Centre for Environment, Fisheries & Aquaculture Science

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CERTIFICATE OF ANALYSIS

PACIFIC OYSTER PSP TOXIN MATRIX CRM

(PO PST CRM 1101) *(Rev1 – April 2013)*

1. Product Specification

1.1. Product Identifier
Product name: Pacific oyster Certified Reference Material for Paralytic Shellfish Poisoning Toxins
Product code: PO PST CRM 1101
Description of product: Sterilised homogenous Pacific oyster tissue with added acidified water and containing known certified concentrations of a range of Paralytic Shellfish Poisoning (PSP) toxins

1.2. Relevant identified uses of the mixture and uses advised against
Use of product: For laboratory use only. This material is not appropriate for any other use.

1.3. Details of the supplier of the CRM
Company name: Centre for Environment, Fisheries and Aquaculture Science (Cefas)
Company address: The Nothe, Barrack Road, Weymouth, Dorset, DT4 8UB, United Kingdom
Telephone: +44 (0)1305 206600
Fax: +44 (0)1305 206601
E-mail: referencematerials@cefas.co.uk www.cefas.defra.gov.uk

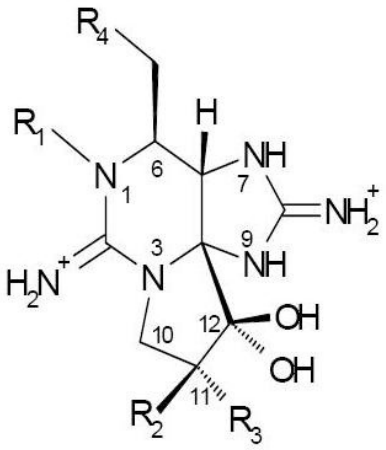
1.4 Product overview
PO-PST-CRM-1101 is a homogenised Pacific oyster (*Crassostrea gigas*) tissue containing a range of important and prevalent paralytic shellfish poisoning (PSP) toxins. This material has been prepared at Cefas using Pacific oysters sourced from Southern England and contaminated with PSP toxins in shellfish feeding experiments. The material was stabilised by the use of pH adjustment, chemical additives and gamma irradiation treatment. Acceptable homogeneity and stability has been demonstrated for each of the PSP toxins present in the CRM. The concentrations of each PSP toxin were quantified with the use of a post-column oxidation liquid chromatography with fluorescence detection method (PCOX-LC-FLD) using external solvent calibration, based closely on AOAC Official Method 2011.02. Confirmation of these results was provided by PCOX LC-FLD with standard addition quantitation and through an inter-laboratory characterisation study of the material using PCOX LC-FLD. Further analysis was conducted using a pre-column oxidation (Pre-COX) LC-FLD method (AOAC OM 2005.06) and by Hydrophilic Interaction Chromatography with Tandem Mass Spectrometric detection (HILIC-MS/MS). The CRM was divided into polypropylene bottles each containing >5.5g of tissue homogenate. Dispensing of 7,600 bottles was conducted in one single processing batch,

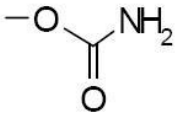
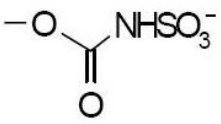
involving the heat sealing of bottles and the fixing of screw-caps. Aliquots have been stored at -80°C since dispensing, other than the period of time during which gamma irradiation was conducted.

Figure 1 shows the chemical structure of the saxitoxin family, specifically listing the toxins present in this CRM.

Note – This revision (Rev 1, April 2013) is released only to update MU values. The property values remain unchanged from those presented on the original certificate (July 2012).

Figure 1. Chemical structures of PSP toxins present in PO CRM 1101.



Toxin	R_1	R_2	R_3	R_4
STX	H	H	H	
GTX2	H	H	OSO_3^-	
GTX3	H	OSO_3^-	H	
NEO	OH	H	H	
GTX1	OH	H	OSO_3^-	
GTX4	OH	OSO_3^-	H	
GTX5	H	H	H	
C1	H	H	OSO_3^-	
C2	H	OSO_3^-	H	
dcGTX2	H	H	OSO_3^-	–OH
dcGTX3	H	OSO_3^-	H	

2. Property values

Table 1 summarises the certified concentrations expressed in µmol per kg tissue for each of the PSP toxins present above the limit of quantitation of the primary quantitative method. Total toxicity was calculated following the guidance in Quilliam (2007) but with toxicity equivalence factors taken from more recent guidance (EFSA, 2009).

Current expiry = **12 months** from date of sale, assuming storage at <-15°C (5°F) from day of sample receipt. **Table 2** summarises other toxins present at trace levels in the CRM, with either non-certified or unquantifiable concentrations.

