UK NRL for Marine Biotoxins

Title: Standard Operating Procedure for Screening for the Presence of Paralytic Shellfish Toxins in Shellfish by pre-column oxidation and HPLC with fluorescence detection

Production Summary

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History of Procedure

Issue	Date issued	Changes
Version 1	27 March 2008	
Version 2	12 March 2009	1.Aim and Scope revised
		4. Environmental control revised
		6. Storage and expiry of reagents clarified
		7. Standards revised to include dcNEO
		12. Storage of standards modified
		14. Additional centrifugation permitted
		19 Reversal of reagent addition steps
		20 Revision of routine procedure
		22. Revision of LRM data collated
		23 Revision of data analysis
		Updating of Annexes
Version 3	7th June 2010	11.4 Update of Toxicity Equivalency Factors
Version 4	7th December 2010	1. Aim and Scope revised
		4. Revision of table 1
		6. Revision of tables
		11-18. Revision of sections to include reference to AOAC 2005.06 and inclusion of dcNEO
Version 5	14th May 2013	Revision of Sections, 1, 13-15, 17, 19 and 21 to include details of modifications which may be required for testing scallops.
		Revision of Tables 3 and 4 to include modifications for testing scallops.
		Inclusion of Turner and Hatfield (2012) in Document section (details of modifications for scallops).

Issue	Date issued	Changes
Version 6	February 2014	Inclusion of optional semi-quantitative approach.
		Section 7 revised to include reference to contingency arrangements.

1. AIM AND SCOPE

The aim and scope of the method is to screen shellfish from the UK statutory biotoxin monitoring programme by HPLC for the presence of the Paralytic Shellfish Poisoning (PSP) toxins.

The method must be able to detect at least the following toxins: saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 2 and 3 (together: GTX2,3), gonyautoxins 1 and 4 (together: GTX1,4), decarbamoyl saxitoxin (dcSTX), gonyautoxin 5 (B-1 or GTX5), N-sulfocarbamoyl gonyautoxins 2 and 3 (together: C1, 2), decarbamoylgonyautoxins 2 and 3 (together dcGTX2,3) and decarbamoylneosaxitoxin (dcNEO or GTX7). Implementation of the method for specific species is dependent upon individual laboratories undertaking the necessary validation work. As a minimum, the method must be applicable to the testing of mussels, oysters (Pacific and native), hard clams, razor clams and cockles. For king and queen scallops, a modified methodology may be required to deal with the matrix suppression effects noted in these species. Internal validation must be conducted to determine whether the AOAC 2005.06 method is suitable for scallop testing, or if the refined methodology is more applicable.

Validation must be in accordance with Annex III of Regulation (EC) No 882/2004 and documented in a validation report. The method must be shown to meet the minimum performance criteria given in table 1 below when used in-house and be accredited to ISO 17025: 2005.

Laboratories are expected to take part in regular proficiency testing exercises (where available) and to perform satisfactorily in these tests.

The identification of the presence of PSP toxins relies on matching the retention time of any oxidation products in samples with those of the corresponding reference standards. If PSP toxins are identified the sample may be subjected to a semi-

quantitative screen analysis. Samples above a specified screen or semi-quantitative threshold are subsequently forwarded for quantitative testing.

Table 1: Minimum Performance Criteria:

Criteria	**Min performance**
Limit of detection	Min. 1/5th action limit (s:n ≥3:1 for diagnostic peak) for each toxin in each species of relevance, plus presence of applicable secondary peaks.
Limit of quantitation	Not applicable
Selectivity	Must show absence of co-extractive components resulting from impurities, degradant or matrix which may compromise the identity of the analytes
Linearity	Not applicable
Recovery	Method recovery: not applicable C18 SPE recovery: ≥ 70%
Precision: Intra-batch	• Precision of each diagnostic toxin peak area ≤10% (n=6) for each species of relevance • Precision of toxin peak retention times ≤2.5% for each species • Repeatability demonstrated for assignment of both +ve (n≥6) and –ve (n≥6) duplicate samples (duplicates analysed in same batch)
Precision: Inter-batch	• Reproducibility of toxin peaks in positive control (LRM or other PSP contaminated samples) demonstrated with RSD% ≤ 20% • Repeatability demonstrated for assignment of both +ve (n≥6) and –ve (n≥6) samples (duplicates analysed on different days)
Uncertainty	Not applicable
Ruggedness	Not applicable

