 AL.

3.3.7. Linearity for STX

Examination of the calibration graphs for STX in both C18 cleaned mussel extract and solvent (figure 18), indicates the largest difference between the two calibrations out of all the peroxide-oxidised toxins, with the resulting slope of the mussel calibration 16% higher than that of the solvent calibration. However, the correlation coefficient of the mussel calibration demonstrates excellent linearity (r2 = 0.993) and the variation in response factors over the entire calibration range is low (RSD = 4%). F-test goodness of fits for both mussel extract and solvent calibrations, together with a visual examination of the residual plots (Appendix 1) indicates a linear relationship between detector response and STX concentration over the given range.

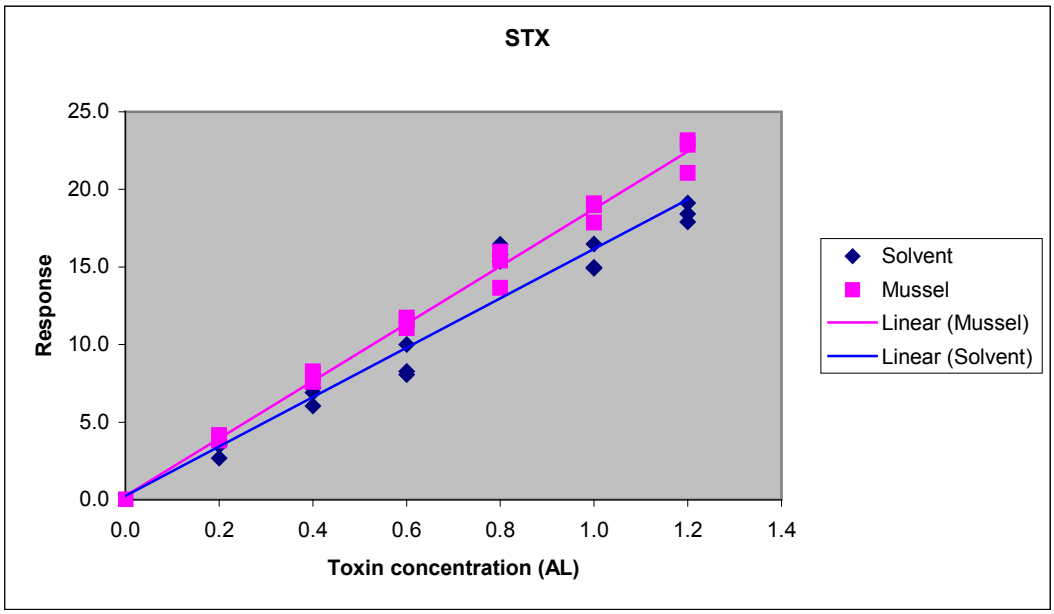


Figure 18. Calibration plots of STX concentration against detector response for standard prepared in cleaned-up tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.3.8. Linearity for C1,2

Visual inspection of both calibration graph (figure 19) and residual plot (Appendix 1) may possibly indicate a slight curvature to the calibration relationship, but an F-test linearity test value of 0.17 is significantly below F-critical, inferring linearity is still associated with the relationship. However, it should be noted that the F-test value is minimised by the relatively high standard deviation associated with the response factors, and this could theoretically hide any subtle variations from linearity in the residual plots, which may be noticeable by eye. The intercept value for the Mussel extract calibration is significantly different to zero, but it is thought this is more an aspect of the random errors and/or possible deviation from linearity at the top of the calibration range, rather than being indicative of any interference effects.

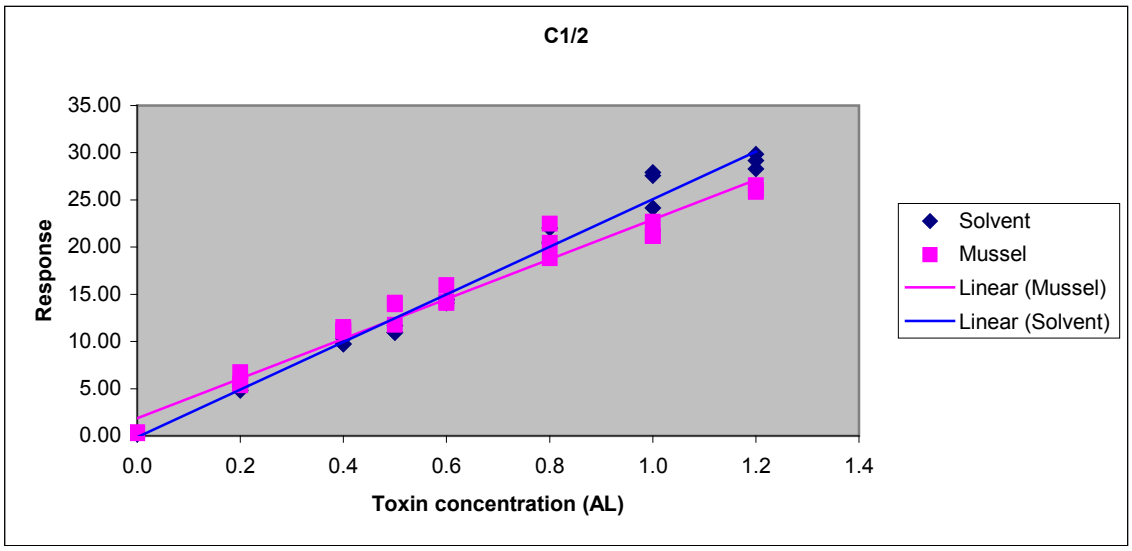


Figure 19. Calibration plots of C1,2 concentration against detector response for standard prepared in C18 cleaned tissue extract and solvent over the working calibration range of 0 to 1.2 AL.

3.3.9. Linearity for dcGTX2,3

The gradient and intercept value in the C18 cleaned mussel extract (figure 20), shows that interfering matrix components have a significant effect on the quantitation of dcGTX2,3 at the lower end of the calibration range. The linearity of the mussel extract calibration appears visually acceptable although is associated with a fairly high RSD in response factors over the calibration range (16%). Residual plots (Appendix 1) show very low residual values for dcGTX2,3 in Mussel extract which results in a very low F-test goodness of fit (0.04 compared with F-critical of 1.984). Linearity is not so well defined in the solvent matrix, with the poor repeatability of the triplicate peroxide oxidation responsible for the differences observed in responses at 1.0 and 1.2 AL.

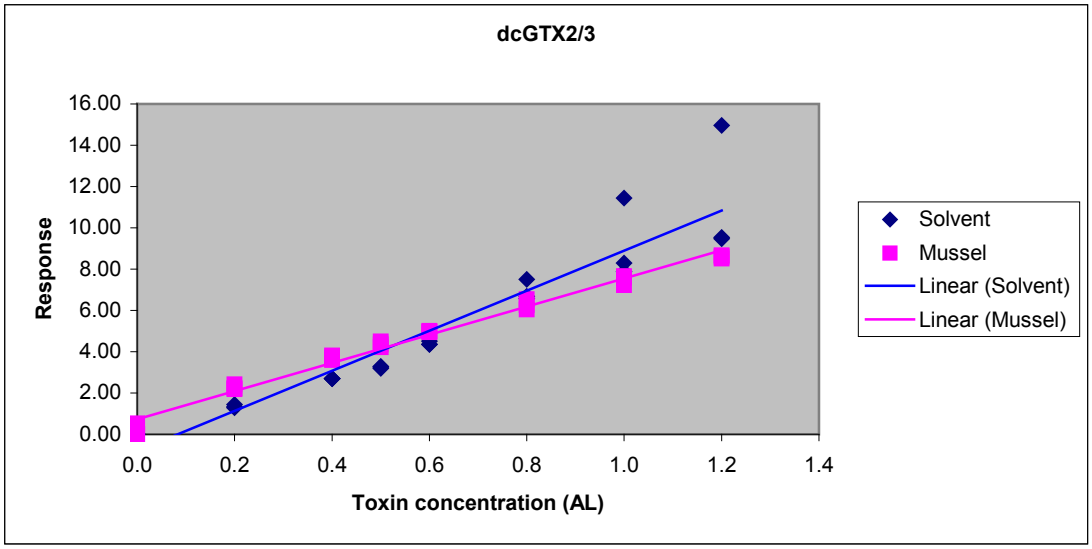


Figure 20. Calibration plots of dcGTX2,3 concentration against detector response for standard prepared in C18 cleaned mussel extract and solvent over the working calibration range of 0 to 1.2 AL.

Table 10. Linear regression gradients, intercepts and coefficients, plus relative standard deviations of response factors calculated for each PSP toxin in mussel extract, solvent and fractions (when applicable) over working calibration range (0 to 1.2 AL; GTX5 0 to 0.12 AL).

Toxin	Matrix	Calibration gradient	Intercept	r^2	RSD% of response factors	F-test	F_{crit}
GTX1,4	Solvent	3.06	0.13	0.968	13%	0.27	1.98
	Fraction F2	2.03	0.21	0.970	14%	0.22	1.98
dcNEO	Solvent	12.61	0.40	0.995	6%	0.22	1.98
	Fraction F3	7.93	-0.15	0.989	8%	0.52	1.98
NEO	Solvent	9.41	-0.09	0.996	8%	0.13	1.98
	Fraction F3	11.83	-0.50	0.963	17%	0.42	1.98
dcSTX	Extract	68.6	0.92	0.994	5%	0.44	1.98
	Solvent	76.6	0.60	0.996	4%	0.36	1.98
GTX2,3	Extract	6.87	0.15	0.983	8%	0.36	1.98
	Solvent	6.64	0.01	0.968	13%	0.33	1.98
GTX5	Extract	108	-0.37	0.993	6%	0.02	1.98
	Solvent	119	-0.12	0.927	17%	0.04	1.98
STX	Extract	18.48	0.26	0.993	5%	0.41	1.98
	Solvent	15.91	0.25	0.956	13%	0.45	1.98
C1,2	Extract	21.06	1.87	0.970	13%	0.17	1.98
	Solvent	25.21	-0.14	0.986	7%	0.53	1.98
dcGTX2,3	Extract	6.82	0.72	0.986	16%	0.04	1.98
	Solvent	9.69	-0.80	0.902	20%	0.60	1.98

3.3.10. Conclusions from linearity studies

Statistical analysis of calibrations using both correlation coefficients and F-test goodness of fit of the residuals, indicated there were no significant systematic deviations from linearity within mussel extracts and fractions examined over the concentration range of 0 to 1.2 AL (0 to 0.12 AL for GTX5) for any of the PSP toxins studied (table 10). The statistical results are enhanced by visual examination of the regression and residual plots, which did not indicate the presence of any systematic deviation of the residuals from zero.

Differences in slope of the calibrations between solvent and mussel extracts were noticeable, particularly in the periodate oxidations of the N-hydroxylated toxins GTX1,4, NEO and dcNEO. However, the relative differences in slope varied between the toxins, so there was no definite enhancement or suppression apparent in Mussel extract for all N-hydroxylated toxins as compared to the solvent calibration. C18 cleaned mussel extract and solvent slope gradients were very similar for all peroxide-oxidised toxins without exception, indicating there are no significant matrix effects in the peroxide oxidation of non-N-hydroxylated toxins

Extension of the calibration study to incorporate higher concentration values (Appendix 2) indicated that for most toxins, the linearity of the can be extended to higher concentrations. The exception is C1,2 in C18 cleaned mussel extract, where the calibration graph, residual plot and correlation coefficient clearly show a deviation from linearity above 1.0 AL (table 11).

Table 11. Comparison of calibration slope gradients, intercepts and correlation coefficients calculated over 0 to 1.2 AL range and 0 to 2.5 AL range for C1,2 in C18 cleaned mussel extract and solvent.

		(0 to 1.2 AL)			(0 to 2.5 AL)		
Toxin	Matrix	Gradient	Intercept	r^2	Gradient	Intercept	r^2
C1,2	Extract	21.06	1.87	0.970	12.62	6.02	0.796
	Solvent	25.21	-0.14	0.986	25.01	-0.16	0.991

3.4. Limits of detection and quantitation for PSP toxins in mussels

3.4.1. Limits of detection for screening method

Instrumental LODs were calculated for the periodate oxidation of all toxins in cleaned-up mussel extract in order to describe the limits of detection for the screening part of the method (2.10.3). This is important as it represents the ability of the method to detect peaks which may easily be identified and quantified by peroxide but which may possibly be missed by any inherent lower sensitivity of the periodate-oxidised initial screening analysis. All LOD values are tabulated (table 12) in terms of mean (± one standard deviation (s.d), 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 are included for both primary (diagnostic quantitation peak) and secondary toxin peaks.

Table 12. Limits of detection (LOD; µg STX eq./g ± 1 s.d.) of the LC-FLD screening method for PSP toxins following periodate oxidation of C18-cleaned Mussel extracts.

Toxin	**Peak assignment**	**Rt (min)**	**LOD** µg STX eq./g)
GTX 1,4	Primary	2.8	0.08 ± 0.014
GTX1,4	Secondary	7	0.08 ± 0.009
dcNEO	Primary	4.6	0.05 ± 0.006

NEO	Primary	5.3	0.03 ± 0.002
NEO	Secondary	9.6	0.03 ± 0.005
dcSTX	Primary	4.5	0.03 ± 0.008
dcSTX	Secondary	5.3	0.03 ± 0.008
GTX 2,3	Primary	6.8	0.04 ± 0.009
GTX 5	Primary	8.8	0.06 ± 0.005
STX	Primary	9.5	0.07 ± 0.020
dcGTX 2,3	Primary	2.4	0.04 ± 0.012
dcGTX 2/3	Secondary	2.8	0.04 ± 0.010
C 1,2	Primary	4.2	0.14 ± 0.025

For the range of toxins investigated here, LODs of ~0.03 to 0.14 µg STX eq./g (0.04 to 0.18 AL) were determined (table 12) for the periodate screening method of C18-cleaned extracts of Mussel tissue. NEO and dcSTX exhibit the highest LODs and C1,2 shows the lowest LOD at 0.14 µg STX eq./g. Whilst these values were not derived from homogenate spiking experiments, these values give an indication of the sensitivity of the screening method.

3.4.2. Limits of detection for quantitation method

Table 13 tabulates method LODs for the quantitation of PSP toxins in mussels. GTX1,4 exhibits the highest LOD (0.16 µg STX eq./g; 0.2 AL), whereas all others show significantly higher analytical sensitivities (0.003 to 0.087 µg STX eq./g; ~0.005 to 0.1 AL). No further dcNEO was available for method LOD determination, so the value of 0.16 µg STX eq./g was used as this concentration was utilized during recovery testing, where results showed a signal to noise ratio of >> 3.0.

