SPECIAL GUEST EDITOR SECTION

Interlaboratory Comparison of Two AOAC Liquid Chromatographic Fluorescence Detection Methods for Paralytic Shellfish Toxin Analysis through Characterization of an Oyster Reference Material

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An interlaboratory ring trial was designed and conducted by the Centre for Environment, Fisheries, and Aquaculture Science to investigate a range of issues affecting the analysis of a candidate Pacific oyster paralytic shellfish toxin reference material. A total of 21 laboratories participated in the study and supplied results using one or more of three instrumental methods, specifically precolumn oxidation (Pre-COX) LC with fluorescence detection (FLD; AOAC *Official Method* 2005.06), postcolumn oxidation (PCOX) LC-FLD (AOAC *Official Method* 2011.02), and hydrophilic interaction LC/MS/MS. Each participant analyzed nine replicate samples of the oyster tissue in three separate batches of three samples over a period of time longer than 1 week. Results were reported in a standardized format, reporting both individual toxin concentrations and total sample toxicity. Data were assessed to determine the equivalency of the two AOAC LC methods and the LC/MS/MS method as well as an assessment of intrabatch and interbatch repeatability and interlaboratory reproducibility of each method. Differences among the results reported using the three methods were shown to be statistically significant, although visual comparisons showed an overlap between results generated by the majority of tests, the exception being the Pre-COX quantitation of *N*-hydroxylated toxins in post ion-exchange fractions. Intralaboratory repeatability and interlaboratory reproducibility were acceptable for most of the results, with the exception of results generated from fractions. The results provided good evidence for the acceptable performance of the PCOX method for the quantitation of C toxins. Overall the study showed the usefulness of interlaboratory analysis for the characterization of paralytic shellfish poisoning matrix reference materials, highlighting some issues that may need to be addressed with further method assessment at individual participant laboratories.

For many years, the official control reference method for the determination of paralytic shellfish poisoning (PSP) toxicity in bivalve molluscs has been the mouse bioassay (MBA), as described by AOAC **959.08** (1). However, with a number of drawbacks relating to the biological assay, in recent years various alternative methods have been investigated and validated, both in single laboratory and interlaboratory studies. AOAC **2005.06** (precolumn oxidation LC with fluorescence detection; Pre-COX-LC-FLD) may be used in the European Union (EU) as an alternative to the MBA (2) for the detection and quantitation of PSP toxins (PSTs), and more recently a postcolumn oxidation method (PCOX-LC-FLD) has been approved as AOAC **2011.02** (3). More recently still, a third PST official method (AOAC **2011.27**) has been published based on a receptor binding assay (4). Other methods are also at various stages of development including the use of LC with MS/MS, although to date a formally validated method has not been published. With the introduction and global expansion in use of analytical methods for direct replacement of biological assays, there is the important need for appropriate reference materials. This includes both standards for instrument calibration and matrix-based materials for analysis of positive controls. If shown to be stable and homogenous, the latter will be useful for both internal QC and external QA, including their use in ring trial and proficiency testing schemes. This small study was designed to develop the understanding of a number of factors relating to the determination of PSTs in laboratory reference materials essential for method validation, internal QC, and external QA. Specific aims were to assess the equivalence of different analytical methodologies, in particular the two AOAC LC-FLD methods, the repeatability and reproducibility of each method, and the

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Table 1. Toxicity equivalency factors used in the study

Toxin	TEF
STX	1.0
NEO	1.0
dcSTX	1.0
GTX1	1.0
GTX2	0.4
GTX3	0.6
GTX4	0.7
GTX5	0.1
C1	0.006
C2	0.1
dcGTX2	0.2
dcGTX3	0.4
dcNEO	0.4

occurrence of any method-related issues concerning the analysis of PSP-contaminated oyster reference materials.

Interlaboratory Study

Thirty-four laboratories known to be experienced with the analysis of PSTs in shellfish materials were contacted during September 2011 to determine interest in participating in the study. A total of 22 participants accepted the invitation and in November 2011 were sent a copy of the experimental protocol together with a sample receipt form and a results submission form, and they were given their unique laboratory code. Subsequently, temperature-controlled packages containing the materials for analysis during the study were sent to each laboratory. On receipt of the package, participants were asked to confirm the contents of the packages and to store samples frozen (≤–15°C) until the day of analysis. Samples were shipped to participants using one of three couriers, and all shipments were made successfully within 5 days of shipping. While samples were reported as arriving in a variety of physical states, it was noted from the temperature records that all packages were still held below room temperature. Noting that Centre for Environment, Fisheries, and Aquaculture Science (CEFAS) stability data showed these materials to remain stable at room temperature for more than 6 days, there were no indications that any of the samples were compromised during the shipment. Before the extraction, samples were to be allowed to defrost until they reached room temperature. Before weighing the samples, each container was to be inverted several times to ensure thorough mixing of the sample contents.

The study materials comprised nine aliquots of a wet frozen shellfish reference material. The sample was prepared from Pacific oysters (*Crassostrea gigas*) sourced from the south coast of England that had been exposed to high concentrations of toxic *Alexandrium fundyense* cells in a laboratory environment (5). The samples contained concentrations of the PSTs gonyautoxins 1 to 4 (GTX1–4), saxitoxin (STX), neosaxitoxin (NEO), and C toxins 1 and 2 (C1&2), with trace quantities of GTX5 and decarbamoylgonyautoxins 2 and 3 (dcGTX2&3). Multiple batches of contaminated oysters were homogenized and combined to produce one large batch that was stabilized through use of pH adjustment and the incorporation of a range of antibiotic and antioxidant additives. After adjusting the water content to approximately 85% and further homogenization, the bulk tissue (approximately 50 kg) was dispensed, sealed, and frozen before being treated by gamma irradiation (approximately 18 kGy). Homogeneity testing was conducted at the CEFAS