2. INTRODUCTION

PSP toxins are potent neurotoxins produced by marine dinoflagellates. Ingestion of shellfish contaminated with these toxins may lead to paralytic shellfish poisoning. The symptoms of PSP include numbness or tingling sensations and muscular paralysis that may result in death through respiratory arrest. The EC regulations specific to PSP testing in shellfish are Regulation (EC) No. 853/2004 and 2074/2005 (as amended).

3. PRINCIPLE OF THE METHOD

The method is an adaptation of the AOAC Official Method 2005.06 (Lawrence *et al.*, 2005). The extracts are cleaned using C18 solid phase extraction cartridges. After periodate (and peroxide oxidation if necessary), they are analysed by HPLC using fluorescence detection. Peroxide and/or periodate oxidations are applied to cleaned extracts derived from an acetic acid extraction. After oxidation the presence of PSP toxins is established by comparing and matching the retention times of specific target chromatographic peaks with those derived from oxidised certified calibration standards. Positive samples may also be assessed using a semi-quantitative determination if required.

4. ENVIRONMENTAL CONTROL

Table 2: Storage criteria for whole shellfish, homogenates and extracts prior to official control analysis

Matrix	**Conditions to apply upon storage**
Whole shellfish	Whole shellfish which meet the criteria of section 4.1 may be stored so that no longer than 72 hours elapses between sample harvest and sample extraction. For example: a) a sample which takes 24 hours to reach the laboratory may be stored for a further 48 hours in the laboratory b) a sample which takes 48 hours to reach the laboratory may be stored for only 24 hours in the laboratory. Unless the shellfish were already in a frozen state when collected,

	shellfish should be stored at 2-8^{o}C. Frozen shellfish that have not yet started to thaw may be stored at ≤-10^{o}C.
Shellfish homogenates	Should not be stored prior to extraction
Acetic acid or HCl extracts and C18 extracts	May be stored at 2-8°C for a maximum of 5 days **in total** from the day of extraction. In the event C18 extracts require storage prior to periodate and/or peroxide oxidation, the cleaned extracts may be stored with or without any pH adjustment. Care should however be taken to ensure this is clearly marked.
Oxidised extracts	Oxidised extracts must not be stored.

5. SAFETY

Reference should be made to individual laboratory risk assessments and COSHH documentation.

6. CONSUMABLES, EQUIPMENT, CHEMICALS AND REAGENTS

6.1 Table 3 lists consumables, equipment, reagents and mobile phases, required to undertake the analysis. All chemicals and solvents are analytical grade or purer unless otherwise stated. All material used must be within their expiry date.

6.2 Equipment listed on Table 3 must be within calibration and fit for purpose before use.

6.3 Expiry dates for reagents and solutions must not exceed that of the manufacturers stated expiry date for the chemicals from which they are prepared. Storage conditions and expiry dates may vary with supplier.

Table 3: Consumables, equipment, chemicals, reagents and mobile phases.