It is therefore clear that whilst the method is capable of detecting most toxins at concentrations significantly below at the target of 0.2 AL per toxin, the sensitivity of GTX1,4 analysis is at this limit. Table 13 also compares the LODs against the values quoted in the AOAC 2005.06 method, similar results are illustrated with the exception of GTX1,4. The LOD for dcGTX2,3 indicates a good level of sensitivity for this toxin but the toxin was not studies within the AOAC method and no direct comparison is possible here.

Table 13. Limits of detection (LOD; µg STX eq./g ± one s.d.) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions and peroxide oxidation of C18-cleaned mussel extracts.

Toxin	**Quantitation method**	**LOD** µg STX eq./g) **± 1 s.d.**	**AOAC 2005.06 LOD** µg STX eq./g)
GTX 1,4	Periodate (F2)	0.16 ± 0.04	0.05
dcNEO	Periodate (F3)	<0.16	Na
NEO	Periodate (F3)	0.068 ± 0.01	0.04
dcSTX	Peroxide extract	0.007 ± 0.003	0.004
GTX 2,3	Peroxide extract	0.087 ± 0.03	0.08
GTX 5	Peroxide extract	0.003 ± 0.001	0.002
STX	Peroxide extract	0.018 ± 0.007	.022
dcGTX 2,3	Peroxide extract	0.053 ± 0.01	Na
C 1,2	Peroxide extract	0.018 ± 0.006	.01

Na = no data available

3.4.3. Determination of the limit of quantitation of the method

The results from the experimental confirmation of calculated LOQs are summarised in table 14**.** The results show that the experimentally confirmed LOQs range from 0.006 to 0.16 µg STX eq./g for the non-N-hydroxylated toxins. As such, the LOQs for all these toxins are at or lower than the target concentration level of 0.2 AL. These results would therefore suggest that the method can operate successfully certainty at concentrations less than or equal to 0.2 AL for each toxin and significantly lower in many cases. Results obtained for the N-hydroxylated toxins show acceptable levels of LOQ for NEO and dcNEO ($\leq$ 0.2 AL), with the exception of GTX1,4 which exhibits an LOQ of 0.38 µg STX eq./g. With GTX1,4, the degree of confidence in the method at 0.2 AL may therefore be questionable. However, results from the medium-term precision analysis of GTX1,4 at 0.2AL show a 16% RSD and a HorRat of 0.76 (table 21) and the long term precision GTX1,4 data taken from the analysis of the LRM also show an acceptable RSD and HorRat (table 24). From these results it is argued that the level of precision is acceptable at 0.2 AL and that quantitation of GTX1,4 can therefore be carried out with a good degree of precision well below the level of quantitation. This fits well with the IUPAC guidelines for single-laboratory validation of methods of analysis, which state that measurements below the limit of quantitation are not devoid of information content and may well be fit for purpose [4].

Table 14. Limits of quantitation (LOQ; µg STX eq./g $\pm$ one s.d.) of the LC-FLD quantitation method for PSP toxins following periodate oxidation of fractions and peroxide oxidation of C18-cleaned Mussel extracts.

Toxin	**Quantitation method**	**LOQ** µg STX eq./g) $\pm$ **1 s.d.**
GTX 1,4	Periodate (F2)	0.38 $\pm$ 0.09
dcNEO	Periodate (F3)	0.16 $\pm$ 0.04
NEO	Periodate (F3)	0.14 $\pm$ 0.04
dcSTX	Peroxide extract	0.01 $\pm$ 0.002
GTX 2,3	Peroxide extract	0.17 $\pm$ 0.03
GTX 5	Peroxide extract	0.006 $\pm$ 0.001
STX	Peroxide extract	0.04 $\pm$ 0.01
dcGTX 2,3	Peroxide extract	0.11 $\pm$ 0.01
C 1,2	Peroxide extract	0.04 $\pm$ 0.01

3.5. Accuracy of the method for the detection for PSP toxins in Mussels

3.5.1 Analysis of NRCC candidate mussel reference materials

3.5.1.1. Preliminary Mussel PSP Certified Reference Material (CRM)

Table 15 shows the comparison of both total toxin level and individual PSP toxins obtained by Cefas (pre-column oxidation) and NRCC (post-column oxidation) and also gives the total toxicity value determined by MBA (analysed by the Canadian Food Inspection Agency (CFIA)). Qualitatively, it is interesting to see that all toxins identified using the AOAC method were shown to be present using the NRCC method, with the exception of the low level dcNEO which was not reported by NRCC. Useful information is attained with a comparison of the relative proportions of toxins identified using the AOAC method in comparison with the NRCC post-column method. Table 15 illustrates a good qualitative correlation between the two methods, with almost identical profiles being observed. Quantitative data indicated that the low levels of NEO, dcSTX and dcGTX2,3 detected using the AOAC 2005.06 method were confirmed by the NRCC values. Concentrations of these toxins fell between 0.01 to 0.05 AL and as such low

accuracy of a method at these very low levels would be expected. However, there was good agreement obtained for the very low levels of dcGTX2,3. The relative over-estimation of dcSTX using AOAC also resulted in the significant under-estimation of NEO due to the subtraction of dcSTX contribution from NEO. Without the subtraction of an estimated dcSTX contribution to the NEO peak, the NEO value quantified was 0.06 µg STX eq./g, exactly the value quoted by the NRCC. This may either further highlight potential issues with quantifying N-hydroxylated toxins in the presence of dcSTX and dcGTX2,3, but is more likely to represent the high degree of method uncertainty to be expected at these very low toxin concentrations using both methods. Values obtained for GTX2,3 and STX using the pre-column oxidation method were both higher than those quoted by the NRCC. However, again, without certification of the NRC values, it is impossible to determine whether the inferred inaccuracy is due to either or both of the two techniques. Interestingly, the MBA result from this reference material is quoted at 2.60µg STX eq./g, which when compared to the AOAC and NRC values shows that the AOAC method gives a more accurate reflection on the tissue toxicity when using the mouse as a reference. This could infer that the higher values obtained for STX and GTX2,3 are more accurate using the AOAC method than when using post-column oxidation, assuming the MBA gives the "correct" result.

Table 15. Concentrations and relative proportions of PSP toxins in NRCC mussel reference material (µg STX eq./g; ± 1 sd) using AOAC method (Cefas), post-column oxidation LC-FLD method (NRCC) and MBA (CIFA).

Toxin	**AOAC (Cefas)**	**Post column (NRCC)**	% accuracy using post-column FLD as reference	MBA (CFIA)	Relative toxin proportions (AOAC)	Relative toxin proportions (NRCC)
GTX 1,4	Nd	Nd	-	-	Nd	Nd
dcNEO	0.03 ± 0.012	Na	-	-	1%	Na
NEO	0.01 ± 0.004	0.06 ± 0.006	19%	-	1%	3%
dcGTX 2,3	0.04 ± 0.005	0.03 ± 0.003	139%	-	2%	2%
C 1,2	Nd	Nd	-	-	Nd	Nd
dcSTX	0.03 ± 0.001	0.01 ± 0.001	284%	-	1%	1%
GTX 2,3	0.30 ± 0.02	0.18 ± 0.01	165%	-	12%	10%
GTX 5	Nd	Nd	-	-	Nd	Nd
STX	1.96 ± 0.07	1.47 ± 0.03	134%	-	83%	84%
Total	**2.38**	**1.75**		**2.60**		

Na = Not analysed for
Nd = Not detected

3.5.1.2. Pilot Mussel PSP CRM

A second candidate CRM currently under developmental at NRCC (PSP-Mus-Pilot) was supplied to Cefas and extracted, cleaned, fractionated and analysed by LC-FLD. Concentration data are given in table 16.

Table 16. Concentrations and relative proportions of PSP toxins identified in NRCC mussel pilot reference material (µg STX eq./g; ± sd) using AOAC method (Cefas), post-column oxidation LC-FLD method (NRCC) and MBA (CIFA).

Toxin	**AOAC (Cefas)**	**Post column (NRCC)**	% accuracy using post-column FLD as reference	MBA (CFIA)	Relative proportions (AOAC)	Relative proportions (NRC)
GTX 1,4	1.75 ± 0.24	1.20	**147%**	-	32%	26%
dcNEO	Nd	Na	-	-	0%	Na
NEO	0.30 ± 0.02	0.49	**61%**	-	5%	11%
dcGTX 2,3	0.76 ± 0.01	0.78	**98%**	-	14%	17%
C 1,2	Nd	Nd	-	-	Nd	Nd
dcSTX	0.02 ± 0.001	Na	-	-	1%	1%
GTX 2,3	1.46 ± 0.02	1.14	**129%**	-	27%	25%
GTX 5	Nd	Nd	-	-	Nd	Nd
STX	1.13 ± 0.01	0.97	**116%**	-	21%	21%
Total	**5.42**	**4.57**		**6.40**		

Na = Not analysed for
Nd = Not detected

The comparability between the AOAC method and the NRCC post-column method is good. Values for dcGTX2,3 are almost identical, with GTX2,3 and STX values derived after pre-column oxidation being 10-30 % higher than those determined approximately by the NRCC. The values for the N-hydroxylated toxins are less in agreement, but bearing in mind the uncertainty associated with each method and the difficulties anticipated with quantifying N-hydroxylated toxins, the level of comparability is still encouraging (Quilliam, personal communication). This CRM is the first example of a sample which contains high levels of dcGTX2,3 and GTX1,4 toxins and as such, required the calculated estimation and subtraction of dcGTX2,3 co-eluting with GTX1,4. Furthermore, selectivity issues with naturally occurring interferences at the same retention time as dcGTX2,3 increased the likelihood of inaccuracies in quantitation for these two toxins. Still, the level of comparison between pre- and post-column derivatisation were good, bearing in mind that the post-column method employed by the NRCC effectively separates these two toxins removing any toxin co-elution issues.

Overall, data derived from the pre-column LC-FLD analysis of these two potential PSP toxin reference materials provided further insight into the behaviour of this method, indicating a good degree of comparability with toxin profiles and concentrations obtained by an alternative analytical method. However, without any formal certification of the “true” PSP quantities, the study can only be viewed as indicative of the performance of the adopted 2005.06 method in the absence of a proper assessment of method accuracy, and as such any subsequent recovery assessments were made with the use of spiked-tissue samples.

3.6. Determination of the recovery of PSP toxins from spiked mussel tissues
3.6.1. Overall recoveries

Recovery results were calculated in terms of expected mean recovery for each toxin in each of the three spiked, extracted, cleaned and derivatised samples. Table 17 presents the mean recovery percentages of PSP toxins from spiked mussels spiked at 0.5 AL and 0.2 AL, with RSDs calculated from the mean recovery of each triplicate oxidation and analysis.

Table 17. Mean percentage recoveries (and RSDs; n=3) of PSP toxins from spiked mussel homogenates spiked at expected concentrations of 0.5 AL and 0.2 AL (GTX5 1/10 conc), plus comparison with range of mean inter-laboratory recoveries reported by AOAC 2005.06 (at variable concentration levels).

	0.5 AL	0.2AL	AOAC
GTX 1,4	81% (4%)	112% (4%)	67% - 79%
dcNEO	53% (4%)	29% (4%)	na
NEO	107% (6%)	136% (2%)	53% - 62%
dcSTX	68% (8%)	85% (1%)	64% - 84%
GTX 2,3	66% (9%)	94% (7%)	76% - 88%
GTX 5 (1/10 conc.)	76% (7%)	82% (1%)	76% - 86%
STX	68% (5%)	122% (0.2%)	74% - 93%
dcGTX 2,3	70% (18%)	82% (1%)	na
C 1,2	63% (10%)	79% (6%)	74% - 78%

Na = not analysed

Table 17 shows the mean recoveries of non-N-hydroxylated toxins spiked at 0.5 AL following peroxide oxidation of C18-cleaned mussel extracts falling in the range of 63% to 76%. RSDs associated with these recoveries indicate a fair degree of repeatability associated with such measurements. It therefore appears that at the 0.5 AL, the recoveries calculated for non-N-hydroxylated PSP toxins are acceptable for such a complex method, where extraction, clean up, fractionation and oxidation steps are all expected to contribute to recovery losses. Such recoveries are not thought to be influenced by the effects of solvent calibrations, as no fluorescence suppression was identified for any of these toxins (figures 15 – 20).
Similarly, the N-hydroxylated toxins (GTX1,4 and NEO) do not show any significantly reduced recoveries at 0.5 AL concentration level, with recoveries of 81 and 107% respectively, although the poorer average recovery of dcNEO (53%) may either relate to the spiking of the toxin as part of a total Mix IV spike, or to the noticeable fluorescence suppression seen in mussel extract as compared with solvent (figure 13).

With the exception of dcNEO, results for the recovery of PSP toxins spiked into mussel matrix at 0.2 AL show mean toxin recoveries at 0.2 AL range from 79% to 136%, with STX and NEO in particular giving rise to recoveries of >100%. Such values may well indicate issues with method uncertainty at these levels of toxin and/or may relate to a degree of toxin peak fluorescence enhancement in the mussel extract matrix or the additive effect of background and interfering baseline noise. DcNEO aside, the results indicate an acceptable level of recovery at 0.2 AL. The poorer recovery for dcNEO may relate to the presence of other toxins in the same sample, or may arise from the increased levels of solvent present in the Mix-IV-spiked Mussel tissues prior to extraction. Differences are therefore observed between the recoveries of each toxin and between different concentration levels. Recoveries for GTX5, dcSTX, dcGTX2,3 and

C1,2 are not too dissimilar for the two concentration levels, with the recoveries at the higher concentration level tending to exhibit the lower recovery.

3.6.2. Investigation of recovery efficiency by exhaustive extraction

Results from the exhaustive extraction of all the eleven naturally contaminated and five spiked mussel samples are shown in table 18 in terms of the percentage of the original AOAC-extracted and quantified toxin concentrations.

Table 18. Apparent extraction recovery efficiencies as expressed by percentage of PSP toxins quantified in re-extracted samples (spiked and naturally contaminated) as compared with levels in original AOAC-method extracted Mussel tissue. Relative standard deviations shown in brackets.