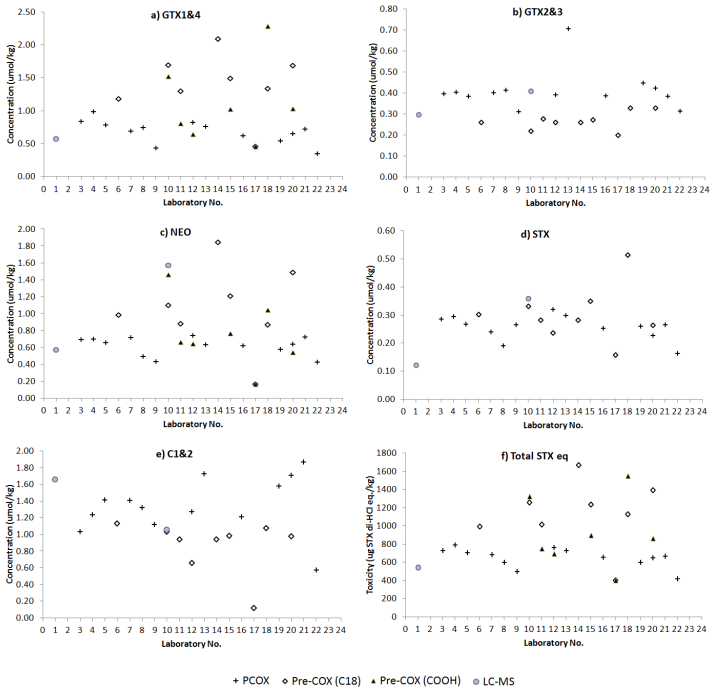

Figure 1. Graphical summary of mean concentrations (μmol/kg; *n* = 9) determined for each toxin (noting different y-axis scales) and total saxitoxin di-hydrochloride equivalents/kg at each laboratory.

Table 2. Summary of PST concentrations (μmol/kg shellfish tissue) and total sample toxicity (μg STX di-HCl eq./kg) generated by participant laboratories using each of the analytical methodologies

Toxin	Pre-COX-LC-FLD (C18 extracts) *n* = 9	Pre-COX-LC-FLD (COOH extracts) *n* = 7	PCOX-LC-FLD (all laboratories) *n* = 13	PCOX-LC-FLD (removed nonstandard laboratories)[a] *n* = 10	LC/MS/MS *n* = 2
GTX 1&4	1.54[b] ± 0.34	0.95 ± 0.37	0.69 ± 0.18	0.76 ± 0.12	0.57[c] ± 0.02
NEO	1.19[b] ± 0.35	0.77 ± 0.40	0.62 ± 0.11	0.66 ± 0.09	0.81 ± 0.78
C 1&2	0.98 ± 0.14	0.94 ± 0.15	1.34 ± 0.37	1.42 ± 0.31	1.36 ± 0.33
GTX 2&3	0.27 ± 0.05	0.27 ± 0.05	0.39 ± 0.04	0.40 ± 0.02	0.35 ± 0.06
STX	0.31 ± 0.08	0.33 ± 0.10	0.26 ± 0.05	0.26 ± 0.05	0.24 ± 0.12
Toxicity	1237[b] ± 239	919 ± 235	650 ± 108	694 ± 65	538[c] ± 30

[a] Laboratories 19 and 22 (use of HCl extraction) and Laboratory 9 (use of additional C18 clean up) removed from data set.

[b] *n* = 7 (two laboratories only reported results from fractions).

[c] *n* = 1.

laboratory and showed acceptable levels of homogeneity and no evidence of dispensing-related trend across the entire batch of samples. After the dispensing of bottles, samples were stored at –80°C until placed into the transportation packaging prior to shipment. For the purpose of this study, samples were selected at random for each participating laboratory. Each aliquot contained approximately 5.7 g of shellfish homogenate, enabling 5.0 g samples to be taken for each analysis.

The nine sample aliquots were extracted and analyzed on 3 separate days, ideally over a period of time >1 week, in three separate calibration batches, each in a single batch of three samples. Participants determined their results from each of the three batches and recorded them on a on a standardized results reporting sheet, along with any supplemental information of interest. Data provided were subsequently used to determine intrabatch and interbatch repeatability at each laboratory, with combined data from all laboratories used to determine intrabatch and interbatch repeatability and interlaboratory reproducibility for each method. Data were also used to summarize differences among the various methods used, using statistical significance testing where appropriate. Issues noted in the data were used to assess the presence of any potential method- or sample-related issues that may affect the analysis of these materials. Results were also assessed to determine assigned values to determine ring trial performance statistics and individual laboratory performance for the trial, although the more laboratory-specific performance data are not discussed further here.

Experimental

Methods of Analysis

The primary methods evaluated were the PCOX-LC-FLD (AOAC **2011.02**) and Pre-COX-LC-FLD (AOAC **2005.06**) methods. The method performance characteristics of both methods had been previously assessed formally in oysters by both single-laboratory (6–8) and interlaboratory (9, 10) validation studies. In addition, the study was open to results conducted by other relevant analytical methodologies, in particular the use of LC/MS/MS. It was requested where practical that laboratories should analyze samples by as many of these methods as possible.

Sample Extraction

Participants were asked to extract study samples using the methodology specified in AOAC **2005.06** (2). This involved a two-step extraction of 5.0 g shellfish tissue in 1% acetic acid (first step in boiling water), with centrifugation after each extraction step and combined supernatants diluted to 10 mL. Extracts were then cleaned up and/or analyzed.

*AOAC **2005.06***

The nine laboratories using this approach were asked to follow the guidance specified in AOAC **2005.06** (2) or in any subsequently published refined versions of the method (11). Aliquots of each extract were cleaned up using C18 SPE cartridges prior to peroxide oxidation and LC-FLD to determine the concentrations of non-*N*-hydroxylated PSTs. *N*-Hydroxylated PSTs were determined following periodate oxidation of either the C18-cleaned up extracts and/or C18-cleaned up extracts further cleaned up using weak ion-exchange (COOH) fractionation. Ideally, results were to be reported for each *N*-hydroxylated toxin following both cleanup methods.