Type	Item
Consumables	Glassware
	C18 SPE cartridges
	0.45 µm membrane filter
	Microvials and caps
	Plastic screw top vials
	0.45 µm syringe filter
	Centrifuge tubes
	Glass or plastic Pasteur pipettes
Equipment	Balances
	Homogeniser
	Centrifuge
	HPLC System
	Pipettes
	pH meter
	Water Bath
Chemicals	Deionised water or bottled HPLC grade water
	Acetic acid, glacial
	Methanol
	Hydrochloric acid
	Acetonitrile
	30% Hydrogen peroxide solution
	Ammonium formate
	Ammonium acetate
	Sodium chloride
	Disodium hydrogen phosphate anhydrous
	Periodic acid
	1M NaOH prepared solution or solid NaOH
	0.5M NaOH
	NaOH solid
Reagents	Ammonium formate 0.3M aqueous solution
	0.1M acetic acid
	0.1mM acetic acid

Type	Item
	Hydrogen peroxide 10% aqueous solution
	Disodium hydrogen phosphate (Na_2HPO_4): 0.3M aqueous solution
	Periodic acid: 0.03M aqueous solution
	Periodate oxidant
	Modified periodate oxidant for scallops
	1M NaOH
	0.1M NaOH
	Acetic acid: 1% aqueous solution
Mobile phase	Mobile phase A: 0.1M Ammonium formate
	Mobile phase B: 0.1M Ammonium format in 5% Acetonitrile
Reference standards (see note 1)	Certified reference standards, including certified reference material (where available) and analytical certified reference toxin standards for at least STX, GTX1,4, NEO, dcNEO, dcSTX, GTX2,3, GTX5, C1,2 and dcGTX2,3

(1): If certified reference standards are temporarily unavailable, where possible, laboratory reference material (LRM) containing the appropriate toxin analogue(s) must be substituted. Analysis may be undertaken where reference standards or suitable LRM are unavailable, however laboratories, in consultation with the competent authority must consider the impact of the absence of any reference standards in their assessment of the toxicity of the sample.

7. REFERENCE MATERIAL AND PROCEDURAL BLANK

7.1 If certified reference standards are temporarily unavailable, refer to the UK NRL document, "Analytical Methods: Ensuring Continuity of the Marine Biotoxin Monitoring Programme", for alternative approaches to assuring method performance.

7.2 When available, a bulk sample of naturally occurring PSP toxin contaminated material will be used as LRM. Ideally this should contain a number of both N-hydroxylated and non-N- hydroxylated toxins.

7.3 The procedural blank consisting of the volume of 1% acetic acid or 0.1M HCl added to the extraction vessel and taken through the extraction procedure.

8. CONTROL CHECKS AND FREQUENCY

8.1 Each analytical run must contain the appropriate certified reference standards or, (where required), a suitable laboratory reference standard preferably containing N-hydroxylayted and non-N-hydroxylated PSP toxins, a procedural blank and an instrumental blank. The positioning and number of control checks must be appropriate to the batch size of samples being run.

Table 4: Control checks

Control	Comment	Criteria
LRM		To be extracted with every batch For preparation see sections 8 and 14/15
Control checks for standards	Standards	For type of standards see Table 8 For preparation of standards see sections 11&12
	Number & concentration of reference standards	At least 1 level of reference standard (including all of the PSP toxins of interest) at individual **absolute concentrations** equivalent to 1/5 of the 'PSP action limit (80 µg [STX eq]/100 g) i.e. 16 µg/ [STX eq]/100 g. *For GTX 5 this may be < 1/5[1] of the PSP action limit (e.g.1.6 µg/STX equiv/100g)* See 10.5 for calculation of STX equivalence
	Standards for semi-quantitation	If assessing screen results semi-quantitatively, a minimum of two levels of reference standards should be running. These should include a low level standard at individual **absolute concentrations** equivalent to 1/5 of the PSP action limit, plus a higher level standard (e.g. equivalent to the PSP action limit.
	Frequency	The standards oxidised using a given oxidant must bracket the corresponding sample extracts. If over 20 sample extracts then additional standards should be run.
Instrumental blank	Water	For LC Column equilibration; at least one injection at the beginning of the batch.
Procedural blank		One per batch (oxidised by periodate and/or peroxide reagents as required). Randomly placed within the analytical batch. This is prepared as described in sections 7.3.
Controls for scallop testing		The LRM, PB, standards and standard control checks must all be run for the scallops method. If running both scallops and non-scallops in one batch, controls for both methods must be included.