	Spiked Mussel (all toxins, n=1)	**STX, GTX2,3 Spiked Mussel (Mean, + RSD% n=4)**	**Natural samples (Mean) + RSD% (n=11)**
GTX 1,4	87%	-	**79% (32%)**
NEO	88%	-	-
dcSTX	78%	-	-
GTX 2,3	77%	77% (29%)	**79% (35%)**
GTX 5	89%	-	-
STX	78%	80% (29%)	**75% (27%)**
dcGTX 2,3	92%	-	-
C 1,2	97%	-	-

The results show that mean extraction efficiencies varied between 75% and 97% for all samples and spiked tissues. This infers that up to 25% of the recovery losses measured previously may be attributable to the extraction process, with the toxin material being held either in the tissue matrix or in the acetic acid solution contained within the pores of the sample. This is perhaps to be expected when considering the relatively low volume of acetic acid to sample weight ratio (6mL to 5g) employed in the AOAC 2005.06 extraction procedure. The apparent extraction efficiency demonstrated here of ~80%, relates well to the range of recoveries calculated for the 0.5AL spiked Mussels. Whilst a significant degree of method uncertainty is expected to contribute to the variability in recovery results, particularly at lower concentration levels, it appears as though, with the possible exception of dcNEO, the recoveries determined are acceptable for such a complex method, with an extraction efficiency of 80% perhaps being the largest contributor to reduced method recovery. Additional work comparing HPLC and MBA results will give further information on whether the extraction efficiency of the AOAC 2005.06 HPLC method is significantly biased or not. Due to a shortage of certified dcNEO standard, no further work can be carried out on this toxin.

3.7 Determination of the precision of the method

3.7.1 Instrumental precision

Instrumental precision of toxin peak retention times is presented in Table 19, below. The table shows that the level of precision of chromatographic retention times is high, hence a high degree of confidence can be placed upon the toxin peaks consistently eluting at repeatable retention times. Table 19 also displays the results obtained from repeat analysis (n=6), in one sequence of one sample of a 0.5 AL Mix IV-spiked Mussel homogenate, extracted, cleaned-up, fractionated and oxidised using both periodate and peroxide oxidants. Relevant quantitation peaks were measured and recorded and the relative standard deviations of the replicate analyses are calculated to

fall between the range of 0.2% to 3.5%, with a mean value of 1.4% RSD. This illustrates that the variability observed intra-batch between peak area responses is low and the precision of short-term instrumental response is high.

Table 19. Instrumental precision, showing variability (RSD%) of toxin retention times and peak area responses.

Toxin Mix	Toxin	Retention time (RSD%, n=7)	Toxin peak area (RSD %, n=6)
Mix 1	GTX 1,4	1%	0.6%
	dcNEO	2%	2.1%
	NEO	2%	3.5%
Mix 2	dcSTX	1%	1.0%
	GTX 2,3	2%	1.0%
	GTX 5	4%	0.2%
	STX	1%	3.0%
Mix 3	dcGTX 2,3	1%	0.3%
	C 1,2	2%	0.6%

3.7.2. Estimation of procedural precision

3.7.2.1. Estimation of short-term repeatability

Table 20 presents the concentrations (+/- 1 s.d.) calculated for triplicate mussel homogenate spikes at 0.2 AL and 0.5 AL for each PSP toxin when extracted, cleaned-up, fractionated, oxidised and analysed within one analytical batch. Relative standard deviations calculated from the resulting concentrations illustrate a short-term method repeatability, albeit on a reduced scale of n=3, that appears to be acceptable. All RSD% values are less than or equal to 10%, except for dcGTX2,3 at 0.5AL where a lower measured concentration for the first of the three samples results in higher than expected short-term variability. The results therefore seem to suggest that at individual PSP toxin concentrations of 0.2 and 0.5 AL, the short term repeatability for the LC-FLD analysis of each toxin is reliable. Further evidence for statistical acceptability of precision comes from the HorRat values, all of which are <2.0 at both concentration levels (table 20). Lawrence and Niedwiadek [8] reported RSD% values <10% for the quadruplicate analyses of PSP toxins. However, these results related to spiked extracts rather than spiked tissue samples, so the results described here are especially encouraging, as they also include any errors associated with the tissue extraction and any possible tissue heterogeneity. Additionally, it should be noted that Lawrence does not state whether the quadruplicate analyses represent replicate injections, oxidations, clean-ups or extract spikes, so a direct comparison of precision values obtained in the two studies is not possible.

Table 20. Calculated mean concentrations (+/- 1 s.d.) of triplicate spiked Mussel homogenate at 0.2 AL and 0.5 AL per toxin (GTX5 1/10 conc), showing estimations of short-term method repeatability in terms of percentage relative standard deviation (n=3; < 2 weeks)

	0.2 AL tissue spikes			**0.5 AL tissue spikes**		
Toxin	**Mean**	**RSD%**	**HorRat**	**Mean**	**RSD%**	**HorRat**
GTX 1,4	0.18 ± 0.007	4%	**0.29**	0.32 ± 0.012	4%	**0.33**
dcNEO	0.05 ± 0.002	4%	**0.29**	0.20 ± 0.011	5%	**0.41**
NEO	0.22 ± 0.004	2%	**0.14**	0.43 ± 0.025	6%	**0.49**
dcSTX	0.14 ± 0.001	1%	**0.07**	0.27 ± 0.023	8%	**0.66**
GTX 2,3	0.15 ± 0.01	7%	**0.50**	0.26 ± 0.024	9%	**0.74**
GTX 5 (1/10)	0.013 ± 0.0002	1%	**0.05**	0.03 ± 0.002	7%	**0.41**
STX	0.20 ± 0.0003	0.2%	**0.01**	0.27 ± 0.014	5%	**0.41**
dcGTX 2,3	0.13 ± 0.002	1%	**0.07**	0.28 ± 0.051	18%	**1.48**
C 1,2	0.13 ± 0.008	6%	**0.43**	0.25 ± 0.025	10%	**0.82**

3.7.2.2. Estimation of medium-term repeatability

3.7.2.2.1 Spiked tissues

Table 21 shows the precision relating to the extraction, clean up, oxidation and analysis of six replicate spiked samples (both 0.2 and 0.5 AL) performed over a longer period of time (> 2 weeks) as described in section 2.10.6.2. For the assessment of medium term precision, a Mix IV spike was used for all non-N-hydroxylated toxins, whilst mussel homogenate was spiked separately with N-hydroxylated toxins to assess the precision of NEO and GTX1,4 analysis.

RSD percentages for the 0.2 AL spiked samples range from 9% for dcGTX2,3 to 41% for C1,2 for the non-N-hydroxylated toxins and lie between 17% and 51% for the N-hydroxylated toxins. At 0.5 AL, RSD percentages range from 4% to 32% for all toxins, with GTX1,4 and NEO in particular showing a high degree of medium term precision at the higher concentration. Overall, the precision at 0.5 AL is higher than at 0.2 AL, with mean RSDs% of 15% and 24% respectively. Acceptable precision is further evidenced from the HorRat values, which are <2.0 for most toxins at both concentration levels (also table 21). Notable results with RSDs > 30% are C1,2 and NEO at 0.2 AL and GTX5 at 0.05 AL, with NEO at 0.2 AL in particular giving a HorRat value of > 2.0. Whilst this illustrates the higher degree of medium term variability associated with the analysis of this toxin, it is noted that HorRat values quoted in the AOAC 2005.06 method were also >2.0 for NEO and the range of RSD values quoted in the method typically range from 10% to > 50% [12]. Considering the potential high variability inherent in such a multi-step method, the majority of these values appear to be acceptable and indicate that the method is repeatable within the laboratory over the medium term.

Table 21. Mean concentrations (µg STX eq./g; +/- 1 s.d.) of six replicate analyses of spiked Mussel homogenate (0.2 AL and 0.5 AL; GTX5 1/10 conc) showing percentage relative standard deviation (n=6).

	0.2 AL tissue spikes			0.5 AL tissue spikes		
	Mean concentration (µg STX eq./g)	**RSD%**	**HorRat**	**Mean concentration (µg STX eq./g)**	**RSD%**	**HorRat**
GTX 1,4	0.17 ± 0.03	17%	**0.82**	0.33 ± 0.01	4%	**0.21**
dcNEO	0.06 ± 0.01	20%	**0.94**	0.17 ± 0.04	26%	**1.41**
NEO	0.15 ± 0.08	51%	**2.42**	0.40 ± 0.10	26%	**1.40**
dcSTX	0.05 ± 0.02	32%	**1.49**	0.23 ± 0.05	23%	**1.23**
GTX 2,3	0.14 ± 0.03	22%	**1.06**	0.28 ± 0.03	10%	**0.53**
GTX 5 (1/10 conc)	0.019 ± 0.005	26%	**0.86**	0.043 ± 0.014	32%	**1.25**
STX	0.09 ± 0.02	16%	**0.77**	0.27 ± 0.01	5%	**0.29**
dcGTX 2,3	0.17 ± 0.02	9%	**0.45**	0.27 ± 0.04	15%	**0.81**
C 1,2	0.07 ± 0.03	41%	**1.94**	0.25 ± 0.02	10%	**0.54**

3.7.2.2.2 Comparison of intra- and inter-batch precision information determined from the analysis of naturally contaminated mussels

A comparison of the intra- and inter-batch repeatability data from the precision study on naturally contaminated mussels is shown in Table 22.

Table 22. Comparison of intra- and inter-batch sample repeatability precision (mean concentrations in µg STX eq./g). Concentrations < 0.2 AL (0.16 µg STX eq./g) are shaded.

		Intra-batch		Inter-batch (long term)	
Sample	**Toxin**	**Mean conc. (µg STX eq./g)**	**%RSD (n=10*)**	**Mean conc. (µg STX eq./g)**	**%RSD (n=10*)**
Sample 811	**C 1,2**	0.017	23%	0.01	33%
	GTX 2,3	0.12	9%	0.14	37%
	GTX 5	0.002	14%	0.0018	32%
	STX	0.55	8%	0.56	12%
	Total	**0.68**	7%	**0.72**	13%
Sample A	**GTX 1,4**	1.05	15%	1.04	18%
	NEO	0.16	12%	0.15	24%
	C 1,2	0.04	22%	0.04	29%
	GTX 2,3	0.61	17%	0.60	24%
	STX	0.58	15%	0.57	17%
	Total	**2.44**	8%	**2.40**	10%
Sample B	**GTX 1,4**	0.14	17%	0.18	22%
	C 1,2	0.03	7%	0.03	33%
	GTX 2,3	0.24	4%	0.23	25%
	STX	0.18	4%	0.17	20%
	Total	**0.60**	2%	**0.63**	10%
Sample C	**GTX 1,4**	0.58	7%	0.51	17%
	NEO	0.07	6%	0.07	15%
	C 1,2	0.04	19%	0.02	48%
	GTX 2,3	0.35	6%	0.35	31%
	GTX 5	0.003	16%	0.0025	30%

	STX	0.34	5%	0.32	14%
	Total	**1.37**	5%	**1.29**	15%
Sample D	**GTX 1,4**	1.23	20%	1.36	16%
	NEO	0.14	9%	0.13	43%
	C 1,2	0.07	12%	0.07	24%
	GTX 2,3	1.06	9%	1.20	22%
	GTX 5	0.003	20%	0.002	41%
	STX	0.88	9%	0.92	19%
	Total	**3.37**	12%	**3.69**	16%
Sample E	**GTX 1,4**	0.69	22%	0.77	21%
	NEO	0.07	21%	0.09	38%
	C 1,2	0.04	39%	0.03	35%
	GTX 2,3	0.50	7%	0.52	29%
	STX	0.38	16%	0.37	25%
	Total	1.68	12%	1.78	22%

*except for sample 811, where n=6 for intra-batch and n=9 for inter-batch

The results indicate that overall the intra-batch repeatability was acceptable for most toxins present at concentration levels >0.2 AL (0.16 µg STX eq./g, with most precision values <20% over the ten analyses in the same batch. Longer term, inter-batch repeatability is, as expected, of higher variability with RSD% values ranging between 10% and 31% for all toxins at concentrations >0.2 AL. Such values appear entirely reasonable given the variability associated with the AOAC method as exemplified by the medium-term repeatability data generated from spiked mussel tissues (section 3.7.2.2.1). A summary of the mean %RSDs (average of all six samples for each toxin) is displayed in table 23**.** For those toxins typically present at levels > 0.2AL (GTX1,4, GTX2,3 and STX) the repeatability of both intra- and inter-batch analyses is good. Even toxins present at levels lower than the 0.2 AL target level still exhibit a good degree of repeatability, as exemplified by the mean RSD% values for C1,2, NEO and GTX5. Further evidence for the acceptability of these values comes from calculation of HorRat ratios for both intra-batch and inter-batch repeatability, less than 2.0 for all toxins (also table 23).

Table 23. Summary of naturally contaminated mussel sample repeatability data showing mean RSDs, range of RSD values obtained and HorRat values, for both intra- and inter-batch repeatability data. Samples showing concentrations < 0.2 AL (0.16 µg STX eq./g) are shaded.

		Intra-batch precision			Inter-batch precision		
Toxin	Mean concentration (µg STX eq./g)	Mean RSD%	Range of RSD%	HorRat	Mean RSD%	Range of RSD%	HorRat
GTX 1,4	0.77	16%	7% - 22%	1.46	19%	16% - 22%	1.14
NEO	0.11	12%	6% - 21%	0.82	30%	15% - 43%	1.34
C 1,2	0.04	21%	7% - 39%	1.23	34%	24% - 48%	1.31
GTX 2,3	0.51	9%	4% - 17%	0.77	26%	22% - 37%	1.47
GTX 5	0.002	17%	14% - 20%	0.63	34%	30% - 41%	0.83
STX	0.49	10%	4% - 16%	0.85	18%	14% - 25%	1.01
Total	1.75	8%	2% - 12%	0.82	14%	10% - 22%	0.95

This experiment was designed to test the repeatability of clean up, fractionation, oxidation and analysis of naturally contaminated samples. Due to lack of availability of naturally, contaminated mussel homogenate, the precision of the whole method (including extraction) was not evaluated. However, the sample repeatability study has demonstrated a good degree of repeatability for the toxins present in the naturally contaminated extracts and the values given in table 23 above show a similar level of repeatability as described by previous spiking-derived precision experiments (3.7.2.2.1). No significant problems were identified with C18 clean-up, fractionation, oxidation or batch calibration and HorRat values infer a good degree of precision associated with the repeatability of the method.

3.7.2.3. Estimation of long-term repeatability

Concentration data from the extraction, clean up and analysis of LRMs were generated over a longer period of time (>2 months), by different analysts and using different batches of consumables and reagents. As such, the data generated represents a practical, long-term precision of the method incorporating variations due to changes in calibration solutions, instrument calibration, calibration states of equipment, variations in ambient temperature, changes in analyst and a general repeatability contribution. This provides a particularly valuable source of information on the overall variability of the method, including extraction precision and is an important part of quality control in future monitoring analysis using this method. The results obtained from LRM analysis to date are tabulated in table 24**.**

Table 24. Mean concentration +/- s.d [RSD (%)] and %RSD data generated from long term extraction, clean-up, fractionation, oxidation and analysis of PSP LRM. Toxins present at concentration levels < 0.2AL are shaded.