*AOAC **2011.02***

Thirteen laboratories used this method during the study. Acetic acid extracts were processed following the guidance of AOAC **2011.02** (3). This included a protein precipitation step and filtration prior to analysis by PCOX-LC-FLD (8). Laboratories were allowed to employ chromatographic variations of the official method as long as the correct extraction and cleanup steps were used.

Hydrophilic Interaction LC (HILIC)/MS/MS

While no formally validated LC/MS methodology is currently published, two participants (Laboratories 1 and 10) expressed interest in testing samples by this methodology. Acetic acid extracts were to be subjected to HILIC prior to MS/MS with

Table 3. Summary of mean intrabatch repeatability data (expressed as RSD, %) from each participant using PCOX-LC-FLD

Laboratory	GTX1	GTX4	NEO	C1[a]	C2[a]	GTX2	GTX3	STX	Toxicity
3	0.04	0.06	0.05	0.03	0.02	0.03	0.02	0.03	0.030
4	0.04	0.06	0.05	0.03	0.09	0.04	0.04	0.04	0.026
5	0.04	0.05	0.06	0.09	0.10	0.02	0.03	0.04	0.032
7	0.05	0.04	0.01	0.06	0.02	0.06	0.02	0.05	0.020
8	0.02	0.05	0.04	0.02	0.02	0.01	0.02	0.02	0.018
9[b]	0.12	0.13	0.10	0.07	0.09	0.09	0.08	0.09	0.093
12	0.04	0.09	0.06	0.05	0.06	0.04	0.02	0.20	0.048
13	0.04	0.03	0.03	0.10	0.15	0.04	0.03	0.04	0.012
16	0.07	0.12	0.06	0.02	0.01	0.05	0.02	0.13	0.042
19[c]	0.07	0.07	0.05	0.01	0.02	0.09	0.02	0.07	0.017
20	0.03	0.07	0.02	0.05	0.03	0.02	0.01	0.02	0.011
21	0.06	0.05	0.07	0.02	0.04	0.01	0.02	0.03	0.039
22[c]	0.09	0.16	0.12	0.13	0.19	0.12	0.06	0.05	0.082
AOAC **2011.02**[d]	0.075	0.097	0.171	0.290	0.920	0.054	0.031	0.044	

[a] C toxin comparison against mussel and spiked materials from AOAC study.

[b] Protocol included additional C18 cleanup step.

[c] Results obtained following nonstandard HCl extraction.

[d] Calculated from mean of repeatability results for oysters in Reference 10; underlined values show results with RSDs higher than the values obtained from AOAC **2011.02**.

or without sample cleanup as dictated by individual laboratory protocol. The method used by Laboratory 1 followed the protocols described by Dell'Aversano et al. (12) but with some modifications. Laboratory 10 used 2 mM ammonium formate (pH 3) with acetonitrile mobile phases and a 150 µL/min flow rate through a ZIC HILIC (Merck, Darmstadt, Germany) 400 × 2.1 mm id, 3 µm particle size column.

Determination and Reporting

Protocols specified in individual participant laboratories were followed for calibrating instrumental methods. Appropriate calibration schemes were to be used throughout each analytical batch, with a minimum of five-point calibrations being utilized for each analyte and with calibration standards prepared from a suitable source of certified PST reference standards obtained from Institute of Biotoxin Metrology, National Research Council Canada (NRCC), Halifax, Canada. For the purpose of quantitation, solvent based standards were generally used. These were prepared in suitable diluents following normal laboratory protocol. For the purpose of LC/MS/MS quantitation, it was recognized that significant matrix effects exist that require the use of matrix-matched standards. The organizers left this decision to the discretion of individual participants but requested that information was to be provided on the method of quantitation performed on the final results sheet.

Participants reported results as individual concentrations of each PST detected and quantitated in terms of µmol/kg sample. The results for each toxin were not to be corrected for recovery in order to maintain consistency among participating laboratories. Individual toxins were to be reported if using either the PCOX-LC-FLD or LC/MS/MS methods. Laboratories using AOAC **2005.06** reported the sum of each quantitated epimeric pair, the compounds of which form identical oxidation products during the sample derivatization process (i.e., C1&2, GTX1&4, GTX2&3, and dcGTX2&3).

Total sample toxicity was reported in terms of µg STX di-HCl eq./kg sample. The procedure for calculating sample toxicity was to follow that recommended by the NRCC PSP supplemental information (13). Toxicity equivalency factors (TEFs) used were those specified by the European Food Standards Agency (EFSA; 14). For those participants calculating sample toxicity using AOAC **2005.06**, the highest TEF for each epimeric pair was to be used to calculate toxicity contribution. For the toxin C1, the TEF described by Oshima (15) was to be used in the absence of a published value from the EFSA (TEFs are given in Table 1).

Results and Discussion

Method

While the majority of participants followed official methods with little or no modifications to the formal protocols, a few laboratories did use protocols significantly different than those requested by the organizers. Laboratory 9 used additional C18 cleanup and no deproteination prior to PCOX analysis, while Laboratory 12 used mainly chromatographic modifications of AOAC **2011.02** methodology. These modifications included the use of 5.5 mM ammonium phosphate in the mobile phase and a 1 mL/min flow rate. Laboratory 17 reported scaling down the extraction to 1 g flesh in 2 × 0.6 mL extractions prior to AOAC **2005.06**. Two participants (Laboratories 19 and 22) conducted sample extraction using 0.1 M HCl rather than acetic acid prior to PCOX analysis, with Laboratory 19 using extraction with no boiling, no deproteination, and centrifugal filtration, with analysis of all samples in one batch. Consequently the organizers determined that results supplied from laboratories that used

Table 4. Summary of interbatch repeatability (RSD, %) over the three separate batches from each participant using PCOX-LC-FLD, plus associated HorRat ratios in parentheses