[1]*The relative toxicity (in relation to STX) reported for GTX5 is 0.1 (see EFSA opinion on relative toxicity of saxitoxin analogues), therefore in order to decrease the quantity of primary ampouled standard used the reference standard may be reduced in concentration provided the response exhibits a ≥ 3:1 signal to noise ratio at the concentration equivalent to 1/5 of the PSP action limit.*

9. HPLC PARAMETERS

Table 5: HPLC Parameters

Parameter	Conditions
Run time	Approx 15 minutes
Injection Volumes	Up to 100µL (periodate extracts). Up to 50µL (peroxide extracts). Dependent upon in-house validation
Suggested Flow Rate	2 ml min^{-1}
Suggested Column	C18 reversed phase. 150 x 4.6 mm id, 5 µm
Guard Column	Same stationary phase as analytical column
Detection	FLD. Excitation = 340 nm, Emission = 395 nm
Column temperature	Dependent on in-house validation conditions
Suggested mobile phase gradient	0-5 % mobile phase B in the first 5 min, 5-70 % B for the next 4 min and back to 0 % B over the next 2 min then at 0 % B for another 3 min before the next injection. This is dependent on in-house validation.

The above conditions may be modified if the laboratory can demonstrate equivalent or improved chromatographic performance as compared to that generated by the above conditions and compliance with the minimum performance criteria stipulated in this SOP.

10. WORKING REFERENCE SOLUTIONS

10.1 Standard solutions for routine screening runs are at 1/5 of the regulatory action level i.e. 16 µg STX equiv/100g. The exception is GTX5 which may be prepared at a concentration of $1/50^{th}$ of the AL[1] i.e., 0.002 µg [STX equiv]/mL (this is equivalent to 1.6 µg [STX equiv. /100 g]). The concentrations are based on Toxicity Equivalency Factors detailed in The EFSA Journal (2009) 1019, 1-76 SCIENTIFIC OPINION, Marine biotoxins in shellfish – Saxitoxin group. Additional standards at higher concentrations will be required if assessing screen results semi-quantitatively or may be utilised for assessment of chromatographic performance.

Table 6: **Examples of reference solution mixes**

Mix	Acetic Acid	Hydrochloric Acid
1	NEO, GTX1,4, dcNEO	NEO GTX1,4
2	STX, dcSTX, GTX5, GTX2,3	STX, dcSTX, GTX5, GTX2,3
3	C1,2, dcGTX2,3	N/A

*dcNEO may either be incorporated into Mix 1, or run as a separate toxin working standard

10.2 Once prepared the mixes should be stored as recommended by the supplier of the reference standards.

11. PREPARATION OF SAMPLES

11.1 Clean the outside of the shellfish with cold running water, if necessary.

11.2 Rinse inside, only if necessary (e.g. the excessive presence of sand or sediment) with fresh, cold running water and drain.

11.3 Remove the tissue from the shell with a suitable knife or scalpel and place in a sieve to drain.

11.4 Transfer to a blender and blend until homogeneous.

12. PREPARATION OF EXTRACTS

12.1 The preparation of acetic acid extracts, sample clean up by solid phase extraction, periodate and peroxide oxidations are as described in the AOAC 2005.06 official method.

12.2 The preparation of HCl extracts (samples and LRM) is as detailed in the UK-NRL document "PSP Extraction SOP". For the procedural blank replace the homogenate with 0.1M HCl.

12.3 The use of matrix modifier is restricted to acetic acid extracts. Matrix modifier for non-scallop samples is prepared as described in the AOAC 2005.06 official method, omitting the precipitation step if required. For scallop samples, the same oyster modifier is used for the samples, but a separate matrix modifier prepared from blank scallop tissue is to be used for standards, as described in Turner and Hatfield, 2012.

13. SAMPLE CLEAN-UP BY SOLID PHASE EXTRACTION

13.1 Sample clean-up for non-scallop samples using SPE C18 cartridges is as detailed in Section F of AOAC 2005.06 official method.