	Mean concentration (µg STX eq./g; +/- s.d.)	%RSD	HorRat
GTX 1,4	**0.40 ± 0.11**	**27%**	**1.49**
NEO	**0.09 ± 0.042**	**45%**	**1.98**
C 1,2	**0.01 ± 0.008**	**69%**	**2.19**
GTX 2,3	**0.34 ± 0.061**	**18%**	**0.96**
GTX 5	**0.002 ± 0.001**	**36%**	**0.89**
STX	**0.55 ± 0.061**	**11%**	**0.63**
Total	**1.41 ± 0.24**	**17%**	**1.10**

Toxins GTX1,4, GTX2,3 and STX were present at concentration levels significantly higher than the 0.2 AL target level and all three analytes exhibited long-term repeatability precision values of between 11% and 27%. These values were similar to those generated from the medium-term repeatability studies (see above) and, as such, give a further degree of confidence in the method for the analysis of these three toxins. Further evidence for the acceptability of this method repeatability comes from the HorRat values (all <2.0; also table 24). For other PSP toxins in the LRM, NEO, C1,2 and GTX5 were present at much lower concentration levels and as such show higher levels of variability (shaded in table 24). Nevertheless, HorRat values are <2.0 for all toxins except C1,2 (mean concentration 0.01 µg STX eq./g) inferring the level of repeatability is still acceptable.

3.8. Ruggedness of the method

3.8.1. GTX2,3 and STX

Each sample was oxidised in triplicate prior to LC-FLD analysis and the results are tabulated below in table 25.

Table 25. Results from ruggedness testing of GTX2,3 and STX.

	Concentration (µg[STX equivalent]/g) for eight experiments							
Expt no.	**s**	**t**	**u**	**v**	**w**	**x**	**y**	**z**
GTX 2,3	0.35	0.36	0.33	0.41	0.30	0.32	0.42	0.38
STX	0.43	0.42	0.41	0.51	0.41	0.39	0.49	0.46
Total	0.78	0.78	0.74	0.92	0.71	0.71	0.91	0.84

Parameter differences were calculated for each experiment by subtracting the means of the two data sets of results relating to the two variables. For example, the parameter difference for extraction temperature (A), is calculated by the following:

$D_A = ((s+t+u+v)/4) – ((w+x+y+z)/4)$

where s, t, u and v are the experimental results relating to an extraction temperature of 100°C, whilst w, x, y and z are the results relating to an extraction temperature of 95°C. Hence a large positive value would indicate that the higher temperature results in a larger final concentration results, whereas a negative value for any of the parameters infers the second parameter (section 2.10.7) results in higher concentrations. Calculated parameter differences are tabulated below in table 26, which lists the parameters in order of importance (magnitude) for both STX and GTX2,3, giving the result in terms of the parameter difference and the parameter difference as a percentage of the spiked concentration (0.4 µg STX eq./g). These results therefore infer that parameter B (Vortex mixing time) has the most significant effect on method stability, with the negative parameter difference confirming that the longer vortex mix results in a higher extraction efficiency.

In order to determine whether such parameter differences are significant and thus result in method instability, the results are compared against method precision using a significance test (t-test). Using the 9 analyses of STX and GTX2,3 run during the short term precision tests, t-test values are calculated (table 26). With t-critical = 4.43 for n=9, the results clearly show that all t-test values are less than t-critical and as such, none of the ruggedness parameters investigated have a significant effect on the method. Therefore, the method is shown to be robust for these two toxins with respect to the parameters studied here, with the assumption that parameters investigated do not interact. However, with a noted increase in recovery associated with a longer sample vortex mixing time, a requirement for a specific vortex time (90 sec) has been instigated into the method.

Table 26. Parameter differences, parameter difference percentages and t-test values from ruggedness testing of GTX2,3 and STX.

GTX2,3				**STX**			
Parameter	**Parameter Difference**	**Parameter difference (%)**	**t test value**	**Parameter**	**Parameter Difference**	**Parameter difference (%)**	**t test value**
D_B	**-0.055**	**-14%**	**-2.61**	**D_B**	**-0.056**	**-14%**	**-2.62**
D_D	**0.039**	**10%**	**1.85**	**D_E**	**-0.032**	**-8%**	**-1.52**
D_G	**0.032**	**8%**	**1.50**	**D_G**	**0.031**	**8%**	**1.46**
D_E	**-0.030**	**-8%**	**-1.42**	**D_F**	**0.023**	**6%**	**1.09**
D_C	**-0.017**	**-4%**	**-0.81**	**D_D**	**0.022**	**5%**	**1.03**

D_A	**0.004**	**1%**	**0.20**	**D_A**	**0.006**	**2%**	**0.30**
D_F	**0.003**	**1%**	**0.16**	**D_C**	**-0.006**	**-2%**	**-0.30**

3.8.2. GTX1,4 and NEO

Each sample was oxidised in triplicate prior to LC-FLD analysis and the results are tabulated below in table 27.

Table 27. Results from ruggedness testing of GTX1,4 and NEO.

	Concentration (µg STX eq./g) for eight experiments							
Expt no.	**s**	**t**	**u**	**v**	**w**	**x**	**y**	**z**
GTX 1,4	0.47	0.31	0.35	0.55	0.32	0.57	0.45	0.30
NEO	0.18	0.21	0.21	0.26	0.18	0.22	0.17	0.17
Total	0.65	0.52	0.56	0.81	0.50	0.79	0.62	0.47

Calculated parameter differences are tabulated below in table 28, which lists the parameters in order of importance (magnitude) for both NEO and GTX1,4, giving the result in terms of the parameter difference and the parameter difference as a percentage of the spiked concentration (0.4 µg STX eq./g). These results therefore show that matrix modifier has the largest effect on the method stability for GTX1,4, whereas the same parameter has a relatively low effect on the stability of NEO quantitation. Oxidation vortex time also appears to have an effect on the quantitation, with the parameter differences illustrating that the longer mixing time results in a greater oxidation efficiency. Similarly, the results indicate that the closer the pH of the periodate reagent is to 8.25, the more efficient the oxidation for both GTX1,4 and NEO.
As above, t-test values calculated using method precision values previously reported (short term precision) are tabulated for each toxin in table 28. Compared with t-critical of 4.43, the results again show that all parameter tests for each toxin are less than t-critical, inferring that none of the parameters have a significant effect on the method and the method is robust for the analysis of these toxins. However, it should be noted that a change in the matrix modifier does result in noticeable differences in quantitation results, which whilst not statistically significant may introduce further uncertainty into the quantitation method. As such, further study should be conducted on this parameter alone to establish appropriate method control limits.

Table 28. Parameter differences, parameter difference percentages and t-test values from ruggedness testing of GTX1,4 and NEO.

GTX1,4				**NEO**			
Parameter	**Parameter Difference**	**Parameter difference (%)**	**t test value**	**Parameter**	**Parameter Difference**	**Parameter difference (%)**	**t test value**
D_G	**0.193**	**48%**	**2.73**	**D_D**	**-0.036**	**-9%**	**-0.20**
D_D	**-0.063**	**-16%**	**-0.90**	**D_C**	**-0.033**	**-8%**	**-0.18**
D_C	**-0.033**	**-8%**	**-0.46**	**D_A**	**0.030**	**8%**	**0.17**
D_E	**0.016**	**4%**	**0.23**	**D_G**	**0.018**	**5%**	**0.10**
D_F	**-0.007**	**-2%**	**-0.10**	**D_E**	**-0.012**	**-3%**	**-0.06**
D_A	**0.006**	**1%**	**0.08**	**D_F**	**-0.006**	**-1%**	**-0.03**
D_B	**0.005**	**1%**	**0.07**	**D_B**	**-0.006**	**-1%**	**-0.03**

3.9. Measurement of uncertainty

The uncertainty inherent in the AOAC 2005.06 method is dependent upon a multitude of variables. Method parameters, environmental factors, uncertainties in weights and volume measurements, reference values, analyst variability and other approximations

and assumptions may all contribute to an accumulative variability of the method resulting in a degree of uncertainty described by the validation characteristics. Whilst assessing uncertainty of measurement, it is important to eliminate duplication of uncertainty components between different parameters. Table 29 describes the major sources of uncertainty associated with the method. Due to the wide ranging behaviour observed between PSP toxin analogues, standardised uncertainty values are calculated for each individual toxin.

Table 29. Uncertainty sources covered by existing data

Uncertainty Source	Data used for determination
Precision – Repeatability	Precision data/spiked tissue matrix/naturally contaminated tissue
Reproducibility	Collaborative study results
Recovery, bias	Recovery data – spiked tissue (uncertainty in determination)
Matrix Effects	Single matrix – Effects of intra-matrix variability not assessed. Uncertainties of each matrix will be compared in final report
Toxicological Data	Evaluation of published data

The uncertainties associated with the toxicological data used to generate the toxicological correction factor, ($F_{tox,}$), can only be assessed using published data, which is not available for the full suite of toxins present within the validation, and may also be of questionable accuracy. Two sets of data are commonly quoted, both describing relative mouse toxicity, the first currently used in this validation, published by Oshima [20], and the second published by Genenah and Shimizu [21] (Table 30)

Table 30. Relative toxicities of PSP toxins from Oshima (1995) and Genenah and Shimizu (1981)

Toxin	**Relative Mouse Toxicity, (Oshima, 1995)**	**Relative Mouse Toxicity, (Genenah and Shimizu, 1981)**
STX	1.00	1.00
NEO	0.92	0.51
dcSTX	0.51	0.60
GTX2	0.36	0.39
GTX3	0.64	1.09
GTX 2,3	0.43	0.56

Additionally, PSP toxins GTX1,4, GTX2,3, C1,2 and dcGTX2,3 each consist of an isomeric pair of toxins which co-elute during HPLC-FLD analysis. As such, samples containing any of these toxins are reported using the highest relative toxicity factor for each pair, which results in the potential for over-estimation of toxicities (table 31).
Both of these issues give rise to an uncertainty in final toxicity measurements, but neither can be formulated statistically into an uncertainty of measurement value. Use of the Oshima toxicities is common globally, utilised by all European monitoring laboratories and by the NRCC, although no guidance is given in the AOAC 2005.06 method. As such, results should be reported with a mention that a conservative approach is taken to relative toxicities, but no toxicity variability can be incorporated into a calculation of total method uncertainty. The potential effects of variable toxicological data and choice of isomer TEFs are described in the context of the parallel testing study (section 4.1.3).

Table 31. Toxicities of isomeric pairs, showing potential overestimation of toxicity using current toxicity calculations.

Toxin	Relative toxicity	Relative toxicity used	Potential overestimation of toxicity
GTX1	0.994	0.994	1.4
GTX4	0.726		
GTX 2	0.359	0.638	1.8
GTX 3	0.638		
dcGTX 2	0.154	0.377	2.4
dcGTX 3	0.377		
C 1	0.006	0.096	16.0
C 2	0.096		

3.9.1. Precision – Repeatability

The measurement uncertainty inherent in the precision component may be evaluated from the statistical distribution of the results of a series of measurements and can be characterised by standard deviations [22]. Uncertainties are calculated for medium term precision (both at 0.2 AL and 0.5 AL concentration levels). Sample repeatability data is not included in preference to spiked sample data as the latter includes precision of extraction efficiency. These RSDs may be pooled to give a total standardised precision uncertainties (table 32):

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

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

Table 32. Precision values (RSDs) and pooled uncertainties calculated for PSP toxins in Mussels.

	Uncertainty in precision		
	Medium term		"Pooled"
	0.2 AL	0.5 AL	Uncertainty
GTX 1,4	0.17	0.04	**0.13**
dcNEO	0.20	0.26	**0.23**
NEO	0.51	0.26	**0.40**
dcSTX	0.32	0.23	**0.27**
GTX 2,3	0.22	0.10	**0.17**
GTX 5	0.26	0.32	**0.29**
STX	0.16	0.05	**0.12**
dcGTX 2,3	0.09	0.15	**0.12**
C 1,2	0.41	0.10	**0.30**

3.9.2 Reproducibility or long term repeatability

The uncertainties associated with long term precision are estimated from the precision data generated by the repeated extraction, clean-up, fractionation and analysis of LRMs. For toxins not present in the current LRM, reproducibility values are

taken directly from the mean of RSD_R data from spiked mussel matrix analysed during the interlaboratory study of the AOAC method (table 33). A mean value of 0.27 was used for toxins dcGTX2,3 and dcNEO not included in the AOAC 2005.06 interlaboratory study.

Table 33. Reproducibility values taken from AOAC 2005.06 interlaboratory study data and long term repeatability values calculated from LRM data.

	Reproducibility	Repeatability
	AOAC	LRM
GTX 1,4	0.28	0.27
dcNEO	0.27	
NEO	0.32	
dcSTX	0.27	
GTX 2,3	0.27	0.18
GTX 5	0.26	
STX	0.35	0.11
dcGTX 2,3	0.27	
C 1,2	0.15	

3.9.3. Recovery

Recovery was calculated using the levels spiked into the tissues as the expected values. The uncertainties present in the determination of recovery were estimated by calculating the RSD for each toxin at each spiking level, thus generating information on the uncertainty in recovery determination. Values are tabulated for each toxin at 0.2 AL and 0.5 AL in table 34 below. Pooled uncertainties are calculated for each toxin using the same formula as in section 3.9.1 (above) and are shown to be of relatively small magnitude.

Table 34. RSDs and pooled uncertainties associated with determination of recovery.