Laboratory	GTX1	GTX4	NEO	C1[a]	C2[a]	GTX 2	GTX 3	STX	Toxicity
3	0.05 (0.21)	0.07 (0.30)	0.06 (0.30)	0.26 (1.18)	0.24 (1.06)	0.12 (0.48)	0.03 (0.10)	0.05 (0.20)	0.039 (0.16)
4	0.05 (0.20)	0.13 (0.52)	0.06 (0.30)	0.07 (0.30)	0.14 (0.62)	0.06 (0.25)	0.07 (0.28)	0.05 (0.19)	0.036 (0.15)
5	0.06 (0.23)	0.06 (0.24)	0.07 (0.32)	0.16[b] (–)	0.18[b] (–)	0.05 (0.21)	0.05 (0.21)	0.04 (0.16)	0.039 (0.16)
7	0.10 (0.40)	0.12 (0.49)	0.08 (0.39)	0.09 (0.41)	0.07 (0.31)	0.09 (0.37)	0.09 (0.34)	0.10 (0.42)	0.084 (0.35)
8	0.09 (0.37)	0.10 (0.39)	0.13 (0.62)	0.19 (0.88)	0.21 (0.89)	0.02 (0.08)	0.16 (0.60)	0.09 (0.34)	0.037 (0.16)
9[c]	0.11 (0.46)	0.33 (1.30)	0.12 (0.59)	0.10 (0.45)	0.35 (1.50)	0.11 (0.44)	0.33 (1.27)	0.14 (0.55)	0.132 (0.55)
12	0.07 (0.31)	0.12 (0.46)	0.10 (0.49)	0.11 (0.50)	0.08 (0.36)	0.06 (0.24)	0.03 (0.11)	0.25 (0.98)	0.059 (0.25)
13	0.06 (0.23)	0.11 (0.42)	0.06 (0.28)	0.17 (0.78)	0.16 (0.68)	0.07 (0.26)	0.06 (0.23)	0.04 (0.17)	0.033 (0.14)
16	0.11 (0.46)	0.14 (0.56)	0.07 (0.32)	0.06 (0.28)	0.06 (0.27)	0.15 (0.61)	0.04 (0.16)	0.17 (0.69)	0.050 (0.21)
19[b,d]	0.12[b] (–)	0.11[b] (–)	0.06[b] (–)	0.01[b] (–)	0.02[b] (–)	0.11[b] (–)	0.03[b] (–)	0.08[b] (–)	0.044[b] (–)
20	0.10 (0.41)	0.16 (0.65)	0.12 (0.59)	0.14 (0.62)	0.16 (0.71)	0.04 (0.16)	0.03 (0.13)	0.04 (0.16)	0.073 (0.31)
21	0.17 (0.71)	0.12 (0.47)	0.07 (0.35)	0.05 (0.21)	0.12 (0.51)	0.02 (0.07)	0.05 (0.18)	0.03 (0.12)	0.060 (0.25)
22[d]	0.21 (0.88)	0.14 (0.58)	0.12 (0.56)	0.24 (1.10)	0.45 (1.95)	0.20 (0.81)	0.17 (0.64)	0.15 (0.60)	0.101 (0.42)
Mean	0.10 ± 0.05	0.13 ± 0.06	0.09 ± 0.03	0.13 ± 0.08	0.17 ± 0.12	0.08 ± 0.05	0.09 ± 0.09	0.09 ± 0.07	0.06 ± 0.03
AOAC **2011.02**[e]	0.287	0.283	0.393	0.510	0.770	0.123	0.120	0.123	0.250

[a] C toxin comparison against mussel and spiked materials from AOAC study.

[b] Analysis of all nine samples conducted in one single batch only.

[c] Additional C18 clean up used.

[d] HCl extraction used.

[e] Calculated from mean of repeatability results for oysters in Reference 10; underlined values show results with RSDs higher than the values obtained from AOAC **2011.02** and underlined HorRat values highlight results >1.3.

different sample preparation protocols (extraction and cleanup procedures) would not be included in the overall consensus values for each method but would be used to calculate repeatability and participant *z*-scores (specifically the PCOX results supplied by Laboratories 9, 19, and 22). Results supplied from other laboratories using slight modifications to protocols, including scaled down extraction (Laboratory 17) or modified parameters relating to the chromatography (Laboratory 12), would be included in all data assessment. Additionally, Laboratory 20 provided further data sets based on HCl extraction of the tissue materials.

Table 5. Summary of mean intrabatch repeatability data (expressed as RSD, %) from each participant using Pre-COX LC-FLD (quantitating *N*-hydroxylated toxins from C18 extracts)

Laboratory	GTX1&4	NEO	C1&2	GTX2&3	STX	Toxicity
6	0.05	0.04	0.04	0.03	0.03	0.02
10	0.02	0.02	0.03	0.02	0.02	0.01
11	0.04	0.05	0.04	0.06	0.03	0.03
12	—	—	0.14	0.12	0.11	0.09
14	0.04	0.05	0.03	0.05	0.02	0.03
15	0.09	0.03	0.07	0.07	0.04	0.05
17	—	—	0.09	0.10	0.48	0.10
18	0.10	0.04	0.08	0.09	0.05	0.07
20	0.02	0.02	0.02	0.05	0.02	0.01
AOAC **2005.06**[a]	—[b]	—[b]	0.32	0.19	0.18	—[b]

[a] Taken from mean of repeatability results for oyster samples in AOAC **2005.06**; underlined RSD values show results with RSDs higher than the values obtained from AOAC **2005.06**.

[b] No data available for oysters.

Method Equivalence

The different methods were compared by assessing total results for each epimeric pair (i.e., GTX1&4, GTX2&3, and C1&2 reported together) given the ability of AOAC **2005.06** to report only these results. Laboratories using the Pre-COX method reported concentrations for the *N*-hydroxylated toxins (GTX1&4 and NEO) determined in either the C18-cleaned up extracts and/or from fractionated extracts (post COOH ion exchange). Figure 1 displays the graphs generated for the spread of results determined for each toxin and for total STX equivalents. Table 2 summarizes the results obtained using each method from all participant laboratories combined.