13.2 For scallops, if the modified scallops method is used, then the procedure detailed in Turner and Hatfield (2012) is to be followed.

14. PERIODATE OXIDATION

14.1 Periodate oxidation is as detailed in Section H(a) of AOAC 2005.06 official method.

14.2 Periodate oxidation for scallop samples using the modified periodate reagent is as detailed in Turner and Hatfield, 2012.

15. PEROXIDE OXIDATION

15.1 Peroxide oxidation is as detailed in Section H(b) of AOAC 2005.06 official method.

16. ROUTINE PROCEDURE FOR THE ANALYSIS OF SAMPLE EXTRACTS, CONTROLS AND RECOVERIES

16.1 Carry out periodate oxidation procedures on all samples, standard mixes and controls (LRM and procedure blank). For some matrices, peroxide oxidation may also be required as defined by individual laboratory SOPs. If so, carry out peroxide oxidation on all required samples, standard mixes and controls.

16.2 For scallop samples, the appropriate method should be used following method verification. If using the refined scallops method, the modified periodate oxidation procedure should be used for all samples, standard mixes and controls (as above). Note that scallop matrix modifier should only be used in the standards, with oyster modifier used for all other samples and controls.

17. HPLC ANALYSIS

17.1 Proceed with HPLC analysis in accordance with each test laboratories HPLC instrumentation procedure.

18. QUALITY ASSURANCE AND SAMPLE ANALYSIS

18.1 Retention time stability

The retention time drift within the run must be ≤ ±2.5%.

18.2 Procedural blank

The chromatogram should ideally be clear of any contamination peak with a signal to noise ratio ≥ 3 at the toxin retention times.

18.3 LRM

The following applies to the interpretation of the quality of the chromatogram of the laboratory reference material (LRM):

- The toxin peaks are identified in the LRM by comparing the match of the retention times with those expected of the standard reference solution (as determined at each individual test laboratory).
- The area responses for the peaks of interest (depending on the toxin content of the LRM) must be recorded on a suitable Shewhart chart. Calculated ratios for the main/target peak of each relevant toxin plotted on the charts and must fall within ± 3 standard deviations (SD).
- Clear procedures must be specified for values falling outside of the action and warning limits.
- If running the separate scallops method, separate Shewhart charts must be used for control of the refined scallops screening method.

18.4 Interpretation of reference standard chromatographic data

The quality of the chromatogram of the reference standard is assessed by the following:

- Where there is just one oxidation product for a particular toxin (e.g., STX), the ratio between the height of the oxidation product signal response to the height of the signal response of background noise of the baseline (s:n) for the main/target peak should be a minimum of 3:1 at toxin concentrations of at least 1/5 the action limit.
- Where there is more than 1 oxidation product (Annex 1), the oxidation product of the main/target peak should demonstrate a s: n ratio ≥3:1 at toxin concentrations of at least 1/5 the action limit.

Additionally, the “most significant” secondary peak must also be detectable

- Sample chromatograms which indicate the main/target peak for each toxin are given in Annex 1.
- The same approach is to be used for all standards oxidised for both scallop and non-scallop samples.

19. DATA ANALYSIS

19.1 Interpretation of sample chromatographic data

Providing the HPLC run has been deemed acceptable; to determine if the sample contains PSP toxins the following procedures apply for both scallop and non-scallop samples:

- For the positive identification of a target/main toxin peak, the ratio between the height of the toxin signal and the height of background noise of the baseline (s:n) for the main/target peak must be 3:1 or greater. For toxins where there is more than one oxidation product the secondary peak must be detectable (where the s:n of the main peak would indicate a detectable secondary peak should be present).