	Uncertainty of recovery measurement		
	Recovery		"Pooled"
	0.2 AL	0.5 AL	Uncertainty
GTX 1/4	0.040	0.037	**0.038**
dcNEO	0.042	0.054	**0.048**
NEO	0.020	0.057	**0.043**
dcSTX	0.009	0.085	**0.060**
GTX 2/3	0.067	0.091	**0.080**
GTX 5	0.013	0.066	**0.048**
STX	0.002	0.052	**0.037**
dcGTX 2/3	0.013	0.181	**0.129**
C 1/2	0.063	0.099	**0.083**

3.9.4. Calculation of combined standard uncertainty

Using method performance data reported earlier in this report and uncertainties pooled as described above, preliminary combined standardised uncertainties for each PSP toxin (Table 35) was calculated from the square root of the sum of squares:

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

where:

u_c = combined standardised uncertainty

$u_1 - u_n$ = individual standardised uncertainties

Table 35. Combined uncertainties calculated from validation data for Mussels showing uncertainties as (a) standardised uncertainty and (b) expanded uncertainty (k=2)

	Combined standardised uncertainty	Combined expanded Uncertainty (k=2)
GTX 1,4	0.30	0.60
dcNEO	0.36	0.72
NEO	0.52	1.03
dcSTX	0.39	0.78
GTX 2,3	0.26	0.52
GTX 5	0.39	0.79
STX	0.17	0.34
dcGTX 2,3	0.32	0.65
C 1,2	0.34	0.68

The standardised combined uncertainties given in Table 35 are preliminary because they will change over time as more method performance data is obtained through routine implementation of the procedure and analytical quality control. The results above show a combined standardised uncertainty for individual toxins, ranging from 0.17 (for STX) to 0.52 (for NEO). Expanded uncertainties range calculated using a coverage factor (k) of 2, subsequently result in a range of values from 0.34 (STX) to 1.03 (NEO). The coverage factor, k was taken to be 2 in order to provide a 95% confidence in the distribution of values, assuming a normal distribution [16].
The manner in which the individual expanded uncertainties are combined to yield a total measurement uncertainty based upon toxic equivalents has not yet been decided: this is still being discussed with the Food Standards Agency but does not detract from the relevance of the method performance data given in this report.

4. Phase II. Analysis of routine and archived mussel samples.

4.1. Analysis of results

The number and availability of fresh mussel samples likely to show signs of PSP toxins were found to be low at the time this part of the study was conducted. Therefore, this phase of work required the extraction and analysis of both fresh and archived mussel tissue. An assessment of the correlation between quantitative LC-FLD results and MBA therefore requires the assumption that low temperature (-20^{o}C) storage of frozen mussel homogenate tissue would not alter the concentrations of PSP toxins within the tissue, although there is little evidence either for or against such an assumption, other than the stability and precision of LRMs analysed previously within our laboratory over a long period of time. Appendix 3a tabulates the results of the LC-FLD qualitative screen and full LC-FLD quantitation of 21 Mussel samples found to be negative for PSP toxins by MBA or by the current HCl-extract LC-FLD screening analysis.

Of these 21 samples, thirteen were found to be PSP positive by the HCl-extract HPLC screen and all subsequently MBA negative. All thirteen samples when analysed by the Lawrence screening method, showed either no PSP (3 out of 13) or low concentrations of toxins (0.06 and 0.11 total µg STX eq./g). Eight samples previously found to be PSP negative by the HCl-extract qualitative HPLC screen, were also found to be PSP-free, both by the periodate screen of and by the full quantitation method. Therefore in the context of this comparison, the AOAC 2005.06 screening step was in agreement with the current LC screening/MBA approach. Interestingly, Appendix 3a does show that 3 of the 13 samples previously found to be HPLC positive (in HCl extract analysis) were deemed negative in the acetic acid extract. Whilst this could indicate slight differences in extraction efficiency between the two extraction methods, the previous positive screening result originated when no minimum signal to noise threshold was employed, resulting in samples containing possible PSP toxin peak at extremely low levels.

Appendix 3b tabulates the results obtained from the quantitative LC-FLD analysis of mussel-tissue found to contain PSP toxins by both the qualitative screen and by MBA analysis. The table illustrates the comparison between 40 such samples, detailing the final calculated total toxin concentrations for both the quantitative LC and MBA methods. Visual inspection of the comparative results indicates that there is a noticeable correlation between the two data sets, with a significant number of samples exhibiting similar values using both methods. Figure 21 plots the correlation between the two sets of data. The correlation data is summarised in table 36 below. 40 MBA positive samples were analysed by HPLC and all MBA positive samples were also found to be positive for the presence of PSP toxins by HPLC. MBA analysis of 12 of the samples resulted in total PSP toxin concentrations higher than the action limit of 80 µg STX eq./100g. 11 of these 12 samples when analysed by HPLC also produced final toxin concentration values higher than the action limit. One sample (sample 811) resulted in an MBA of 89 µg STX eq./100 g, whereas the HPLC concentration determined was just below the action limit at 77µg STX eq./100 g. Such variability can be explained by the precision of the method.

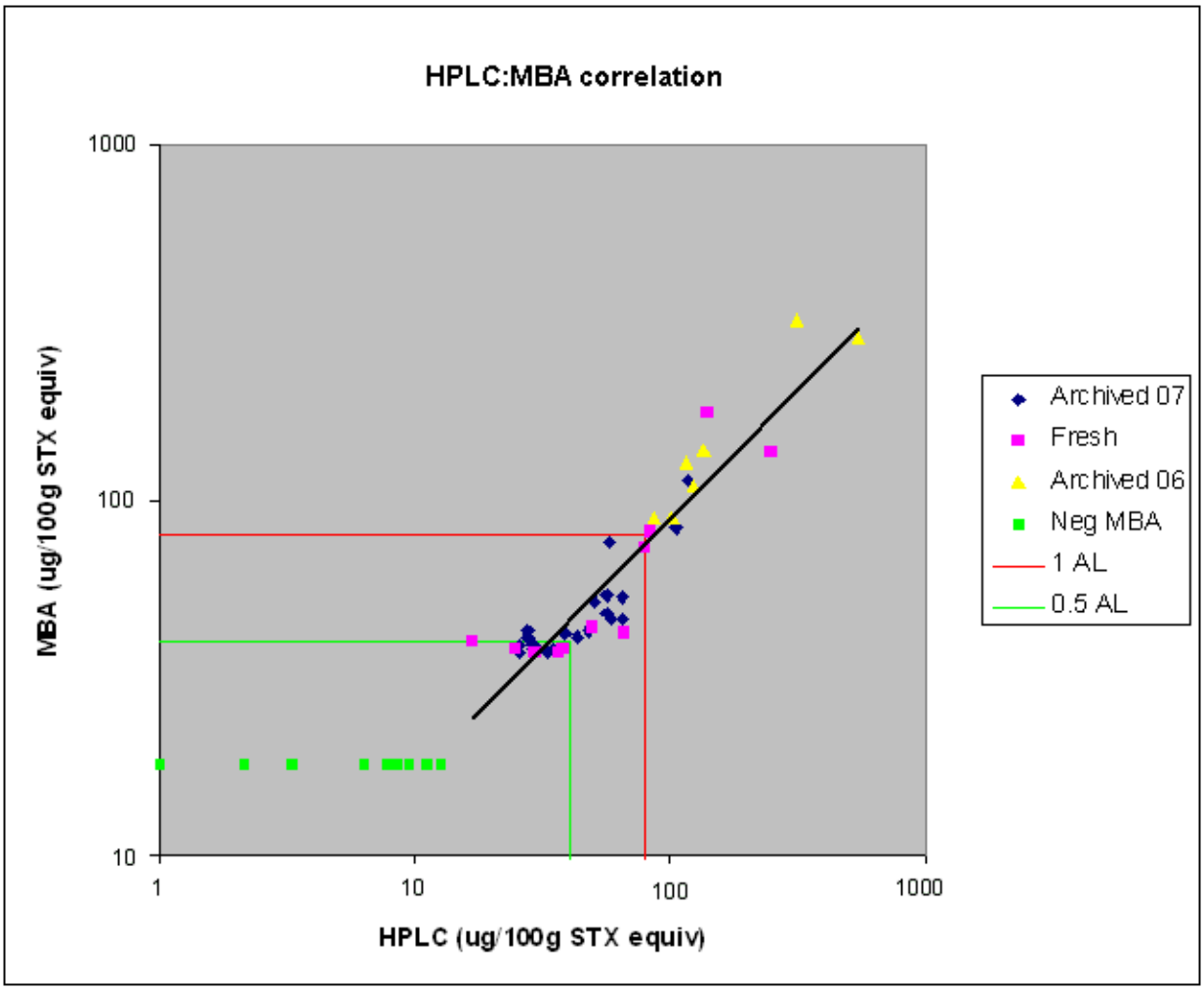


Figure 21. Comparison of total PSP toxin concentration results obtained by both MBA and quantitative HPLC-FLD method (natural log scale). 1.0 and 0.5 AL limits are highlighted. Oshima [20] TEFs used for toxicity calculations. Positive samples colour coded depending on source, MBA negatives/not tested shown as MBA result at half MBA detection limit (18 μg STX ev./100g).

The overall HPLC/mean MBA results ratio is 101% inferring that the HPLC method agrees closely, on average, with the results obtained by the MBA. The percentage relative standard deviation of the HPLC/MBA results ratios is 30%, which illustrates the degree of correlation scatter shown visually in figure 21. A two-tailed t-test calculated results with a t-value of 0.76 which when compared with the t-critical value (n=40) using a two-tailed t-test at 95% confidence of 1.80, infers that there is no significant statistical difference between the two analyses as they are statistically both from the same distributions. An analysis of the rank correlation between the two data sets (correlation coefficient, r = 0.92) is displayed graphically in figure 22. This further illustrates a good degree of correlation between the two techniques, especially interesting as the two techniques employ different extraction procedures and measure toxicities in very different ways.

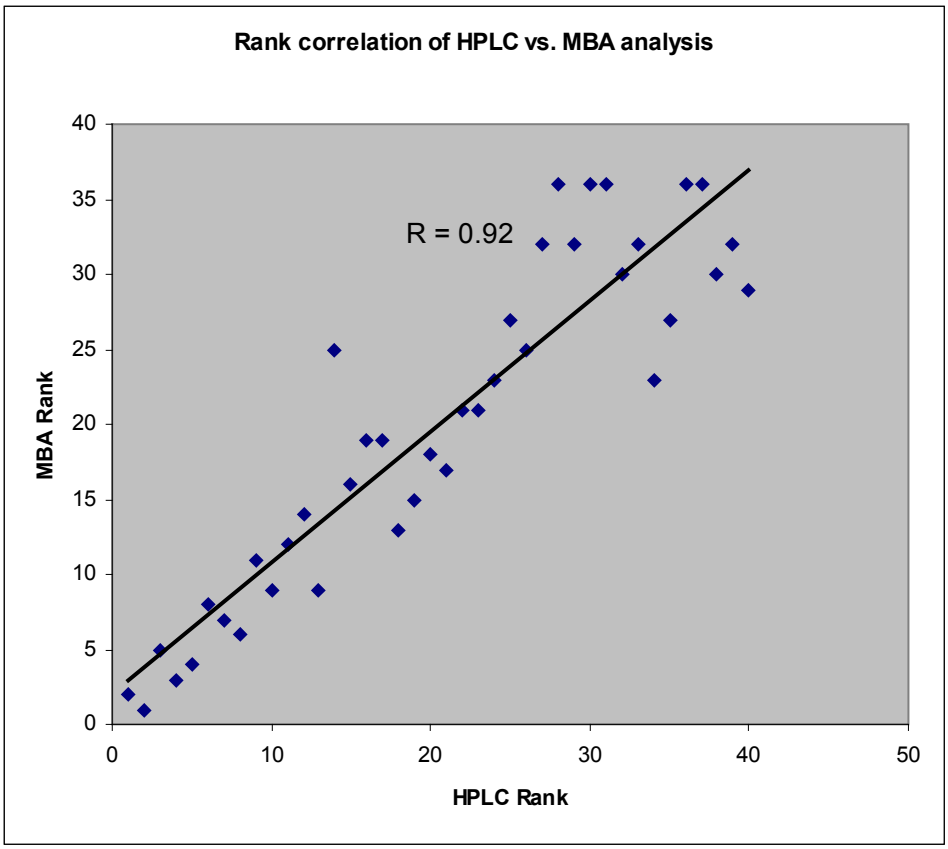


Figure 22. Rank correlation of HPLC and MBA analysis results for 40 MBA PSP-positive Mussel samples.

Table 36. Summary of results from quantitative HPLC and MBA analysis of naturally contaminated Mussel samples.

Total number of Mussel samples	61
Number of MBA +ve samples	40
Number of MBA +ves assigned HPLC +ve	40 (100%)
Mean HPLC concentration	83 μg/100g STX equiv
Mean MBA concentration	75 μg/100g STX equiv
Mean HPLC/MBA (n=40)	101%
RSD of HPLC/MBA results	30%
Correlation coefficient (r; all positives)	0.93
r; Fresh +ve samples only; n=11	0.88
r; 2007 archive +ve samples only; n=22	0.86
r; 2006 archive +ve samples only; n=7	0.94
T test of HPLC/MBA results	0.76
t-critical*	1.80
MBA > AL, HPLC < AL	1 (2.5%)
MBA < AL, HPLC > AL	0 (0%)

* t-critical using two-tailed t-test at 95% confidence

The results also show little difference between the correlations of results obtained from fresh vs. archived samples. Mean values are similar for the data set, and the percentage relative standard deviation of the analysis ratios is acceptable. As discussed earlier, unoxidised samples were run alongside periodate and peroxide oxidised

samples in order to determine the presence of any naturally fluorescent matrix co-extractive compounds. In a number of samples, levels of dcGTX2,3 and to a lesser extent GTX1,4 interferences, were detected, thus requiring subtraction of the un-oxidised peaks from the oxidised peaks. It was noted that the presence of these components was variable throughout the data set. Performing this subtraction requires the assumption that the peak response of a naturally fluorescent peak will be constant in both the un-oxidised and oxidised samples. Such an assumption contains a degree of uncertainty, as shown by the variability in chromatographic profiles between unoxidised and oxidised blank samples (section 3.2). However, the general approach is thought to be valid (Lawrence, personal communication), as without the use of interference subtraction, significant levels of dcGTX2,3 would have been falsely quantified in a large number of positive samples, giving rise to the potential for a number of false positive results.
The aim of this parallel testing was to demonstrate that the HPLC method performs adequately as an analytical procedure for the quantitative analysis of PSP toxins in Mussels, successfully identifies contaminated and non-contaminated samples and identifies positive samples at half or below the regulatory action limit (0.5 AL).
Overall, the results presented here confirm the success of this testing, with visual and statistical results from this study indicate that there is no significant difference between the results produced by the MBA and the quantitative HPLC analysis. These results suggest therefore, that implementation of the quantitative HPLC method could be recommended in terms of the ability of the method to replicate the values achieved by the current laboratory official control method for quantifying PSP toxins in harvested shellfish.

4.1.1. Effects of toxicity factor variability on results correlation

As described in section 3.9, there is a lack of information regarding the use of toxicity equivalence factors (TEF), which may have potential significant effects on the final toxicity results calculated. Table 37 shows the relative toxicities quoted by Oshima (1995) and Genenah and Shimizu (1981). In addition to this potential variability, use of the AOAC 2005.06 method requires the summation of isomeric pairs (due to isomer co-elution) and the use of the highest TEF for each pair (table 37).