The weight of the homogenate present in the bottles is not certified.

Table 1. Certified concentrations for Cefas PO PST CRM 1101.

Analyte	CAS	Concentration (95% confidence)
Saxitoxin (STX)	35554-08-06†	0.22 ± 0.02 µMol/kg
Gonyautoxin 4 (GTX4)	64296-26-0‡	0.33 ± 0.05 µMol/kg
Gonyautoxin 3 (GTX3)	60508-89-6‡	0.23 ± 0.02 µMol/kg
Gonyautoxin 2 (GTX2)	60537-65-7‡	0.20 ± 0.02 µMol/kg
Gonyautoxin 1 (GTX1)	60748-39-2‡	0.41 ± 0.05 µMol/kg
Neosaxitoxin (NEO)	64296-20-4‡	0.64 ± 0.09 µMol/kg
N-sulfocarbamoyl-gonyautoxin-2 (C1)	80173-3-4‡	0.66 ± 0.15 µMol/kg
N-sulfocarbamoyl-gonyautoxin-3 (C2)	80226-62-6‡	0.74 ± 0.09 µMol/kg
Total toxicity	*Not applicable*	668 ± 57 µg STX di-HCl eq./kg

†CAS of dihydrochloride form; ‡CAS of free base form

Table 2. Other toxins present in Cefas PO PST CRM 1101 (non-certified).

Analyte	CAS	Approximate concentration
Gonyautoxin 5 (GTX5 or B1)	64296-25-9	Trace
Decarbamoylgonyautoxin 2 (dcGTX2)	86996-87-4‡	0.08 µMol/kg (<LOQ)
Decarbamoylgonyautoxin 3 (dcGTX3)	87038-53-7‡	0.01 µMol/kg (<LOQ)

‡CAS of free base form; LOQ = limit of quantitation

3. Preparation of material

This material was prepared following a series of preliminary feasibility studies to investigate the preparation and stabilisation of a range of shellfish matrix reference materials for PSP toxins (Higman and Turner, 2010; Turner *et al.*, 2010). The target was the preparation of a matrix material containing a suitable range of toxins, each present at concentrations useful for method validation and routine quality control purposes. At the same time a total sample toxicity between half and one and a half times the current regulatory action limit (800µg STX di-HCl eq./kg) was targeted. Pacific oysters (*Crassostrea gigas*) were sourced locally from the South Coast of England and used for a series of shellfish feeding trials during 2010 and 2011. During each feeding trial, live oysters were exposed to high concentrations of toxic *Alexandrium fundyense* cells in a laboratory environment. After 5 days feeding, each batch of oysters was homogenised, tested for PSP toxin content and stored at -20°C until further use. After completion of the feeding, the multiple batches of contaminated oysters were thawed, re-homogenised and combined to produce one large batch of tissue, held on ice for the remainder of the preparation and bottling procedure. Once the mixture was equilibrated to +6°C, the content was adjusted through the addition of appropriate volumes of PSP-free Pacific oyster homogenate, de-ionised water and hydrochloric acid. This resulted in the production of a homogenous tissue with a water content of approximately 85% and with a pH of 3.8 ± 0.2. In addition, a number of chemical additives were added (0.02% by weight), namely ethoxyquin, oxytetracycline and erythromycin. The mixture was homogenised further before final toxicity, water content and pH checks. In total 49.5kg of tissue was produced, the bulk of which was utilised for production of this CRM. Once prepared the homogenate tissue was continuously stirred and held on ice, with the maximum temperature recorded during the entire processing and bottle-filling step being +8°C. Bottle dispensing was all conducted in one single day and in one continuous batch. An automated peristaltic pump was primed for 10 minutes before being set to dispense 5.7g

of tissue into each bottle. Once filled with tissue, the bottles were fitted with a foil cap which was heat sealed and a screw cap applied. Sealed samples were placed into polythene bags, labelled with a bag number and 1-2 bottles taken from each bag for homogeneity determination throughout the entire processing batch. The whole bottling process was conducted in approximately 12 hours and resulted in the production of 7,600 bottles of CRM, each being assigned a unique number. Once each polythene bag was filled and labelled, it was heat sealed and placed into -20°C storage overnight before being transferred to a -80°C freezer. All samples were subsequently transferred into multiple freezer boxes containing frozen ice packs and transported from the Cefas Weymouth Laboratory to the Gamma Irradiation Processing Plant (Isotron, Swindon, UK). The transport took 2 hours, during which all samples remained in a frozen state. A bag of travel controls was also sent along with the samples, but not subjected to irradiation. The sealed bags of CRM aliquots were placed into a large container together with eight dosimeters equally distributed within the bags to record dose levels. The samples were treated overnight with a target dose of 18 kGy. Records subsequently obtained from the dosimeters confirmed the irradiation at 18.4 ± 0.4 kGy (95% confidence). After processing, sample bags were placed back into frozen storage and transported back to Cefas, along with the untreated travel controls. Samples were placed back into 20°C storage overnight before being transferred to a -80°C freezer for long term storage. Five of the ten travel controls were extracted and analysed in a single batch together with 5 samples of treated CRMs. Results confirmed no differences were apparent between the treated and non-treated samples, as expected from previous preliminary investigations (Turner *et al*., 2010). Bio-burden testing on the remaining 5 travel controls and 5 irradiated samples confirmed the removal of biological activity in the gamma irradiated samples.