19.2 Semi-quantitation of screen results

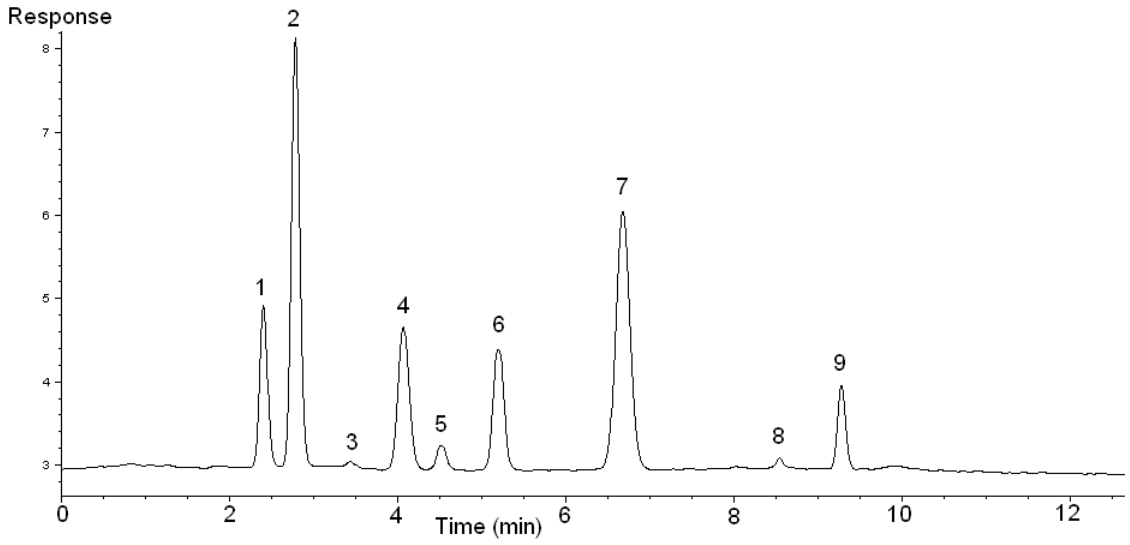
- A minimum of a two-point calibration is constructed for each toxin based on the low and high level standards analysed during the instrument sequence. Peak area responses from toxin oxidation product peaks identified in sample chromatograms are to be compared against the calibration gradients.
- Each peak is assessed against the response factor calculated for the toxin with the highest toxicity equivalence factors (TEF) and/or lowest fluorescence response factor (**Table 7**).
- Each toxin peak is semi-quantified in terms of µg STX eq/kg, with all peaks (**Table 1**) summed to give an estimated total STX eq/kg.

Table 7. Chromatographic peaks detected by pre-column oxidation LC-FLD following the analysis of commercially-available PST CRMs showing main peaks used for semi-quantitation.

Peak number	Potential toxins	Semi-quantified against
1	dcGTX2&3	dcGTX2&3 (Mix 3)
2	GTX1&4*, dcGTX2&3, C3&4	GTX1&4 (Mix 1)
3	NEO, dcSTX	Peak not used
4	C1&2	C1&2 (Mix 3)
5	dcNEO*, dcSTX*	dcSTX (Mix 2)
6	NEO*, dcSTX	NEO (Mix 1)
7	GTX2&3*, GTX1&4	GTX2&3 (Mix 2)
8	GTX5	GTX5 (Mix 2)
9	STX*, NEO	STX (Mix 2)

*Primary quantitative peaks

Figure 1 Example chromatogram of standard containing each of the commercially available toxins, highlighting peak numbers referenced in Table 1.



19.3 Use of screen results

- Screen positive samples may either be progressed directly to full quantitation, or further assessed with the semi-quantitation approach
- Samples showing semi-quantified PSP at levels ≥ 400 g STX eq/kg should be progressed to full quantitation.
- Samples showing semi-quantified PSP at levels < 400 g STX eq/kg may be reported as <0.5 MPL.