Values are reported as mean values (calculated from all data points supplied from all nine samples analyzed over the three batches) together with the associated SD of the mean. For the purpose of this overview of equivalence, outliers were removed from the data following use of the Grubbs test for outliers. In addition, a further assessment was made using PCOX results provided only by those laboratories utilizing the correct methodology. Specifically, data supplied by those laboratories utilizing an HCl extraction (Laboratories 19 and 22), plus Laboratory 9 that incorporated an additional C18 cleanup step, were removed. However, both sets of data are summarized in Table 2 for comparative purposes.

Table 6. Summary of interbatch repeatability (RSD, %) over the three separate batches from each participant using Pre-COX-LC-FLD (C18 extracts), plus associated HorRat ratios in parentheses

Laboratory	GTX1&4	NEO	C1&2	GTX2&3	STX	Toxicity
6	0.07 (0.40)	0.04 (0.19)	0.09 (0.34)	0.07 (0.29)	0.05 (0.22)	0.04 (0.18)
10	0.03 (0.15)	0.04 (0.23)	0.06 (0.21)	0.03 (0.14)	0.03 (0.11)	0.02 (0.11)
11	0.11 (0.63)	0.10 (0.52)	0.06 (0.23)	0.06 (0.25)	0.04 (0.15)	0.08 (0.36)
12	—	—	0.17 (0.64)	0.16 (0.68)	0.15 (0.60)	0.11 (0.49)
14	0.10 (0.57)	0.12 (0.65)	0.10 (0.39)	0.05 (0.23)	0.07 (0.27)	0.10 (0.46)
15	0.16 (0.89)	0.08 (0.45)	0.09 (0.34)	0.08 (0.35)	0.05 (0.19)	0.10 (0.46)
18	0.22 (1.20)	0.12 (0.63)	0.07 (0.28)	0.14 (0.58)	0.22 (0.90)	0.10 (0.43)
17	—	—	0.13 (0.50)	0.17 (0.70)	0.59 (2.37)	0.14 (0.61)
20	0.05 (0.28)	0.11 (0.57)	0.03 (0.12)	0.08 (0.34)	0.03 (0.10)	0.06 (0.27)
Mean	0.11 ± 0.07	0.08 ± 0.04	0.09 ± 0.03	0.09 ± 0.05	0.15 ± 0.21	0.08 ± 0.04
AOAC **2005.06**[a]	0.68	—[b]	0.25	0.25	0.29	—[b]

[a] Taken from mean of repeatability results for oyster samples in AOAC **2005.06**; underlined RSD values show results with RSDs higher than the values obtained from AOAC **2005.06**; underlined HorRat values highlight results >1.3.

[b] No data available for oysters.

Visually there were some clear differences in the results provided by the different methodologies. Table 2 shows that while the results obtained for STX were generally in good agreement, differences were notable for the other toxins present in the samples. The significance of these differences was determined through analysis of variance (ANOVA), which confirmed significant statistical differences among concentrations reported using the three methods. This was found for both the full PCOX data set and the modified data set with the data removed from laboratories employing a nonstandard protocol. Given the low number of laboratories reporting LC/MS/MS data and the fact that this method is more in the development stage as compared with the two LC-FLD methods, a further test was conducted to determine the significance of any differences apparent between the two LC-FLD methods alone. Results obtained following a *t*-test (two samples assuming equal variance, two-tailed, 95% confidence) again confirmed the significance of the apparent differences for each of the toxins, including STX. An additional *t*-test confirmed a significant difference between the concentrations reported using either post-C18 or post-ion-exchange extracts prior to periodate oxidation and Pre-COX- LC-FLD.

The results in Table 2 show a clear relative overestimation of toxin concentration given by the Pre-COX method in C18-cleaned up extracts for GTX1&4 and NEO as compared to the PCOX method and the Pre-COX post-ion-exchange fractionation. Conversely, the values given by PCOX are higher on average for C1&2 and GTX2&3 than for either of the Pre-COX data sets. Consequently, the mean values for total sample toxicity are higher in both sets of Pre-COX results as compared to the PCOX results. The modified PCOX data set, containing only the results from laboratories using the specified protocols, shows a better agreement with the Pre-COX method results, with the concentrations and associated uncertainties (SDs) overlapping for the majority of toxins. Pre-COX- LC-FLD results in this study are affected by higher levels of interlaboratory method variability as shown by the higher SDs summarized in Table 2. On the whole, the results obtained from the participants reporting LC/MS/MS results appear to agree more closely to the PCOX results than the Pre-COX results.

Method Repeatability

*AOAC **2011.02***.—The mean intrabatch repeatability of the PCOX-LC-FLD results from each laboratory (n = 13) together with those described by AOAC **2011.02** is summarized in Table 3. The table highlights participant results showing SDs higher than those from the official method. Intrabatch repeatability data were generally very good for each of the participants, with only three individual results higher than 0.15. Those results underlined relate primarily to toxins for which very low values were reported for the official method (e.g., STX and GTX3) as opposed to unacceptably higher values reported by participants. For C1 and C2 toxins poor data were reported for the official method due to an absence of suitable matrix materials; these data are good additional evidence for acceptable intrabatch precision of the PCOX method for those toxins in each of the participant laboratories.