4. Recommended use

4.1 Sample receipt

PO CRM (1101) is sent from Cefas in a frozen physical state inside packaging containing frozen ice blocks which have been validated for CRM shipment. Please note that from transportation validation trials, if the shipment takes more than 2 days, it is likely that the samples may arriving in a semi-frozen or even thawed state. However, the packaging used will keep the samples cool and the short term stability trials have indicated that CRM stability is maintained for up to 6 days at room temperature. Therefore receipt of CRMs which are not frozen should not affect the stability of the materials or the certification of the products, as long as they are received within 8 days of shipment.

4.2 Storage

Once received, it is recommended that PO-PST-CRM (1101) is stored in a freezer (< -15°C, 5°F) until required for use. When the materials are stored unopened under these conditions, the certified concentration of PO PST CRM 1101 is valid for 12 months from the date of sample receipt. On the day of use, the aliquots should be removed and allowed to thaw to room temperature, without the use of any form of heating. Before opening the bottle, it should be shaken for a few seconds to thoroughly mix the contents. Approximately 5.7g tissue is present in each bottle, enabling 5.0g of material to be accurately measured and taken for each test.

4.3 Handling

PO-PST-CRM contains shellfish tissue, hydrochloric acid and a range of PSP toxins, harmful neurotoxins responsible for PSP intoxication. Although the toxins are present at low concentrations by weight, the materials should only be handled by personnel who are suitably trained. The Safety Data Sheet (SDS) supplied with these CRMs should be consulted before any of the CRMs are opened.

4.4 Specific use

PO-PST-CRM (1101) is suitable for a range of analytical testing functions. The material is certified following a number of within laboratory and between-laboratory characterisation studies, each with a number of different analytical methods. The material is ideal for the determination of accuracy and repeatability of analytical methods, so can be a valuable tool to aid method validation experiments. Furthermore it would enable the regular determination of method performance in a monitoring environment, providing higher levels of internal quality control to a laboratory's quality management systems. Regular routine use of the material, which has been subjected to processing methods to increase stability and homogeneity, will give a more appropriate ongoing assessment of trends in measurement performance, where use of uncharacterised laboratory reference materials are more likely to be affected by instability and heterogeneity.

5. Homogeneity

48 CRM samples were taken for homogeneity testing. These were selected throughout the entire period of bottle dispensing to give a representative cross section of samples dispensed over the entire production batch. Samples were extracted using the normal AOAC 2005.06 extraction process (1% acetic acid) and cleaned up by automated C18 SPE (Anon, 2005). Each sample was pH adjusted and oxidised by periodate and peroxide oxidation prior to LC-FLD analysis.

Samples were run in a random order to avoid the instrumental analysis sequence following the same order of preparation. Consequently the measurements were conducted in such a way that any potential drift in the measurement was separated from any potential trend in the sample batch. The data was assessed as having a normal distribution with no visual indication of skew (asymmetry), kurtosis (changes in shape) or common extreme values and outliers. No visual trends were evident for any of the toxins in relation to the order of dispensing. Statistical assessment of the results confirmed there was no slope associated with any of the linearity plots generated for each toxin in relation to order of preparation and therefore no evidence for any trend in the batch. Further statistical assessment was conducted using one way analysis of variance (ANOVA) on results obtained from the analysis of 16 triplicate samples. Results were used to generate the between-bottle contributions to the uncertainties associated with the homogeneity of CRM following the approach described in section 8 and ISO Guide 35.

6. Stability

6.1. Short term stability

Stability studies were conducted to determine the presence of any potential degradation or transformation of toxins during sample transport. 75 aliquots of CRM were subjected to a stability assessment using a reverse isochronous model, resulting in the analysis of all CRMs under repeatability conditions. Specifically five temperature conditions (-20°C, +4°C, 10°C, 22°C, +40°C) were investigated at five time points (0, 3, 6, 9 and 14 days) and analysed together with samples stored at -80°C. Each aliquot was analysed in triplicate using PCOX LC-FLD, with further confirmation being provided by Pre-COX LC-FLD. Results demonstrated stability for 14 days at -20°C, +4°C and +10°C and for a minimum of 6 days at room temperature (approximately +22°C). After 6 days at

room temperature a degree of epimerisation was observed for GTX1&4 and GTX2&3, although results indicated continued stability of total epimeric pairs for up to 14 days at room temperature. Subsequently, with samples sent frozen and with reasonable delivery times, there is no evidence of any stability issues over the short term.