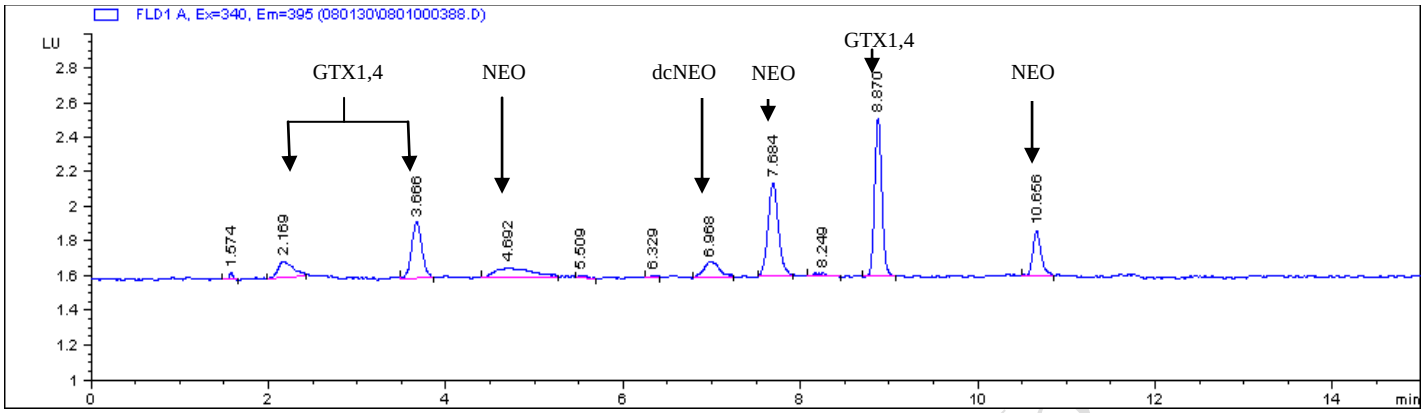
20. REFERENCES

- The EFSA Journal (2009) 1019, 1-76 SCIENTIFIC OPINION, Marine biotoxins in shellfish – Saxitoxin group, Scientific Opinion of the Panel on Contaminants in the Food Chain (Question No EFSA-Q-2006-065E) Adopted on 25 March 2009
- Lawrence et al 2005. AOAC Official Method 2005.06. Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection.
- Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Union L226/22
- Regulation EC 2074/2005 laying down implementing measures for certain products under regulation EC No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004) of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulation (EC) No 853/2004 and EC No 854/2004 Official Journal of the European Union L338/27
- Turner, A.D. and Hatfield, R.G. (2012) Refinement of AOAC Official Method 2005.06 Liquid Chromatography-Fluorescence Detection Method to improve performance characteristics for the determination of paralytic shellfish toxins in king and queen scallops. J. AOAC International. 95(1): 129-142

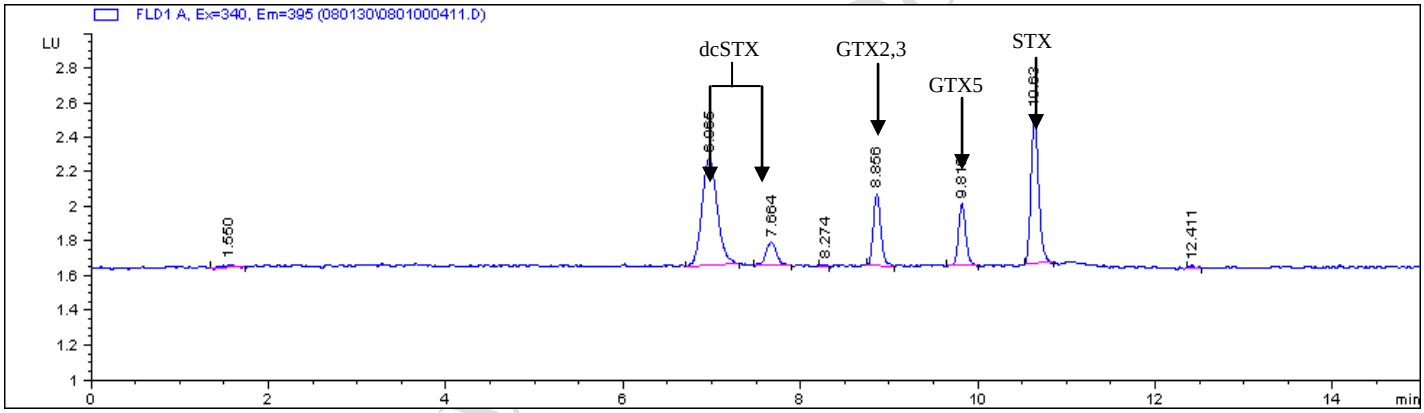
- Supplemental Information for PSP Toxin CRMs. Do not use the toxicity equivalence factors stated in this document.
- Calibration solutions of paralytic shellfish poisoning (PSP) toxins
- Certificates of analysis for PSP toxins
- UK-NRL PSP Extraction SOP
- UK NRL Analytical Methods: Ensuring Continuity of the Marine Biotoxin Monitoring Programme.

