UK NRL for Marine Biotoxins

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

Production Summary

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History of Procedure

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1. AIM AND SCOPE

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

The method must be able to quantify 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), and 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 in each species and each toxin of relevance. As a minimum, the method must be applicable to the testing of mussels, hard clams, razor clams and cockles.

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 ISO17025:2005.

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

Table 1:

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	Min. 2/5th action limit (s:n ≥10:1 for diagnostic peak) for each toxin in each species of relevance, or evidence for acceptable precision of analysis at concentrations >LOD and <LOQ

Selectivity	Must show absence of co-extractive components resulting from impurities, degradant or matrix which may compromise the identity of the analytes
Linearity	To show detector linearity over working range (minimum LOQ to 1.2 AL)
Recovery	To show similar or improved recoveries to those reported in the AOAC 2005.06 and subsequent validation reports
Precision: Intra-batch	RSD ≤20% per toxin for each species
Precision: inter-batch	RSD ≤25% per non-N-hydroxylated toxin and RSD ≤30% per N-hydroxylated toxin for each species
Uncertainty	Expanded uncertainty of measurement is applied at 95% confidence level to all values obtained by HPLC. The uncertainty associated with the measurement of each toxin analogue, in each sample matrix must be established.
Ruggedness	Evidence shown that method remains unaffected by small variations in method parameters or where the variation of a parameter is shown to be significant, it is tightly controlled.

The method may be used in conjunction with the HPLC screening procedure described in the UKNRL screening by HPLC SOP to determine if PSP toxins are present in the acetic acid extract of the sample. Samples may also be progressed straight to quantitation without the preliminary screening step.

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.

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 and only applies to samples extracted following the acetic acid procedure.

Using the protocol described in the UKNRL PSP screening by HPLC SOP, a homogenised bivalve molluscan shellfish sample is mixed with acetic acid in a boiling water bath to extract all water-soluble compounds including PSP toxins. The resulting extract is then taken through this procedure where it is first cleaned up using solid phase extraction (SPE) C18 cartridge. Samples are subjected to ion exchange clean up (fractionation) and periodate and peroxide oxidation prior to HPLC-FLD analysis. Some of these steps may be omitted if a preliminary screen has established the toxin profile of the sample.

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 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

Table 3 list consumables, equipment, reagents and mobile phases, required to undertake the analysis. The equipment listed must be within calibration and fit for purpose before use.

Table 3: Consumables, equipment, chemicals, reagents & mobile phases

Type	Item
Equipment/Apparatus	Gloves and safety glasses C18 SPE cartridges SPE ion exchange cartridges Graduated 5mL plastic tubes and snap on caps Glass volumetric flasks

Type	Item
	Various calibrated automatic pipettes (5µL-5000µL) pH paper Plastic micro centrifuge safelock tubes Laboratory film Autosampler vials and caps Plastic syringes 0.45 µm nylon syringe filters Calibrated timer Calibrated pH-meter Vortex mixer HPLC column: Reversed phase C18 (150 x 4.6mm id, 5µm) HPLC-FLD system
Chemicals/Reagents/Controls	Methanol Acetonitrile Analytical certified reference standards for STX, GTX1,4, NEO, dcNEO, dcSTX, GTX2,3, GTX5, C1,2 and dcGTX2,3 Glacial acetic acid 1% acetic acid 0.1M acetic acid 0.1mM acetic acid 5M NaOH 1M NaOH 0.5M NaOH Periodate oxidising agent 10% (w/v) aqueous hydrogen peroxide 0.1% ammonium acetate 0.03M sodium chloride 2.0M sodium chloride Matrix modifier
Mobile phase	Mobile phase A: 0.1M Ammonium formate
	Mobile phase B: 0.1M Ammonium formate 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, where possible, laboratory reference material (LRM) containing the appropriate toxin analogue(s) must be substituted.

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 will consist of the volume of 1% acetic acid added to the extraction vessel and taken through the extraction procedure.

8. CONTROL CHECKS AND FREQUENCY

Table 4 lists the controls which must be incorporated within each HPLC batch.

Table 4: Controls

Control	Comment	Criteria
LRM		To be extracted and analysed with every batch
Control checks for standards	Standards	For list of compounds see Table 3
	Number & concentration of reference standards	At least 5 levels 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. (see note 1) *For GTX 5 this may be < 1/5 (see note 2) of the PSP action limit (e.g.1.6 µg/STX equiv/100g)*
	Frequency	The standards oxidised using a given oxidant must bracket the corresponding sample extracts. A set of standards should be run every 20 injections.
Instrumental blank	Water	For LC Column equilibration; at least one injection at the beginning and end of an analytical batch
Procedural blank		One per batch (oxidised by periodate and peroxide reagents). Randomly placed within the analytical batch.

(1): Concentrations based on Toxicity Equivalency Factors detailed in The EFSA Journal (2009) 1019, 1-76 SCIENTIFIC OPINION, Marine biotoxins in shellfish – Saxitoxin group

(2): 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 describes the conditions of HPLC analysis.

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 meet or exceed the minimum performance criteria described in Table 1.

10. WORKING REFERENCE SOLUTIONS

The following is presented as an example, the concentration of intermediate standards and working standards may vary but should cover the range from the limit of quantification to 0.48µM.