6.2. Transport stability

An additional short term stability experiment was conducted to test the suitability of two types of transport packaging to provide appropriate temperature control during sample shipment of CRMs. Replicate aliquots of CRMs were packaged into transport containers which were held in two different temperature-controlled environments for differing periods of time. The two regimes were chosen to model both winter and summer temperatures, incorporating expected high temperatures during the day, followed by maximum lows (highest expected temperature) during the night. Packaged containers were exposed for 7 days, 72hr, 48hr, 24hr and 0hr (control), with an isochronous design being used to enable subsequent LC-FLD analysis of CRMs to be conducted under repeatability conditions. Results confirmed stability of each toxin for the full 7 days under each of the temperature regimes employed and in the two different packaging materials tested. Transport conditions chosen are therefore suitable so that no additional uncertainty with respect to instability due to transport needs to be taken into account. Consequently the uncertainty associated with short term stability under transport conditions is taken as zero.

6.3. Long term stability

Long term storage stability studies have demonstrated excellent stability of each individual toxin when samples are stored at below -15°C for 12 months. Aliquots of CRM were subjected to a stability assessment again using a reverse isochronous model. The two temperature conditions investigated were -20°C and +4°C each at six time points (0, 1, 2.5, 6, 9 and 12 months) and analysed in comparison with samples stored at -80°C. Each aliquot was analysed in triplicate using PCOX LC-FLD, with further confirmation being provided by Pre-COX LC-FLD. Results demonstrated excellent stability for 12 months at -20°C, including no evidence of epimerisation. At +4°C stability was previously determined for up to 2 months, after which there were slight decreases in C2 and GTX3, plus increases in GTX2 and GTX1 relative concentrations. However, with samples stored frozen there was no evidence of any stability issues over the 12 month period. The variability associated with the long term stability was incorporated into the overall uncertainty budget for the CRM.

6.4. Shelf-life and CRM availability

7,600 samples of PO CRM were produced, are available for purchase and are currently stored at Cefas at a temperature of -80°C. Feasibility studies conducted at Cefas on similar oyster reference materials have demonstrated excellent stability over several years when these samples are stored at -80°C. However, ongoing shelf-life studies will be conducted on PO CRM 1101 to demonstrate long-term stability and to ensure the certified values reported here are maintained. Any changes which may occur will be reflected in an updated certificate of analysis.

7. Determination of property values

Pre-certification trials were conducted to determine the optimum extraction protocol for the CRM, which balanced the need to maximise the recovery of toxins from the tissue whilst minimising the size of the extraction dilution factor. Results indicated that a fully exhaustive extraction in acetic acid did not provide a significantly higher efficient extraction as compared with the standardised two step AOAC 2005.06 extraction, possibly as a result of the high proportion of water present in the tissue. Comparisons were also conducted between extractions performed with both acetic acid and hydrochloric acid. Data showed similar mean toxin concentrations in replicate extracts of both solvents, but with some evidence for slightly lower concentrations of C1&2 and higher concentrations of GTX2&3 in the HCl extracts. This was to be expected given the potential hydrolysis of N-sulfocarbamoyl toxins in the presence of HCl. Consequently during the characterisation procedure, a Cefas-modified two step acetic acid extraction was utilised for extracting CRMs for the purpose of toxin quantitation. Specifically this involved an extraction of gravimetrically determined aliquots of approximately 5g of oyster tissue in 5.0mL 1% acetic acid, with vortex mixing (90s) before being placed in boiling water for 5 minutes. After cooling, samples were re-vortex mixed and centrifuged (4,500 rpm for 10 minutes) before decanting the supernatant. The remaining tissue was subjected to a second extraction using 3mL 1% acetic acid (no boiling) before further centrifugation, after which the supernatants were combined and total volume determined.

Property values were determined following the guidance of ISO Guides 34 and 35. Results from the characterisation studies conducted are summarised in **Table 3**. The primary method of analysis used was the PCOX LC-FLD method (AOAC 2011.02) using external toxin calibrations prepared in solvent from traceable certified reference standards. Pre-certification checks had shown there were no significant differences in the quantitation returned following the use of either solvent or matrix-matched calibration standards. Analysis was performed over multiple batches and different days on replicate (n=54 to 63) deproteinated extracts of CRMs extracted using the Cefas-modified acetic acid extraction method.