According to EC Decision 2002/657 (16): "For analysis conducted under intralaboratory reproducibility conditions, the intralaboratory CV shall not be greater than the reproducibility CV." Table 4 summarizes the interbatch repeatability results reported from each laboratory, together with the associated HorRat ratios. HorRat values were calculated using the predicted RSD (= CV) calculated from individual toxin concentrations (expressed as mass fraction), with the total toxicity HorRat values calculated from an expected RSD of 25%. Again, the data were generally good, with mean interbatch repeatability ranging between 0.08 and 0.17 depending on the toxin, only a low number of results showing repeatability higher than the values reported by the official method, and only two results returning HorRat values >1.3. The poor values reported by AOAC **2011.02** for the C toxins have been improved upon here with good intralaboratory reproducibility data for both C1 and C2 toxins in the majority of laboratories. Overall, therefore, the results show a good potential for use of this method for characterization of this candidate reference material through interlaboratory study.

Table 7. Summary of mean intrabatch repeatability data (expressed as RSD, %) from each participant post-ion-exchange using Pre-COX-LC-FLD (n = 7)[a]

Laboratory	GTX1&4	NEO
10	0.01	0.01
11	0.05	0.06
12	0.13	0.15
15	0.11	0.08
17	0.11	0.74
18	0.16	0.04
20	0.07	0.07

[a] No data available for oysters in AOAC **2005.06**.

*AOAC **2005.06** from C18-cleaned up extracts*.—Table 5 summarizes the mean intrabatch repeatability of the Pre-COX-LC-FLD results reported by participants quantitating *N*-hydroxylated toxins in the C18-cleaned up extracts (n = 7 to 9). Intrabatch repeatability results taken from oyster samples analyzed during the AOAC **2005.06** interlaboratory study are also shown for comparison purposes. The table demonstrates very good levels of intrabatch repeatability data associated with the majority of results, with only one individual result higher than 0.10. As evidenced from the raw data, the one instance of high repeatability for STX (Laboratory 17) appears to relate to problems with both the qualitative and quantitative determination of STX in that laboratory as opposed to low values within the official method (data not shown). Table 6 summarizes the interbatch repeatability results reported from each laboratory, together with the associated HorRat values. The data were good, with only one (again, STX from Laboratory 17) returning a repeatability higher than the value reported by the official method and with HorRat values >1.3. Mean values ranged between 0.09 and 0.15 depending on the toxin. Overall, the results from the repeated analysis of the oyster material indicate improved levels of intrabatch and interbatch repeatability as compared to the values reported by AOAC **2005.06**, perhaps related to the greater levels of experience of participants in this study as compared with the original interlaboratory method validation.

*AOAC **2005.06** from COOH-cleaned up fractions*.—Table 7 summarizes the mean intrabatch repeatability of the Pre-COX-LC-FLD results supplied by participants quantifying *N*-hydroxylated toxins in the post-ion-exchange fractions (n = 7). No comparison was possible with AOAC AOAC **2005.06** due to the absence of PSP-contaminated oyster samples containing either GTX1&4 or NEO (2). Results highlight generally acceptable levels of intrabatch repeatability, with two individual results having values >0.15 and one notable example of poor repeatability of analysis within the same batch (Laboratory 17, NEO). Raw values supplied by this laboratory (data not shown) seemed to indicate specific method performance issues, with NEO being clearly identified and quantitated in some samples and not detected in others. Table 8 summarizes the interbatch repeatability results reported from each laboratory, together with the associated HorRat values. The data were good for the majority of laboratories, but with two showing repeatability higher than 0.25. Out of the four results showing HorRat values >1.3, two were very close to the 1.3 limit, while the other two were >2.0.

Overall, while the results showed generally acceptable levels of repeatability as compared to the values reported by AOAC **2005.06**, values were mostly higher than those reported from analysis of C18-cleaned up extracts. The noted instances of variability being higher than expected may indicate specific issues with fractionation methodology employed within some laboratories.

Table 8. Interbatch repeatability (RSD, %) over the three separate batches from each participant using Pre-COX-LC-FLD (post-ion-exchange), plus associated HorRat ratios in parentheses

Laboratory	GTX1&4	NEO
10	0.01 (0.07)	0.02 (0.13)
11	0.13 (0.70)	0.12 (0.63)
12	0.12 (0.65)	0.17 (0.91)
15	0.17 (0.97)	0.13 (0.67)
17	0.23 (1.31)	0.93 (4.87)
18	0.39 (2.15)	0.25 (1.33)
20	0.19 (1.07)	0.07 (0.38)
Mean	0.18 ± 0.12	0.24 ± 0.31
AOAC **2005.06**[a]	0.68	NA

[a] Calculated from mean of repeatability results for oyster samples in AOAC **2005.06**; underlined HorRat values show results >1.3.

LC/MS/MS

Although only two laboratories returned results using the LC/MS/MS method, assessment of method repeatability within each of the laboratories was still possible. Problems with detection and quantitation of GTX1 and GTX4 at Laboratory 10 prevented the full assessment of precision and repeatability for this laboratory for these toxins and for total sample toxicity. Table 9 summarizes the intrabatch and interbatch repeatability data calculated in both laboratories. There was good evidence for acceptable repeatability of analysis using this method by both participant laboratories. All intrabatch RSDs are <0.10%, with the exception of NEO in Laboratory 1 as a result of NEO detection issues. Results for interbatch repeatability are generally good for each toxin where reliable toxin identification has been achieved. For these toxins, all HorRat values are <0.8, including the values calculated for GTX5 at Laboratory 1. Problems with the identification of NEO at Laboratory 1 in the first six samples and the identification of GTX1 and GTX4 at Laboratory 10 prevented the calculation of HorRat values for these toxins. Overall, generally acceptable levels of repeatability were evidenced, given the caveats relating to problems with detection of some toxins.