10.1 The toxins may be prepared in 4 mixtures:

Mix 1: N-Hydroxylated toxins (currently GTX 1,4, Neo and dcNEO if required*)

Mix 2: Non-N-hydroxylated toxins (STX, dcSTX, GTX 2,3 and GTX 5)

Mix 3: Additional non N-hydroxylated toxins (C1,2 and dcGTX2,3)

Mix 4: Separate mix of dcSTX and dcGTX2,3 for periodate oxidation for the assessment of dcSTX and dcGTX2,3 contributions to NEO/dcNEO and GTX1,4 respectively. * dc NEO may either be incorporated into MIX 1 working standards or prepared as a separate toxin standard.

11. PREPARATION OF SAMPLES

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

11.2 Rinse the inside, only if necessary, 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 and 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 matrix modifier is prepared as described in the AOAC 2005.06 official method, omitting the precipitation step if required.

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

13.1 This protocol applies to filtered acetic acid extracts deemed positive following the screening analysis detailed in the UKNRL HPLC screening SOP. Note that samples may be progressed straight to quantitation without the preliminary screening step. In this case, all samples should be submitted to the ion exchange clean up (fractionation) step prior to periodate and peroxide oxidations.

13.2 If the screening results of a sample are shown to potentially contain any of the N-hydroxylated toxins with primary toxin peaks present at a signal to noise ratio ≥ 3, the filtered extract is subjected to ion exchange clean up (fractionation), following the procedure described in either AOAC 2005.06 or Turner et al., 2009.

13.3 If the screening results of a sample do not potentially contain N-hydroxylated toxins, the filtered extract is not fractionated and is progressed straight to peroxide oxidation and HPLC-FLD analysis.

13.4 Carry out periodate and if necessary peroxide oxidation procedures on all samples, standard mixes and controls (LRM and procedural blank), as described in the AOAC 2005.06 official method.

14. HPLC ANALYSIS

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

14.2 An aliquot of unoxidised filtered extract is run alongside periodate and/or peroxide oxidised samples to check for and take into account any presence of naturally fluorescent co-eluting extractives.

14.3 Control checks and frequency should be as detailed in Table 4.

15. QUALITY ASSURANCE AND SAMPLE ANALYSIS

15.1 Instrument sensitivity

The main target peak of the LOQ standard for Mix 1, 2 and 3 must display a signal to noise ratio ≥ 3.

15.2 Retention time stability

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

15.3 Procedural blank

The chromatogram should ideally be clear of any contamination peak with a signal to noise ratio ≥ 3 at the toxin retention times. However, due to the use of matrix modifier during the oxidation of the procedural blank, any peaks present in the procedural blank with a signal to noise ratio ≥ 3 must be recorded and the peak area subtracted from any PSP toxin peaks

15.4 LRM

The calculated toxin concentrations for the peaks of interest (depending on the toxin content of the LRM) must be recorded for each toxin analysed. Clear procedures must be specified for values falling outside of the action and warning limits.

15.5 Standards

15.5.1 Peak area responses must be measured for each toxin quantitation peak in the initial calibration of each of the toxin mixes. Correlation coefficients (r^2) must be calculated for the quantitation peak of each toxin in toxin mixes over a minimum of four different concentration levels and should be ≥ 0.97.

15.5.2 Where appropriate, a continuing calibration check (CCC; a level 3 calibration standard for toxin mixes 1, 2 and 3 using the appropriate oxidation method) must be run through the sequence after every 20 injections. CCC results are generated by quantifying the response of the CCC against the initial calibration, enabling the determination of whether the initial calibration is still applicable. CCC results analysed during the sequence must fall within 30% of the expected value.

15.6 In addition, laboratories are expected to take part in regular proficiency testing exercises (where available) and to perform satisfactorily in these tests.

16. DATA ANALYSIS

16.1 Providing the HPLC run has been deemed acceptable, each sample chromatogram must be assessed individually for the presence or absence of each PSP toxin. This must include the instrument blank, procedural blank, calibration standards, samples and LRM.

16.2 Carefully check the integration of toxins ensuring the baselines drawn accurately represent the most likely true baseline of the peak. Care should be taken when integrating early or closely-eluting peaks, particularly on sloping baselines.

16.3 Use the peak areas recorded to generate calibrations for each of the toxin mixes analysed, using the results to calculate both correlation coefficients and calibration gradients. Additionally, use the results from the analysis of Mix 4 to calculate the ratio of dcGTX2,3 peaks.

16.4 Record the shellfish species for each sample and use the appropriate values for expanded measurement uncertainty for each sample.

16.5 Toxin concentrations may be expressed in either µmol or in µg STX equivalence per toxin per gram of flesh, but the total toxicity of the sample may be expressed in µg STX eq./kg flesh or µg STX eq./100g .

16.6 Refer to AOAC 2005.06 for instructions on the calculation of NEO in the presence of dcSTX. Refer to the CRL WG guidance for the calculation of dcNEO in the presence of dcSTX.

16.7 Individual toxin concentrations are only included in the total toxicity if the value is ≥ the toxin LOQ. Three final values are to be reported. The actual toxicity value, a higher value based upon the addition of measurement uncertainty to the

final value and a lower value based on the subtraction of measurement uncertainty. These values must be calculated using the correct species-specific values for Measurement Uncertainty generated during the in-house method validation.

16.8 Results are reported without any correction for toxin recovery.

17. REFERENCES

AOAC Official Method 2005.06. Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. Gaithersburg, MD, USA: AOAC International.

Cefas contract report (2008). 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.

Turner A.D., Norton D.M., Hatfield R.G., Morris S., Reese A.R., Algoet M and Lees D.M. (2009). Refinement and extension of AOAC 2005.06 to include additional toxins in mussels: single laboratory validation. J. AOAC International. 90(1) p. 190-207.

NRC documents:

- 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

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

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