Secondary analysis was performed using replicate standard addition quantitation following PCOX analysis of CRM extracts fortified with a range of known concentrations of toxins taken directly from freshly-opened ampoules of certified reference standards (NRCC, Canada). Graphs were constructed for each toxin plotting the measured peak area and concentrations together with a linear regression line and 95% confidence interval for the fitted means. Regression lines were extended to peak=0 and the confidence intervals on the predictor were projected onto the concentration axes.

Additional confirmation was provided following an interlaboratory assessment of the PO CRM by PCOX LC-FLD at a total of 10 laboratories (Cefas, 2012). Results were included in the characterisation only from those laboratories performing the exact specified extraction method (acetic acid extraction).

The results were supported further by analyses with Ultra-Performance Liquid Chromatography (UPLC) with positive-mode electrospray ionisation and tandem mass spectrometry (MS/MS). Hydrophilic interaction liquid chromatography (HILIC) was employed and toxin quantitation performed by standard addition to account for the significant effects of matrix suppression.

Results obtained from the PCOX LC-FLD analyses with external solvent calibration were used as the primary analytical method for certification. Toxin concentrations determined using standard addition quantitation and from the interlaboratory ring trial were used as additional methods and compared with the primary method to demonstrate equivalence. **Figure 2** illustrates the comparison of results obtained using these four approaches. Data obtained from the LC-MS/MS analyses was

used as additional confirmation and are also reported. All instrumentation and measurement equipment utilised in certification studies is regularly serviced, maintained, calibrated and the performance verified. All analytical balances and other measurement devices used during the characterisation studies were calibrated over the full measurement range of interest and subjected to daily checks prior to use. Quantitation of toxins was conducted against calibrations prepared using certified reference standards obtained from the National Research Council of Canada (NRC), Institute of Biotoxin Metrology.

Table 3. Summary of toxin concentrations in PO CRM from characterisation studies as determined by a) PCOX LC-FLD with external calibration b) PCOX LC-FLD with standard addition c) HILIC-MS/MS with standard addition d) interlaboratory determination by PCOX LC-FLD with external calibration.

Toxin	a) PCOX LC-FLD (external calibration)	b) PCOX LC-FLD (standard addition)	c) Interlaboratory determination by PCOX LC-FLD	d) HILIC-MS/MS (standard addition)
GTX1	0.41 ± 0.04	0.39 ± 0.02	0.43 ± 0.09	0.43 ± 0.09
GTX4	0.33 ± 0.03	0.31 ± 0.02	0.33 ± 0.04	0.34 ± 0.07
NEO	0.64 ± 0.04	0.64 ±0.04	0.66 ± 0.07	0.67 ± 0.13
C1	0.66 ± 0.06	0.64 ± 0.03	0.73 ± 0.10	0.62 ± 0.12
C2	0.74 ± 0.05	0.72 ± 0.05	0.73 ± 0.14	0.68 ± 0.14
GTX 3	0.23 ± 0.01	0.23 ± 0.01	0.20 ± 0.02	0.21 ± 0.04
GTX 2	0.20 ± 0.01	0.22 ± 0.02	0.19 ± 0.02	0.24 ± 0.05
STX	0.22 ± 0.02	0.22 ± 0.01	0.26 ± 0.04	0.24 ± 0.05
Toxicity	668 ± 30	662 ± 37	694 ± 58	694 ± 139

Using a two-tail Student's t-test at 95% confidence, results generated from the two in-house PCOX LC-FLD methods were found to exhibit no significant differences. Measurement accuracy of the methods was further verified through the interlaboratory ring trial conducted. There was excellent overlap between the results generated by these three data sets and the additional supporting results provided by the LC-MS/MS analysis.