Method Reproducibility

A summary of the reproducibility of each method, as determined by the total RSDs for each toxin and by each method across all laboratories, is given in Table 10. These were calculated from all data points following removal of outliers from each of the data sets using the Grubbs test. Results show comparable reproducibility data between the PCOX- and Pre-COX-LC-FLD methods for some toxins (GTX1&4 and STX), with better reproducibility for C1&2 using the Pre-COX

Table 9. Summary of mean intrabatch and interbatch repeatability data (expressed as RSD, %) from each participant following LC/MS/MS analysis (*n* = 2), plus associated HorRat values in parentheses

Laboratory	Batch type	GTX1	GTX4	NEO	C1	C2	GTX2	GTX3	GTX5	STX	Toxicity
1	Intra	0.03	0.02	0.03[a]	0.02	0.05	0.06	0.02	0.05	0.06	0.01
	Inter	0.03 (0.13)	0.06 (0.27)	0.03[b]	0.02 (0.11)	0.12 (0.58)	0.10 (0.39)	0.04 (0.17)	0.27 (0.77)	0.15 (0.58)	0.06 (0.22)
10	Intra	—	—	0.03	0.08	0.09	0.09	0.06	—	0.03	—
	Inter	—	—	0.05 (0.22)	0.09 (0.41)	0.10 (0.49)	0.11 (0.42)	0.07 (0.28)	—	0.04 (0.14)	—

[a] NEO detected only in third batch with none detected in first six samples.

[b] HorRat value not reported given variable detection throughout batches.

method and improved reproducibility for NEO and GTX2&3 following PCOX. Reproducibility was also improved in the PCOX results after removal of the results from laboratories utilizing nonstandard sample preparation protocols. As expected, the repeatability and reproducibility of the Pre-COX method was worsened through use of the ion-exchange fractionation step, with HorRat values for both *N*-hydroxylated toxins >2.0. Examination of the raw data provided strong evidence for a lack of robustness in the method among the various participant laboratories. The cause of this wide variation and subsequent high level of interlaboratory variability was not clear from these results alone. It may result from a combination of a number of factors potentially including variable performance of different ion-exchange cartridges, differences in matrix effects where different chromatographic methods are used, and/or potential issues with QC with the ion-exchange method. This highlights the need for individual laboratories to thoroughly assess the recoveries of *N*-hydroxylated toxins determined following ion-exchange fractionation and to ensure intrabatch and interbatch repeatabilities are consistently falling within prescribed limits. LC/MS/MS repeatability data are shown for comparison, but no HorRat values or significant conclusions are given due to the low number of laboratories utilizing this method.

The overall interlaboratory reproducibility of each method highlighted the acceptable degree of variability for each of the methods. Again, this also provided good evidence for acceptable reproducibility for the determination of C1 and C2 toxins following PCOX-LC-FLD. All data were found to fall into an acceptable range of reproducibility, with the exception of the data for GTX1&4 and NEO following ion-exchange fractionation.

Method-Related Issues

The ring trial results have highlighted a number of interesting points relating to the analysis of PSP toxins in this oyster tissue. While there may not be any evidence for the issues encountered here to be applicable to the performance of analytical methods in other species of PSP-contaminated bivalve molluscs or in samples with different PSP toxin profiles, there is cause to be aware of some performance issues that could be investigated further.

Relative Method Performance

Results have shown some notable differences in performance among the various methods. Specifically there is evidence for generally good agreement between the PCOX and Pre-COX methods for certain toxins (GTX2&3 and STX), but with apparent overestimation in concentrations of GTX1&4 and NEO following Pre-COX analysis of C18-cleaned up extracts and some evidence for higher concentrations of C1&2 as determined by the PCOX method. Although SDs associated with the mean values for each of the results determined with each method were found to overlap, there are statistically significant differences between the sets of data returned by the different methods. Differences in concentrations determined by Pre-COX in comparison with PCOX are to be expected to an extent given the inability of the former method to distinguish between the responses of the oxidation products of individual epimers. Different toxin epimers (e.g., GTX1 and GTX4) may have different instrumental molar responses following LC-FLD, so the overall response and

Table 10. Summary of RSD, % values from each method (outliers removed), showing HorRat values in parentheses

Toxin	Pre-COX-LC-FLD (C18 extracts)	Pre-COX-LC-FLD (COOH extracts)	PCOX-LC-FLD	PCOX-LC-FLD[a]	LC/MS/MS
GTX1&4	0.22 (1.21)	0.39 (2.17)	0.26 (1.06)	0.15 (0.63)	0.03
NEO	0.30 (1.56)	0.52 (2.74)	0.17 (0.82)	0.13 (0.63)	0.96
C1&2	0.14 (0.52)	—	0.28 (1.25)	0.22 (1.00)	0.24
GTX2&3	0.18 (0.76)	—	0.11 (0.42)	0.06 (0.24)	0.18
STX	0.26 (1.02)	—	0.20 (0.79)	0.18 (0.71)	0.51
Toxicity	0.19 (0.86)	—	0.17 (0.69)	0.10 (0.39)	0.06

[a] Removal of Laboratories 9, 19, and 22 as outliers.

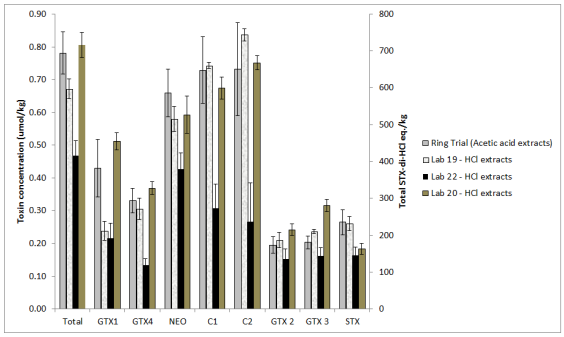

Figure 2. Comparison of PCOX-LC-FLD quantitative results from Laboratories 19, 20, and 22 after HCl extraction with quantitative results from other participants after acetic acid extraction.