Table 37 summarises the comparative results between HPLC and MBA when these factors are varied. Use of the Genenah and Shimizu toxicities results in a higher mean HPLC result, with the mean HPLC/MBA of 121% describing a clear positive HPLC bias as a result. A t-test analysis of the HPLC and MBA data sets fails for these results, indicating that use of these values results in data sets that are significantly different. Additionally, 3 of the 40 samples exhibit false positives (7.5% of data set). Use of the lower toxicity TEF for each isomer results in a significant negative HPLC bias as compared with the normal use of the higher TEFs. 10% of the data set shows false negatives as a result. Results therefore indicate a degree of variability inherent in the use of TEFs and the importance of using the most accurate values when modelling toxicity using non-animal methods. However, the results indicated that the best agreement between HPLC and MBA results was obtained when using TEFs quoted by Oshima and when using the highest TEF for each isomeric pair. Both these practices are maintained in monitoring laboratories world-wide. It should be noted that these comparisons depend significantly on the overall toxin profile of the samples. The relative low presence of C1,2 and dcGTX2,3 toxins reduces the overall influence of high TEF variability (between isomers) on overall sample toxicity.

Table 37. Effects of toxicity factor variability on HPLC:MBA results comparison (a) using standard Oshima TEF values and highest toxicity value for each isomeric pair (b) using Genenah and Shimizu and TEFs and (c) using lowest toxicity values for each isomeric pair.

	(a) Standard values	(b) TEF variability	(c) Isomer choice
Number +ve samples	40		
Mean MBA concentration (ug STX eq./100g)	75		
Mean HPLC concentration (ug STX eq./100g)	83	99	58
Mean HPLC/MBA (n=40)	101%	121%	72%
RSD% of HPLC/MBA results	30%	31%	32%
Correlation coefficient (r2; all positives)	0.87	0.86	0.60
T test of HPLC/MBA results	0.76	2.33	-1.64
t-critical	1.8		
MBA > AL, HPLC < AL	1 (2.5%)	0	4 (10%)
MBA < AL, HPLC > AL	0 (0%)	3 (7.5%)	0

4.2. Comparison with the method currently in use in the monitoring programme

4.2.1. Assessment of ease of method implementation

The methods employed during the HPLC quantitation of PSP toxins in the acetic acid extracts of Mussel tissues represents some major changes from the protocols employed presently in the laboratory for the qualitative HPLC screen and MBA analysis of HCl extracts on Mussels. In terms of extraction, the logistics of the two operations appear to be fairly similar. Other than the health and safety issues relating to the use of a boiling water bath, the use of the acetic acid extraction method is simpler than carrying out the HCl extraction where constant monitoring and additional filtering is a requirement. C18-SPE clean up of acidic extracts is identical for both methods. Occasional method failures previously identified have highlighted the need for additional data to be recorded during the C18 and subsequent pH-adjustment phase. Whilst this will require further work from analysts, it is an essential step to eliminate the possibility of failed sample analysis. Periodate oxidation and LC-FLD analysis in both the current screening approach and the full 2005.06 method are similar. The Lawrence method however, requires the use of matrix modifier in the oxidation process. This will require only a minor level of in-house training together with the additional work required on a regular basis to process and prepare the matrix modifier solution. Any detection of peaks at the retention times of PSP toxin standards in the periodate screen will result in the sample being oxidised by peroxide and fractionated prior to periodate oxidation of fractions F2 and F3. These processes represent a significant departure to the current laboratory procedure and significant additions to the overall work load are expected, for example. Work in this study has indicated that for the quantitation of Mussel samples using HPLC, for each sample, a periodate screen will be followed by a peroxide and second periodate oxidation of the C18-cleaned extract, plus periodate oxidations of two fractions (section 2.1) Together with the oxidation of an un-oxidised sample aliquot to test for the presence of naturally fluorescent compounds, these analyses will significantly add to the work-load of the analyst undertaking this method. It is therefore certain that the additional work required for this method will have a significant effect on turnaround times and unit costs per sample.

4.2.2. Assessment of results turnaround times

The current HPLC-screen/MBA method for PSP toxin analysis in the routine monitoring programme enables the analysis of up to a maximum of 40 samples per day.

Experience in carrying out such analyses over the past year shows that whilst in general results become available within 36 hours of sample receipt [14].

Using the fully quantitative HPLC method, it is anticipated that a similar number of samples could be extracted, cleaned-up and oxidised by periodate for screening purposes on day one, assuming the samples arrived early enough for two batches of extraction and centrifugation to take place. However, the overall turnaround time of the quantitation will depend heavily on the number of samples identified as positive in the initial screen and hence on the numbers of oxidations and analyses required to carry out full quantitation. Assuming an average percentage of positive screens of 25%, a resulting 10 quantitative analyses would be required by HPLC. Un-oxidised samples would also have to be analysed along with extracts and fractions of an LRM and procedural blank. Such a throughput could be analysed on one instrument with the laboratory. However, a worst case scenario of 50% positive samples, would result in the need to utilise further instruments and add further time to the overall procedure.

The AOAC 2005.06 method highlights problems with toxin oxidation product stability, advising the stability should not be assumed beyond 8 hours [12]. This limitation would result in either a restricted sequence length (with a reduced number of samples), or the use of further standards throughout the sequence. However, an analysis of standard stability in our laboratory using a temperature-controlled HPLC autosampler (set at 4^{o}C) has shown that the oxidation products of all toxins are stable for up to 24 hours (data shown in Appendix 5). Therefore, there are no restrictions on the length of analytical sequence, up to 24 hours, which will improve sample throughout.

Any samples exhibiting levels of toxins above the range of linearity described in this validation study could be reported as “> AL”, or may have to be diluted for purposes of accuracy and analysed again in order to bring the diluted cleaned-up extract concentration within linear range for quantitation. In addition, any problems associated with quality control, would result in the need to repeat the oxidation and analysis of the samples run on the previous day. Such a repeat would cause a major problem to the back-log of samples if a significant number of samples were testing positive in the screening step. Hence it is especially important to provide contingency for such an occurrence.

In the scenario where the quantitation sequence ran smoothly and there were no instrument or QC errors associated with the batch, it is feasible that on day three, data interpretation could be carried out and the results reported later that day. However, the time required to interpret the data is significantly longer than the time to analyse MBA results, so again, this would almost certainly result in positive results being reported towards the end of day three, resulting in an overall minimum turnaround time of 52 hours. If instrument or QC problems did occur, then the turnaround time for a batch of samples would rise to at least 80 hours, considerably higher than the turnaround currently experienced with the HPLC ‘screen’/MBA analysis.

4.2.3. Assessment of equipment and process reliability

During the course of this validation study, a very low number of instrument problems were encountered. However, an HPLC or automated SPE unit failure will lead to a significant pressure on the application of the method to routine monitoring. Such failures would lead to extensive delays in use of instrumentation, even though instruments are covered by a service contract providing, typically, a 48 hour response.

Such a response is, however, not a guarantee of repair, as parts may need to be ordered, a process which could significantly extend the period of instrument downtime.

4.2.4. Assessment of contingency

The application of the fully quantitative HPLC method for the routine analysis of PSP toxins clearly requires a higher level of instrumental use, both in terms of HPLC-FLD and automated SPE instrumentation. As such, the method requires the use of two HPLC-FLD systems in order to run both the screen and quantitative parts of the method on subsequent days. At present, C18-clean-up is carried out on an Aspec “XL4” SPE unit and fractionation is carried out on the single arm Aspec instrumentation (also used as back-up for the current XL4 system). As a result on the additional load on these instruments, it is essential that contingency is put in place for further availability of additional HPLC and SPE instrumentation.

As such our laboratory has recently purchased both a new HPLC-FLD instrument (Agilent 1200 series) and a new Gilson Aspec XL4 unit. The new HPLC will provide contingency in case of failure in either the screening or quantitation HPLC, and also for ASP analysis. The new Aspec XL4 will enable fractionation to be transferred to a four-arm transfer unit, thus considerably improving the speed of the fractionation step and providing a back-up instrument (XL) if the new unit fails.

Similarly, contingency measures will be put in place for loss of key staff and it is recognised that ongoing training and staff development is a key requirement for the ongoing successful implementation of the quantitative HPLC method for the analysis of routine shellfish samples.

4.2.5. Ease of results interpretation

Data interpretation associated with the full quantitative HPLC method for the detection of PSP toxins in shellfish tissue is a more complex process than that required for both the current qualitative screen and interpretation of MBA results. As discussions throughout this report highlight, there are numerous factors to be taken into consideration when calculating toxin concentrations in mussel tissue, including the potential presence of mussel tissue co-extractive matrices and procedural interferences. With the additional requirement to calculate the estimated concentrations of co-eluting non-N-hydroxylated toxins and remove these from any detected N-hydroxylated toxins, the overall approach to the data interpretation is complex and time-consuming, with the interpretation of sample and QC data associated with a 10 sample batch run estimated to take between 2 and 3 hours of analyst time.

In order to aid the interpretation of data from the chromatographic results, training is provided to all analysts. A repeatable approach has been defined regarding integration of toxin peaks and in particular, with attention focussing on integration methods to be used when measuring peak areas of the chromatogram significantly affected by matrix interferences. A routine method of data interpretation and concentration calculation has been implemented which is both accurate and relatively easy to use given the appropriate training.

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

In accordance with EU legislation on the use of Official Control Methods [2], the "Lawrence" AOAC 2005.06 HPLC-FLD method was subjected to an in-house program of validation and applied in tandem alongside the OC MBA. Extensive testing was carried out on mussel tissues using the 2005.06 method to assess the selectivity, linearity, limits of detection and quantitation, accuracy, recovery, precision, repeatability, ruggedness, fitness for purpose and robustness of the method. Additionally, the method validation was extended to include toxins (dcGTX2,3 and dcNEO) not included in the AOAC 2005.06 method. The aim of the study was an assessment of the overall applicability of the method to the analysis of PSP toxins in mussels as a potential future part of the national biotoxin monitoring program in the UK. The validation study was conducted on mussels collected under the UK monitoring programmes and included all PSP toxins currently available as certified reference standards. A comparison of results obtained using the quantitative method with those obtained using MBA was also performed. Quantitative results from each of the validation tasks where appropriate are summarised in table 38 for each PSP toxin and results of this assessment are provided below.

Initial work focussed on the chromatographic post-oxidation behaviour of dcNEO and dcGTX2,3 toxins, toxins not being described by the AOAC 2005.06 method. Additional work was undertaken on C1,2, toxins not currently analysed as part of our routine HPLC screening analysis. Results indicated the requirement for periodate oxidation for dcNEO and showed the greater sensitivity resulting from the peroxide analysis of C1,2 and dcGTX2,3, as observed previously with other non N-hydroxylated toxins [1,14]. Analysis of retention time characteristics of the entire suite of toxins enabled an assessment to be made on the necessary approaches to be taken for toxin quantitation, and the need for three separate toxin standard mixes to be employed for quantitation purposes.

Qualitative chromatographic observations showed that whilst significant matrix components are present in the fluorescence chromatograms of cleaned-up mussel extracts and fractions, most of these do not interfere with the selectivity of the analysis of individual PSP toxins. Hence, the quantitation of most toxins is not expected to be biased by selectivity effects. The exceptions to this were peaks of dcGTX2,3 and GTX1,4 where matrix interferences were found to be present at the same retention times as toxin quantitation peaks. Such interferences when present could bias accurate quantitation and supports the need for running unoxidised samples alongside periodate and peroxide oxidised samples as described in the official method. Phase II parallel sample testing illustrated the variable nature of these non-toxin components and showed that without subtraction of these co-extractives from potential toxin peaks, a number of false positives would have been assigned. Whilst such interference subtraction involves a degree of assumption and uncertainty and significantly adds to the overall turnaround of sample analysis, it reduces the likelihood of false positives.

Investigations of linearity showed that for the suite of PSP toxins studied, the calculated calibration functions demonstrated linearity over the working range of 0 to 1.2 AL per toxin. Evidence for this was obtained through visual inspections of both calibration curves and residual plots, and the use of additional f-test goodness of fit analysis. Some differences were observed between the slopes of calibration curves when comparing matrix (mussel)-matched and solvent spiked calibration standards. However, there was

no significance attached to these differences, as the relative slope differences varied from toxin to toxin, and differences were only observed in the calibration curves of the N-hydroxylated toxins, GTX1,4, and dcNEO.

In relation to regulatory limits, a good level of instrumental sensitivity was illustrated for the periodate screen with detection limits of approximately 0.03 to 0.14 µg STX eq./g (0.04 to 0.18 AL). Whilst these values are not derived from homogenate spiking experiments, these values give an indication of the sensitivity of the screening method when employing acetic acid extracts. Experimentally-determined method LODs ranged from (0.007 to 0.087 µg STX eq./g; ~0.01 to 0.1 AL) for all PSP toxins, with the exception of GTX1,4, (0.16 µg STX eq./g; 0.2 AL). This illustrates the sensitivity of the method and the ability of the method to detect all toxins at levels less than or equal to 0.2 AL, with most toxins at levels well below this limit. Sensitivities are similar to those quoted in the AOAC 2005.06 method, again with the exception of GTX1,4 where a poorer sensitivity is described. Limits on the availability of the dcNEO reference standard restricted LOD determinations for this toxin. Therefore the LOD is quoted as <<0.16 µg STX eq./g, as recovery testing of dcNEO at this concentration level had previously shown peak responses with a signal to noise ratio >> 3.0. Limits of quantitation were shown experimentally to vary between 0.01 to 0.16 µg STX eq./g (0.01 to 0.2 AL) for all toxins except GTX1,4 (0.38 µg STX eq./g; 0.47 AL). As such, the LOQs for all these toxins except GTX1,4 are at or lower than the target concentration level of 0.2 AL. These results would therefore suggest that the method can operate with an acceptable level of precision and certainty at less than or equal to 0.2 AL for each of these toxins. The higher LOQ for GTX1,4 relates to the poorer sensitivity of the instrumental method for the diagnostic peak of this toxin (and most likely a product of inefficient oxidation by periodate), and results in a reduced confidence in the degree of precision obtainable for the analysis of GTX1,4 in the 0.2 to 0.5 AL concentration range. However, the medium term precision data (from spiked homogenate) and long term LRM data for GTX1,4 indicates the precision for these toxins is good at 0.2AL (≤25% RSD%; HorRat < 2.0). Thus it can be agreed that quantitation for GTX1,4 can be carried out with a good degree of confidence down to the limit of detection (0.16 µg STX eq./g; 0.2 AL). Such an argument is not unacceptable, given the recognition that analytical measurements below the LOQ are not devoid of information content and may well be fit for purpose [4].