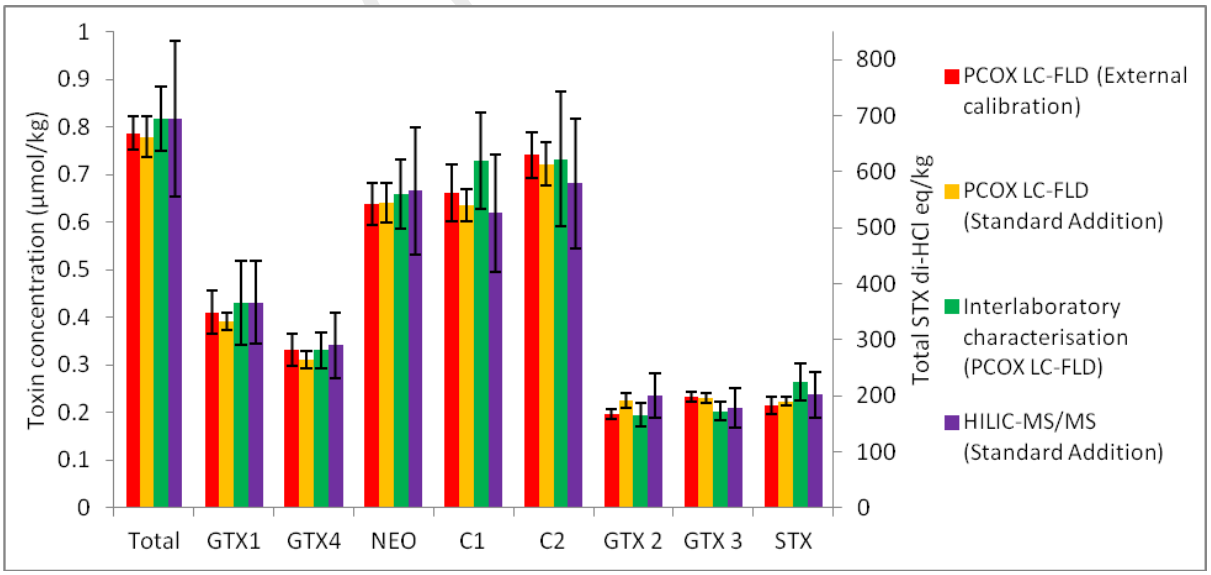


Figure 2. Summary of characterised toxin concentrations as determined by each of the four methods

A fifth method, Pre-COX LC-FLD method based on AOAC 2005.06, was used as a further analytical tool, but results from these analyses did not contribute to the material characterisation given the inability of this method to distinguish between epimeric pairs. **Table 4** summarises the results obtained following repeated analysis by Pre-COX LC-FLD of peroxide-oxidised C18-cleaned extracts and periodate-oxidised post-ion-exchange fractions and is reported here as additional information only.

Table 4. Summary of non-certified toxin concentrations in PO CRM from characterisation studies as determined by Pre-COX LC-FLD with external calibration.

Toxin	Pre-COX LC-FLD with external calibration
GTX 1&4	0.95 ± 0.05
NEO	0.49 ± 0.03
C 1&2	0.84 ± 0.10
GTX 2&3	0.23 ± 0.04
GTX 5	0.03 ± 0.01
STX	0.25 ± 0.04

8. Uncertainty of measurement

An assessment of the uncertainties associated with the assigned property values has been conducted. Specifically this involved combining the standard uncertainties associated with material homogeneity, stability and with the characterisation.

With no evidence of short term instability in transported samples sent frozen, the transport conditions are suitable to enable the uncertainty associated with the short term stability to be zero. Whilst no instability was determined over long term storage conditions, the standard uncertainties were calculated from the standard deviations of the toxin concentrations determined during the stability experiments conducted under repeatability conditions.

Between-bottle homogeneity data was calculated for each toxin following ISO Guide 35. Given the dominant contribution of the measurement method to the size of the uncertainty, as a result of the larger standard deviations associated with between-batch analysis, the alternative method for calculating the uncertainty estimate proposed by ISO Guide 35 was followed:

$$u_{bb} = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{\nu_{MS_{within}}}}$$

Given the agreement in results between the characterisation methods used, the uncertainties associated with the two main PCOX LC-FLD analyses were utilised for uncertainty calculation. Specifically the characterisation uncertainties were calculated for each toxin in accordance with:

$$u_{char} = \frac{\sqrt{\sum [u_c]^2}}{l}$$

Where u_C is the method-specific characterisation uncertainties and l is the number of methods (Pauwels *et al*., 1998).

Standard uncertainties were subsequently combined:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{LS}^2 + u_{SS}^2}$$

where u_{char} = uncertainty associated with characterisation, u_{bb} = uncertainty associated with between-bottle homogeneity, u_{LS} = uncertainty associated with long term (storage) stability and u_{SS} = uncertainty associated with short term stability (taken here as zero).

A coverage factor (k) of 2 was used to calculate expanded uncertainties for each property value with 95% confidence, given the normal distribution determined for the property values during the homogeneity study. Final calculated expanded uncertainties were applied to the final properties as summarised in Table 1.