ANNEX 1: Sample chromatograms
(Concentrations have been increased to ensure all oxidation products are visible)

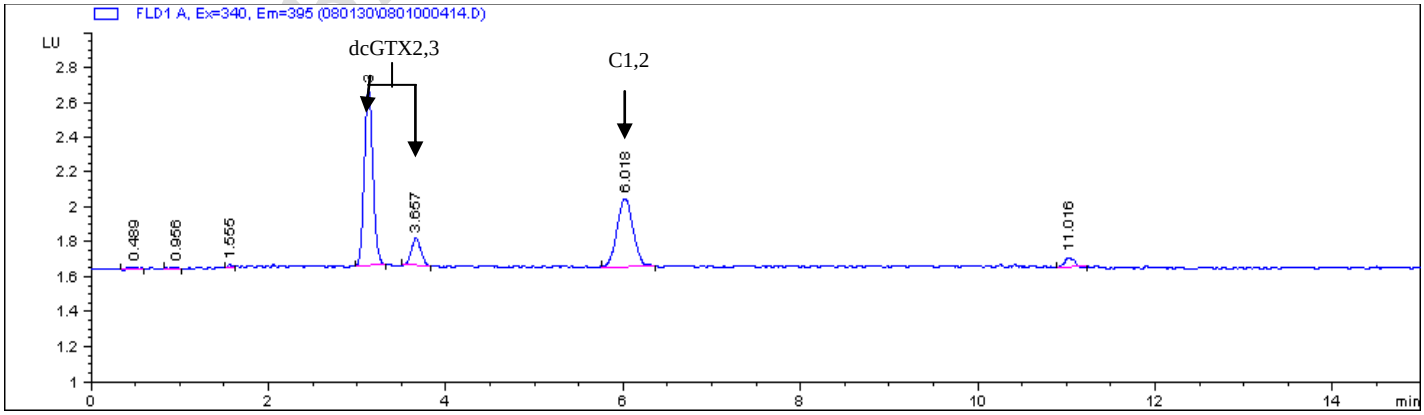
(a) Periodate oxidation of hydroxylated toxins (Mix 1)



(b) Peroxide oxidation of non-hydroxylated Toxins (Mix 2)



(c) Peroxide oxidation of non-hydroxylated toxins (Mix 3)



ANNEX 2: Toxicity equivalency factors (TEFs) of STX-group toxins proposed by CONTAM Panel (to be applied on a molar basis).

Toxin	TEF
STX	1.0
Neo STX	1.0
GTX1	1.0
GTX2	0.4
GTX3	0.6
GTX4	0.7
GTX5 (B1)	0.1
GTX6 (B2)	0.1
C2 (GTX8)	0.1
C4	0.1
dcSTX	1.0
dcNeo STX (GTX7)	0.4
dcGTX2	0.2

Figure 2: (Extracted from AOAC Official Method 2005.06) "Chromatographic patterns showing oxidation products formed after periodate and peroxide oxidations of toxins included in this study (refers to AOAC 2005.06). The same quantity of toxin was used for each oxidation reaction. Arrows indicate peaks used for quantification.