The accuracy of the method was examined by the analysis of two, naturally contaminated and potential reference materials supplied by the NRCC. Whilst not formally certified, these materials enabled the comparison of AOAC 2005.06 method results with results obtained using post-column HPLC-FLD analysis, as performed by NRCC analysts. In both samples, the comparison between methods was good, with a very close agreement in toxin profiles between the two methods being evident. Comparative results for the pilot CRM were particularly encouraging as the concentrations of most toxins were above the target concentration of 0.2 AL and a more complex mixture of toxins was detected. In particular, the presence of both GTX1,4 and dcGTX2,3 enabled a successful assessment of the concentrations of co-eluting toxins. The very close agreement for dcGTX2,3 concentration is particularly positive, in light of the potential for selectivity issues highlighted in this study. Examination of total PSP toxicity results between the AOAC HPLC method and post-column HPLC method as compared with the MBA result, highlighted the higher degree of accuracy of the AOAC method for these two samples.

Method recovery experiments were undertaken on spiked tissue homogenates at both 0.2AL and 0.5 AL concentrations and for all toxins. The results have shown that recovery is similar to the levels described in the AOAC 2005.06 method, with results demonstrating some differences between the values for each toxin and between different concentration levels. dcNEO exhibited lower recoveries as compared with the other toxins, but this toxin is not present within the AOAC 2005.06 so comparisons were not possible in this study. The lower values calculated for this toxin may relate either to the behaviour of the toxin or relate to the experimental design. dcGTX2,3, also not included in the AOAC 2005.06 method showed recoveries comparable to those of other toxins.
Repeat extraction experiments have demonstrated an apparent extraction efficiency of approximately 75-80 %, which relates well to the range of method recoveries calculated for the 0.5 AL spiked mussels. Whilst a significant degree of method uncertainty is expected to contribute to the variability in recovery results, particularly at lower concentration levels, it appears, with the possible exception of dcNEO, the recoveries determined were acceptable for such a complex method, with the 20-25 % losses on extraction being the largest contributor to method recovery.

Instrumental precision investigations showed that variation of peak retention times and peak areas was particularly low. Precision data showed RSDs all $\leq$4 % for retention time variability over a two month period and the replicate injections of a cleaned up, spiked sample extract showed peak area precision falling between the range of 0.2 % to 3.5 %, with a mean value of 1.4 % RSD. Short-term repeatability studies involved the triplicate extraction, clean up, fractionation, oxidation and analysis of spiked mussel tissues at 0.2 and 0.5 AL per toxin. Percentage RSDs calculated were less than 10% for each toxin, with the exception of dcGTX2,3, which appears consistent with values quoted in previous studies by the method authors [9,10,11]. Furthermore, an acceptable level of precision at the 0.2 AL (RSDs for all toxins $\leq$7 %), gives a good indication that the method is capable of reliably quantifying all PSP toxins at a concentration level significantly lower than the action limit. HorRat values were shown to be acceptable at both concentration levels giving further evidence for acceptability of precision.

Medium term repeatability was assessed with the replicate (n=6) spiking, extraction, cleanup, fractionation, oxidation and analysis of mussel tissues spiked at 0.2 and 0.5 AL, all carried out over a period time greater than two weeks and with different analysts. The mean percentage RSD for all PSP toxins is 29 % at 0.2 AL and an improved 20 % at 0.5 AL. HorRat values were <2.0 for all toxins at both concentration levels, with the exception of NEO at 0.2 AL, further evidencing the degree of acceptability associated with the precision of the method. Considering the high variability inherent in such a multi-step method, these values seem reasonable and seem to indicate that the method is repeatable within the laboratory over the medium term.

A long-term precision assessment of repeatability was conducted over several months, with the repeat extraction and analyses of a laboratory reference material (LRM), consisting of a naturally contaminated mussel sample. The analysis was carried out using multiple analysts, different consumables wherever possible, and on different HPLC-FLD instruments. The three toxins (STX, GTX2,3 and GTX1,4) present at concentration levels exceeding the target 0.2 AL level exhibited very good levels of method precision (RSDs 11-25 %; HorRat ratios <2.0), with the GTX1,4 toxin quantitation giving further confidence in the long term repeatability of the fractionation step in addition to the extraction, C18 clean up and oxidation. The values obtained give

good evidence of a highly repeatable method and provide the foundation for important QC monitoring in future routine analysis programs.

The ruggedness of the method was assessed with two separate experiments focussing on a range of parameters thought to have an impact on method stability. Two N-hydroxylated (GTX1,4 and NEO) and two non-N-hydroxylated (STX and GTX2,3) toxins were chosen as model toxins and parameters affecting extraction, clean up, fractionation, oxidation and analysis were all varied according to a strict experimental design. Results indicated that whilst the variation of certain parameters improved method recovery, none of the parameters monitored had any statistically significant effect on method stability. Therefore the method was shown to be robust and stable.

The validation results obtained throughout the study were used to calculate standardised and expanded uncertainties for the analysis of PSP toxins in mussels. The contributions to uncertainty were assessed to ensure all factors were incorporated whilst eliminating measurement uncertainty duplication. Results showed a preliminary combined standardised uncertainty for individual toxins, ranging from 0.17 (for STX) to 0.52 (for NEO with preliminary expanded uncertainties (k=2) ranging from 0.34 (STX) to 1.02 (NEO).

Following laboratory validation, the analytical method was trialled in comparison with the OC MBA, with the analysis of mussel samples obtained from the UK biotoxin monitoring programme. Samples had been previously extracted using HCl extraction, and if deemed positive by our HPLC screening method, had been submitted for MBA analysis. Acetic acid extracts of samples were subjected to both the screening and quantitation elements of the AOAC 2005.06 method and comparisons were carried out between results obtained from the HPLC screening method utilising HCl and acetic acid extracts and between the fully quantitative 2005.06 and the MBA. The correlation between the two screening methods was excellent, with very few differences observed between the screening results obtained using either HCl or acetic acid extraction. The MBA and HPLC total toxicity results showed a good overall correlation, with visual and statistical results from the two methods ($r = 0.93$; t-test $t = 0.74$ (t-critical = 1.80); mean HPLC/MBA = 101%; RSD = 30%) indicating that there is no significant difference between the two methods. However, whilst there was a close correlation between the two methods, it is noted that the data set analysed is limited and ideally further comparative work would be undertaken prior to any possible future total reliance on the HPLC technique alone. One sample initially found to give an HPLC result <50% of the MBA result was repeated and shown to give a closer agreement. This initial discrepancy was found to relate to a C18 clean-up error, as a result of which additional QC steps have been implemented to prevent re-occurrence. Analysis of the toxin profiles present in the mussels analysed, showed the prevalence of GTX1,4, GTX2,3 and STX toxins (accounting for around 90% of total toxin content), with minor contributions of most other toxins. Analysis of the effects of changing toxin equivalency factors (TEFs) and utilising the lower toxicity TEF for each isomeric pair showed significant effects for both. Use of Genenah and Shimizu TEF values resulted in a 120% positive HPLC bias, a failed correlation t-test ($t = 2.3$) and three false HPLC positives out of the 40 sample data set. Use of the lower toxicity values for each of the four isomer pairs (GTX1,4, GTX2,3, C1,2 and dcGTX2,3) conversely resulted in a strong negative HPLC bias (HPLC/MBA = 72%), with four false HPLC negative values. Statistical analysis of these results alone therefore indicated that current use of Oshima TEFs results in the highest correlation between HPLC and MBA results.

In terms of method implementation, the fully-quantitative HPLC method is challenging and costly to implement. A high level of training is required and a significant increase in sample turnaround times will be noticeable if this method is adopted. Assuming an absence of instrumental and/or QC failures, quantitation data interpretation would be completed on day three, resulting in an overall minimum turnaround time of 52 hours. If instrument or QC problems did occur requiring a repeat screening or quantitation analysis, then the turnaround time for a batch of samples would increase by an additional 24 hours (80 hours total), significantly higher than the turnaround currently experienced with the HPLC 'screen'/MBA analysis. Whist contingency measures have been put into place, occasional instrument and/or QC failures are inevitable.

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

	Linearity	LOD	LOQ	Recovery %		Short term precision RSD%		Medium term precision RSD%		Long term precision	Ruggedness	Standardised Uncertainty
	(r^2)	ug/g STX equiv	ug/g STX equiv	0.2 AL	0.5 AL	0.2 AL	0.5 AL	0.2 AL	0.5 AL	RSD%	Stability	
GTX 1,4	0.970	0.16	0.38	112%	81%	4%	4%	17%	4%	25%	Yes	0.30
dcNEO	0.989	<0.16	0.16	29%	53%	4%	5%	20%	26%	nd	nd	0.36
NEO	0.963	0.068	0.14	136%	107%	2%	6%	51%	26%	nd	Yes	0.52
dcSTX	0.994	0.007	0.01	85%	68%	1%	8%	32%	23%	nd	nd	0.39
GTX 2,3	0.983	0.087	0.17	94%	66%	7%	9%	22%	10%	17%	Yes	0.26
GTX 5	0.993	0.003	0.006	82%	76%	1%	7%	26%	32%	nd	nd	0.39
STX	0.993	0.018	0.04	122%	68%	0.2%	5%	16%	5%	11%	Yes	0.17
dcGTX 2,3	0.986	0.053	0.11	82%	70%	1%	18%	9%	15%	nd	nd	0.32
C 1,2	0.970	0.018	0.04	79%	63%	6%	10%	41%	10%	nd	nd	0.34
Mean	0.982	0.067	0.123	91%	72%	3%	8%	26%	17%	18%	na	na

Nd = not determined; na = not applicable

6. References

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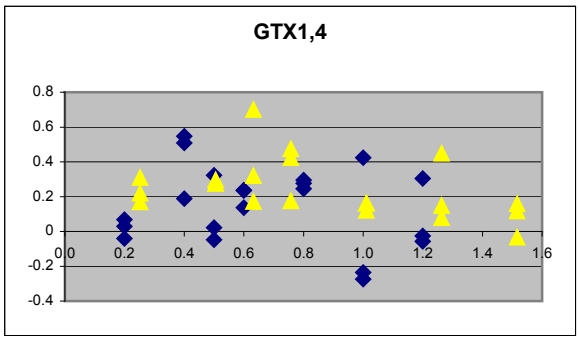
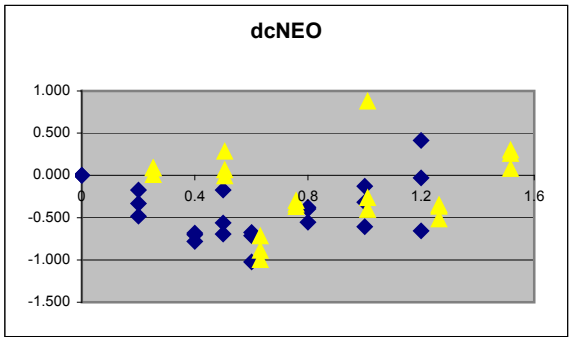
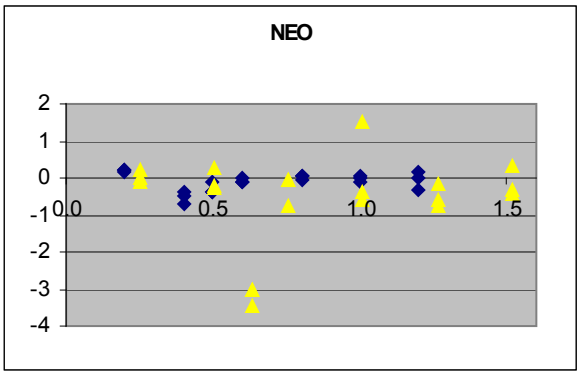
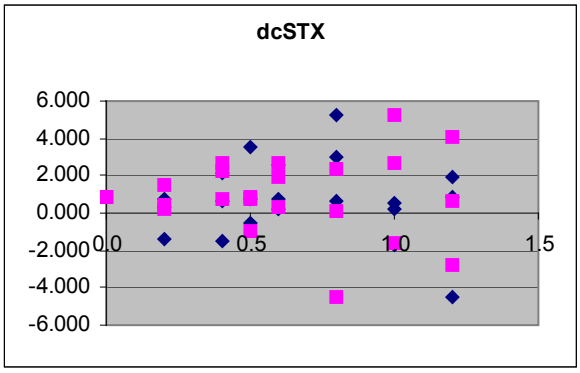
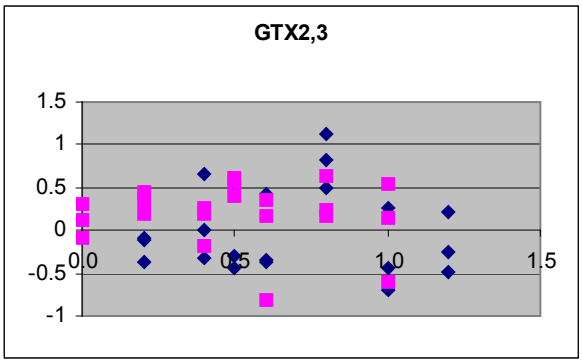
Appendix 1. Residual plots for individual PSP toxins in mussel extract, fractions (where applicable) and solvent calibrations over the working calibration range (0 to 1.2 AL)

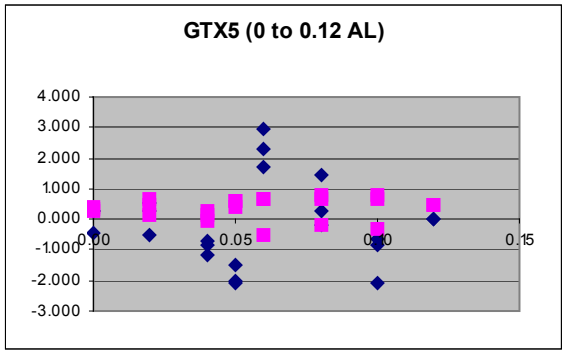


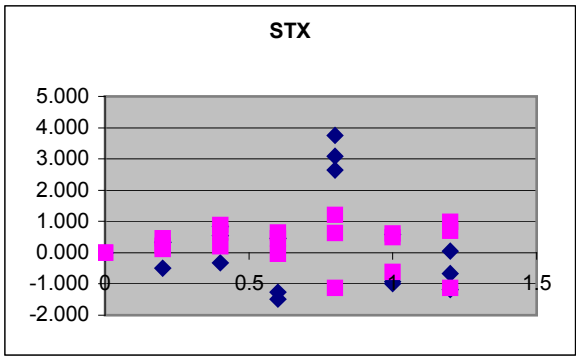


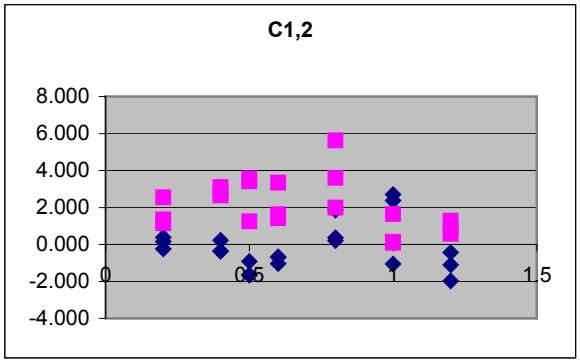


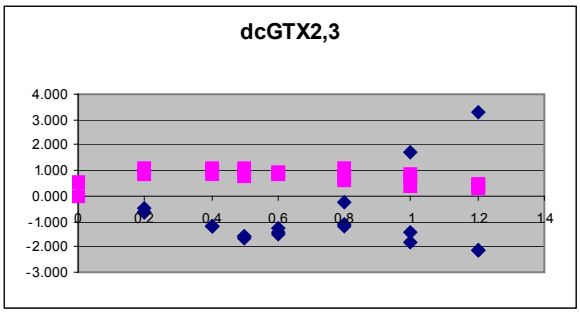


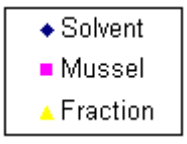












Appendix 2. Linear regression gradients, intercepts and coefficients, plus relative standard deviations of response factors calculated for each PSP toxin in Mussel extract, solvent and fractions (when applicable) over extended calibration range (0 to 2.5 AL; GTX5 = 0 to 0.25 AL).