9. Figures

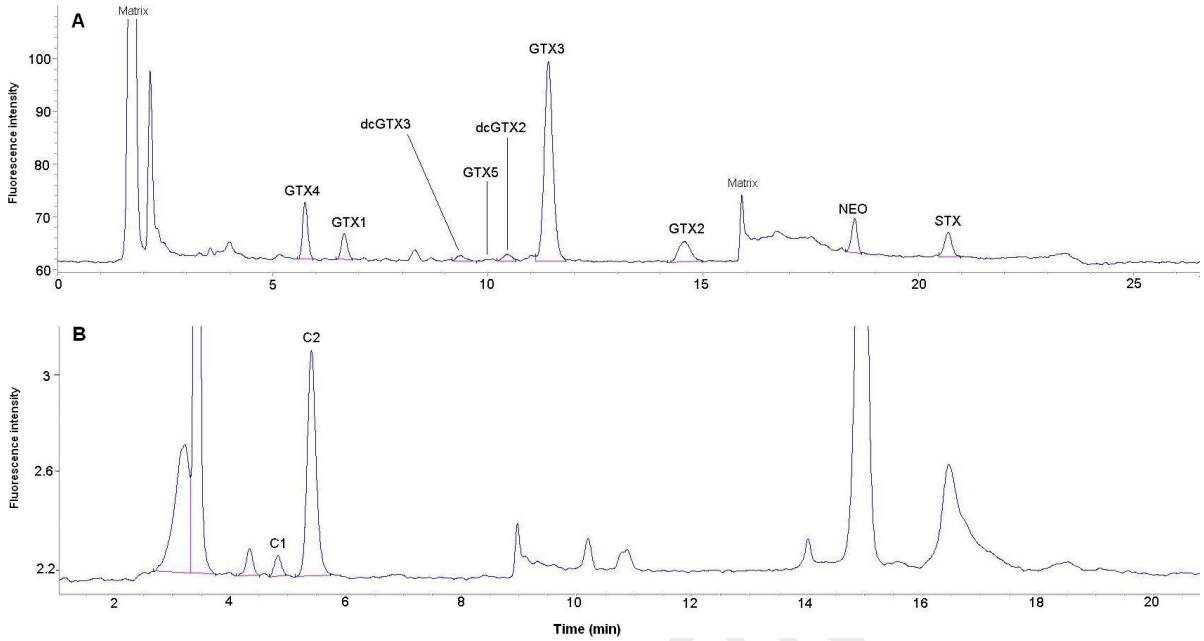


Figure 3. Analysis of deproteinated acetic acid extracts of PO PST CRM 1101 by PCOX LC with FLD for (A) STX/GTX toxins and (B) C toxins.

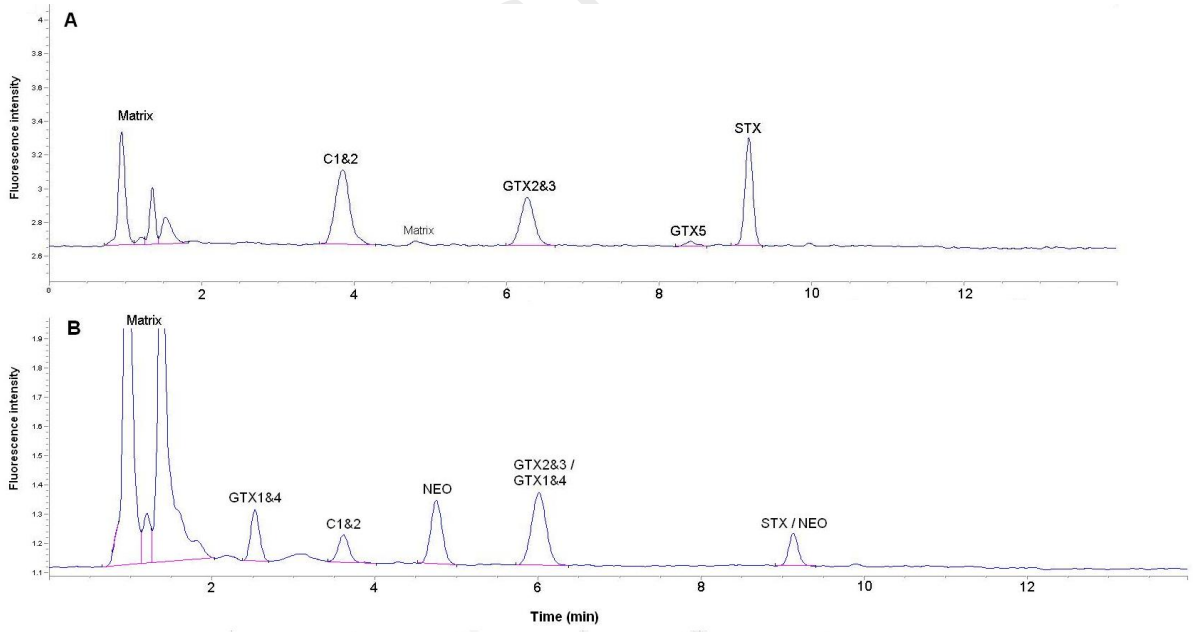


Figure 4. Analysis of C18 SPE-cleaned acetic acid extracts of PO PST CRM 1101 by Pre-COX LC with FLD following (A) peroxide oxidation and (B) periodate oxidation.