Toxin	Matrix	Calibration gradient	Intercept	r^2	RSD% of response factors	F-test	F critical
GTX1,4	Extract	4.96	0.20	0.982	11%	0.78	2.64
	Solvent	3.13	0.01	0.989	8%	1.19	2.64
	Fraction F2	2.06	0.15	0.990	13%	0.69	2.64
dcNEO	Extract	8.76	0.01	0.995	7%	0.78	2.64
	Solvent	11.76	0.29	0.989	5%	1.59	2.64
	Fraction F3	7.74	-0.49	0.996	8%	1.11	2.64
NEO	Extract	16.51	0.23	0.991	7%	1.14	2.64
	Solvent	9.33	-0.03	0.993	4%	3.66	2.64
	Fraction F3	11.32	-1.12	0.991	19%	0.44	2.64
dcSTX	Extract	66.63	1.18	0.991	5%	2.57	2.64
	Solvent	75.43	1.15	0.991	5%	3.34	2.64
GTX2,3	Extract	6.25	0.41	0.984	8%	1.80	2.64
	Solvent	7.67	-0.48	0.975	12%	1.77	2.64
GTX5	Extract	14.22	-0.82	0.986	13%	0.84	2.64
	Solvent	15.44	-1.21	0.983	16%	0.65	2.64
STX	Extract	16.96	0.82	0.993	6%	1.46	2.64
	Solvent	19.07	-1.15	0.983	12%	1.06	2.64
C1,2	Extract	12.62	6.02	0.796	27%	1.07	2.64
	Solvent	25.01	-0.16	0.991	8%	1.37	2.64
dcGTX2,3	Extract	6.55	0.74	0.993	10%	0.35	2.64
	Solvent	10.33	0.87	0.944	23%	1.21	2.64

Appendix 3a. Results obtained from qualitative and quantitative (LC-FLD) analysis of PSP toxins in acetic acid extracts of mussel samples (BTX/2007/ sample numbers given) with those obtained from previous qualitative (positive LC-FLD) and quantitative (negative MBA) analysis of HCl extracts.

Toxin	2425	2330	2340	1667	1669	1670	1469	1286	958	960	2306	2308	2310	454	459	463	464	476	481	2318	2273
	Fresh	**Fresh**	**Fresh**	**A 07**	**A 07**	**A 07**	**A 07**	**A 07**	**A 07**	**A 07**	**Fresh**	**Fresh**	**Fresh**	**A 07**	**A 07**	**A 07**	**A 07**	**A 07**	**A 07**	**Fresh**	**Fresh**
GTX 1/4	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dcNEO	0.00	0.01	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NEO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dcGTX 2/3	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C 1/2	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dcSTX	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2/3	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.05	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
STX	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	0.08	0.03	0.13	0.00	0.00	0.00	0.06	0.08	0.00	0.00	0.11	0.02	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total HPLC (ug/100g)	**8**	**3**	**13**	**0**	**0**	**0**	**6**	**8**	**0**	**0**	**11**	**2**	**9**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
MBA result (Neg/No test)	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**Neg**	**No test**	**No test**	**No test**	**No test**	**No test**	**No test**	**No test**	**No test**
Original HCl HPLC screen	Pos	Pos	Pos	Pos	Pos[a]	Pos	Pos	Pos	Pos[a]	Pos[a]	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Acetic acid HPLC screen	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

A07 = archived sample from 2007, Fresh = fresh sample.

Individual toxin concentrations given in µg STX eq./g. Total PSP toxin HPLC results and MBA results given in µg STX eq./100 g.

[a] Sample originally scored positive due to presence of peaks, but prior to implementation of minimum signal to noise threshold.

Appendix 3b. Comparison of results obtained from (LC-FLD) analysis of PSP toxins in acetic acid extracts of mussel samples (µg STX eq./g; Oshima TEFs) with quantitative (positive MBA) analysis of HCl extracts.

Sample code[a]	998	973	1188	924	980	769	768	790	683	788	791	868	907	1007	1063	1075	1434	1780	1047	1109
	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07	A 07
GTX 1/4	0.17	0.28	0.20	0.21	0.16	0.23	0.12	0.25	0.12	0.10	0.12	0.65	0.28	0.20	0.17	0.26	0.28	0.19	0.29	0.29
dcNEO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NEO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
dcGTX 2/3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C 1/2	0.00	0.02	0.01	0.02	0.00	0.01	0.01	0.02	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.03	0.04	0.02	0.02	0.02
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GTX 2/3	0.09	0.13	0.20	0.23	0.07	0.08	0.07	0.08	0.14	0.08	0.11	0.27	0.14	0.21	0.07	0.13	0.24	0.24	0.18	0.17
GTX 5	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.001	0.000	0.003	0.000	0.000	0.000	0.002	0.004	0.003	0.002	0.002
STX	0.03	0.04	0.20	0.11	0.03	0.03	0.05	0.08	0.10	0.06	0.09	0.18	0.05	0.08	0.03	0.21	0.48	0.20	0.08	0.08
Total	0.29	0.48	0.62	0.57	0.26	0.35	0.26	0.43	0.39	0.26	0.33	1.18	0.49	0.51	0.29	0.65	1.05	0.65	0.57	0.57
Total HPLC (ug/100g STX eq)	**29**	**48**	**62**	**57**	**26**	**35**	**26**	**43**	**39**	**26**	**33**	**118**	**49**	**51**	**29**	**65**	**105**	**65**	**57**	**57**
av MBA (ug/100g STX eq)	**39**	**43**	**46**	**54**	**39**	**38**	**37**	**41**	**42**	**37**	**37**	**113**	**44**	**52**	**38**	**53**	**84**	**46**	**76**	**48**

Sample code	1293	1535	2272	2314	2316	2319	2357	2360	2419	2432	2444	2445	2451	807	811*	1547	1605	1637	1696	1745
	A 07	A 07	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	Fresh	A 06	A 06	A 06	A 06	A 06	A 06	A 06
GTX 1/4	0.07	0.00	0.26	0.55	0.21	0.05	0.11	0.15	0.00	0.18	0.12	1.01	0.12	0.15	0.00	2.00	1.71	0.34	0.60	0.38
dcNEO	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NEO	0.00	0.00	0.03	0.10	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.16	0.17	0.04	0.05	0.00
dcGTX 2/3	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.01	0.01	0.00	0.00	0.19	0.00	0.31	0.00	0.07	0.00	0.09
C 1/2	0.01	0.00	0.01	0.01	0.00	0.00	0.14	0.00	0.00	0.02	0.00	0.03	0.00	0.05	0.01	0.37	0.14	0.12	0.03	0.11
dcSTX	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
GTX 2/3	0.13	0.00	0.31	0.51	0.11	0.08	0.25	0.08	0.13	0.28	0.12	0.79	0.27	0.18	0.15	1.85	0.70	0.51	0.35	0.59
GTX 5	0.001	0.002	0.000	0.001	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.003	0.000	0.004	0.002	0.004	0.003	0.001	0.003	0.001
STX	0.06	0.27	0.16	0.24	0.03	0.03	0.24	0.06	0.08	0.16	0.11	0.52	0.11	0.45	0.61	0.72	0.42	0.26	0.12	0.17
Total	0.27	0.28	0.79	1.41	0.37	0.17	0.84	0.30	0.25	0.66	0.38	2.50	0.50	1.02	0.77	5.42	3.14	1.35	1.15	1.34
Total HPLC (ug/100g STX eq)	**27**	**28**	**79**	**141**	**37**	**17**	**84**	**30**	**25**	**66**	**38**	**250**	**50**	**102**	**77**	**542**	**314**	**135**	**115**	**134**
av MBA (ug/100g STX eq)	**41**	**43**	**73**	**175**	**37**	**40**	**81**	**37**	**38**	**42**	**38**	**136**	**44**	**89**	**89**	**287**	**321**	**138**	**127**	**109**

A07 = archived sample from 2007, A06 = archived from 2006, Fresh = fresh sample, results highlighted in red are > 80µg STX eq./100 g.

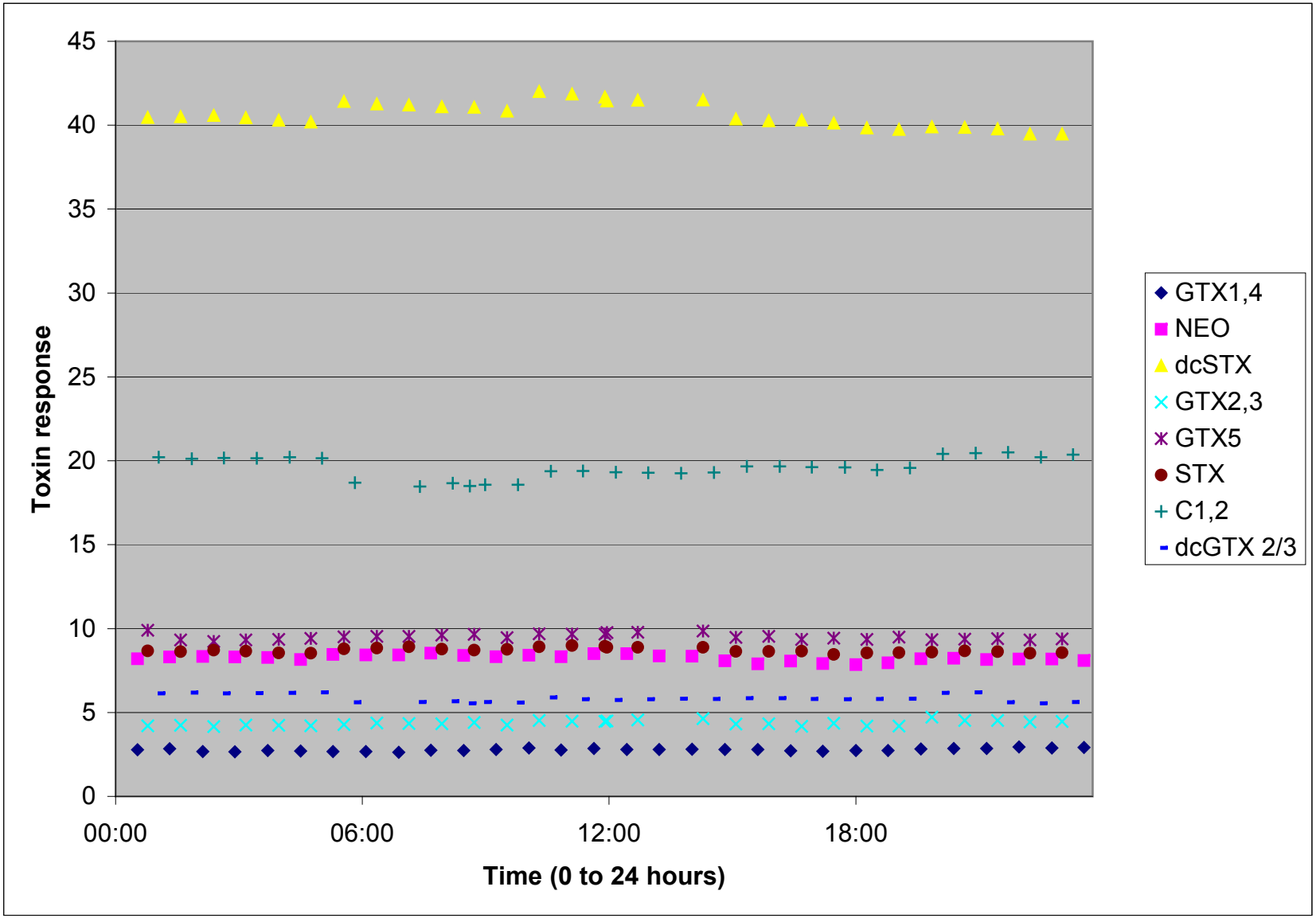
*811 HPLC result is mean of repeat analyses.

[a] Sample code is BTX/2007/ except for A06 samples which are BTX/2006/

Appendix 4: Relative toxicity factors for PSP toxin analogues

Toxin	Relative toxicity	Toxins	Relative toxicity used
GTX1	0.994	GTX 1/4	0.994
GTX4	0.726		
dcNEO	0.513	dcNEO	0.513
NEO	0.924	NEO	0.924
dcSTX	0.513	dcSTX	0.513
GTX 2	0.359	GTX 2/3	0.638
GTX 3	0.638		
GTX 5	0.064	GTX 5	0.064
STX	1.000	STX	1.000
dcGTX 2	0.154	dcGTX 2/3	0.377
dcGTX 3	0.377		
C 1	0.006	C 1/2	0.096
C 2	0.096		

Appendix 5: Stability of oxidation products of PSP toxins shown by the peak area responses of diagnostic peaks measured over a twenty-four hour period.



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