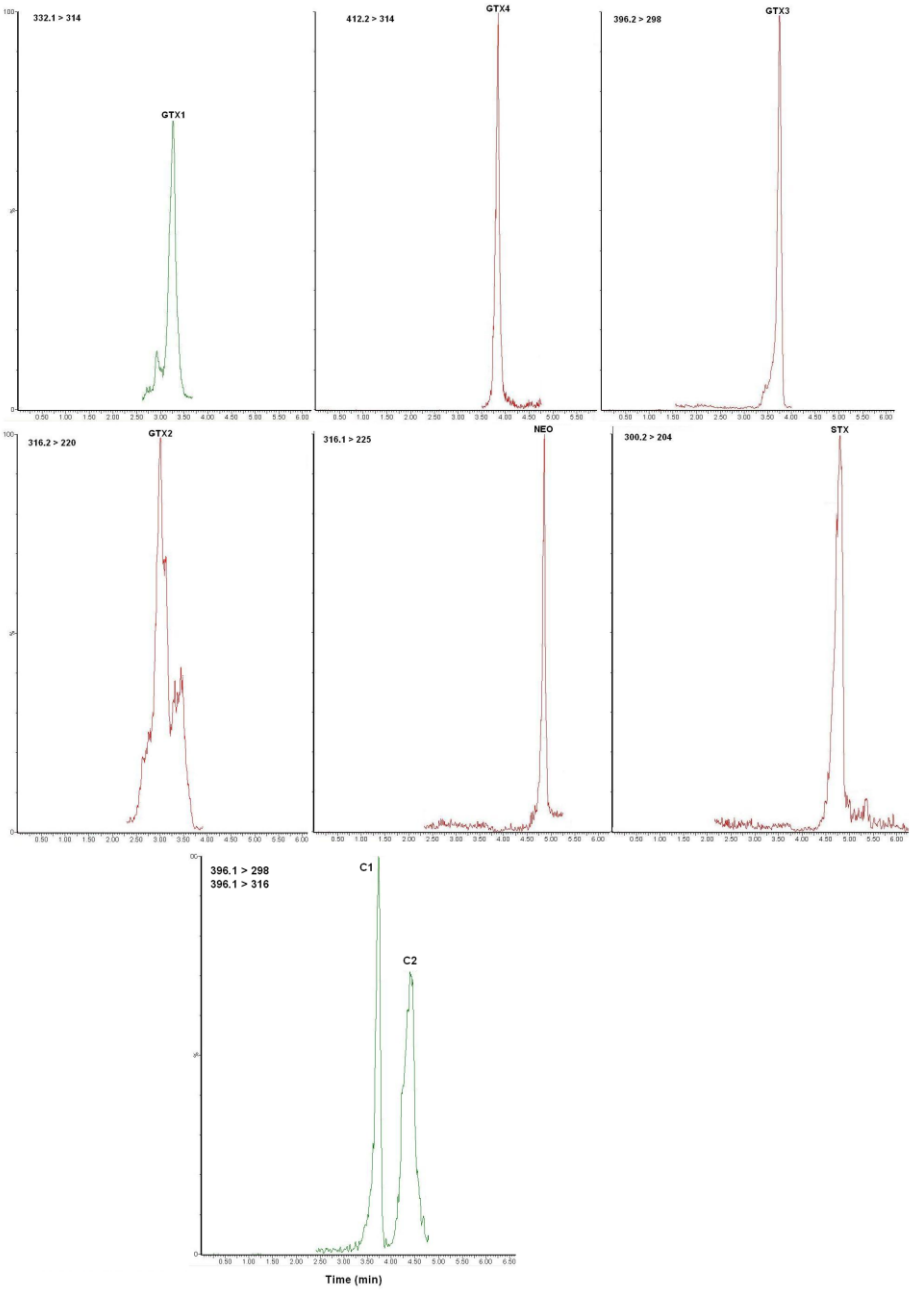


Figure 5. HILIC-MS/MS analysis (Waters Acquity UPLC BEH Glycan 1.7µm 2.1 x 100mm column) of acetic acid extracts of PO PST CRM 1101.

10. Safety

This material is for laboratory use only. Please refer to Safety Data Sheet provided separately for specific guidance.

11. References

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12. Acknowledgements

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13. Contact

Inquiries are welcomed from any users or potential customers. Please contact

Dr Andrew Turner/Dr Wendy Higman
Cefas
Barrack Road
Weymouth
Dorset, DT4 8UB
UK

Website: www.cefastechnology.co.uk/shop/acatalog/biotoxin_reference_materials.html
Email: andrew.turner@cefas.co.uk/wendy.higman@cefas.co.uk
Tel: +44 1305 206600

Certificate date: April 2013

Name and signature of certifying officer:

Dr Andrew Turner

Principal Chemist and Biotoxin Reference Materials Technical Manager