subsequent calculations of concentration may differ from those of the Pre-COX method for which oxidation products formed from the two different epimers are identical and one single molar response applies to both epimers. With this in mind, differences in toxin concentrations determined by PCOX and Pre-COX (following ion-exchange fractionation) are not too unexpected. The differences in total sample toxicity quantitated by the two LC methods, therefore, appear to relate not only to differences in quantitation of individual toxin molar concentrations, but also due to the known overestimation in total STX equivalents calculated when assuming the sole presence of the most toxic of each epimeric pair. Another notable effect evident from these results appears to relate to the differences in *N*-hydroxylated toxin concentrations quantitated following both C18-cleanup and ion-exchange fractionation. For reasons currently unknown, concentrations determined following fractionation are closer to those determined by PCOX as evidenced in previous studies with UK oysters (17, 18) while C18-cleaned up extracts give rise to concentrations of GTX1&4 and NEO that are notably higher (more than double the values returned by PCOX). A recent European Union Reference Laboratory (EU RL) study investigating the recovery of the Pre-COX method in a range of EU National Reference Laboratories (NRLs) also found some recovery issues with these toxins, although these effects were found to be variable (Ben-Gigirey, B., personal communication, 2012). It is not clear from these results why these differences exist, and it must be noted that there is no published evidence for this issue to occur in other shellfish species or in other oyster samples from different spatial or temporal sources. Nevertheless, this highlights some evidence for the potential overestimation of toxicity with Pre-COX-LC-FLD following quantitation of *N*-hydroxylated toxins in C18-cleaned up extracts. While it may be assumed that this relates to the effects of matrix co-extractives present in the C18-cleaned up extracts that are subsequently removed following ion-exchange fractionation, further work would be required to establish the exact cause.

Overall, this has demonstrated the potential effective use of the AOAC **2011.02** PCOX method for the characterization of candidate reference materials such as the one used in this study.

Extraction Methods

Previous work conducted on UK oysters demonstrated a fairly close similarity between toxicity results determined from both acetic acid and hydrochloric acid extracts, with on average slightly higher toxicities determined following HCl extraction (17). The results obtained from Laboratories 19 and 22, which used HCl extraction procedures as opposed to acetic acid, provide further insights into the differences in results using the two extraction methods. In addition, Laboratory 20 submitted an additional data set generated following HCl extraction of study tissues. The HCl extract results are illustrated in Figure 2 and show some notable differences. Data from Laboratories 19 and 20 showed good agreement with other laboratories using acetic acid, with the possible exception of GTX1 from Laboratory 19 that was generally lower in concentration than other results. Furthermore, the concentrations returned for C1 and C2 showed that no noticeable conversion of C toxins to other more toxic PSTs. However, Laboratory 22 returned results showing notably lower concentrations for the majority of toxins as compared to the results obtained following acetic acid extraction and from the other two laboratories reporting HCl extraction data. While the lower values reported from Laboratory 22 may indicate problems with the quantitative methodology, it is not clear from these results alone whether the method of extraction results in significant differences in final toxin concentrations or not. Further work will be required to determine these effects more robustly.

Use of C18 Cleanup Prior to AOAC 2011.02

Laboratory 9 used an additional C18 cleanup step prior to the analysis of samples by PCOX-LC-FLD. Such an approach may be of interest to some laboratories, particularly where matrix components present in the raw acidic extracts have been found to result in significant levels of fluorescence enhancement or suppression, or where unacceptable retention time shifts are experienced in some shellfish species. However, results determined from this participant showed generally lower values than the consensus means. While there is currently only data from this one laboratory using this approach, there is the indication of potential recovery losses through the use of this additional sample preparation step. This highlights the potential need for further work to assess the relative advantages and disadvantages of using such additional cleanup procedures during the analysis of certain shellfish species.

Matrix-Modified Standards

All participants using the PCOX method used solvent-based standards, with the exception of Laboratory 7, which used calibrants prepared in diluted oyster matrix. In addition Laboratory 7 provided quantitative results obtained using a single-point calibration prepared in mussel extract, and Laboratory 20 provided quantitative data obtained using standards prepared in C18-cleaned up acetic acid extracts of PSP-negative Pacific oysters. A comparison of the PCOX results generated by these two laboratories using matrix-modified standards against the values obtained by solvent standards is shown in Figure 3. The results indicate no visual differences between the values obtained using either mussel or oyster extract matrix calibrants for the majority of toxins and for total STX equivalents. Exceptions include concentrations of GTX4 and GTX3 following quantitation against oyster matrix standards at Laboratory 20 that are lower and higher, respectively, than the mean ring trial values, and both are outside the SD limits. However, there were no apparent differences between the quantitative values obtained

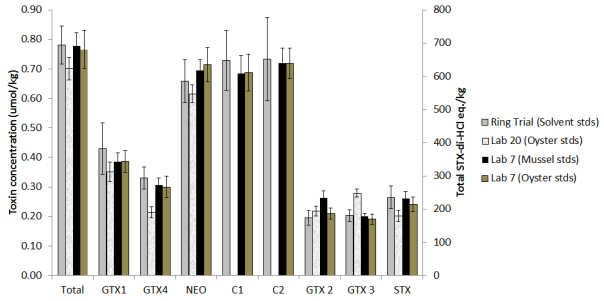

Figure 3. Comparison of PCOX-LC-FLD quantitative results from Laboratories 7 and 20 using oyster and mussel-extract calibrants with quantitative results from other participants using solvent-based standards.

by Laboratory 7 in either mussel or oyster matrix in comparison with the ring trial data. Overall, while this small data set indicates generally low matrix effects, there may be the potential for method accuracy to be affected in some laboratories with the use of matrix-modified standards.

LC/MS/MS Methods

Laboratories 1 and 10 supplied results following analysis by HILIC/MS/MS that provided some further interesting insights into the quantitation of PSTs in this reference material and highlighted some issues that may warrant further investigation. The mean results obtained using this method compared well with the results derived from the PCOX analysis for some specific toxins (C1&2, STX, and GTX2&3, most notably). In addition, the data provided by Laboratory 1 showed the presence of toxin GTX5 in each analysis, a toxin known to be present at trace levels within the material but which most of the other laboratories did not report as being detected. Although there is relatively little data to examine in this study using this method, there is nevertheless good promise for the potential use of the technique for the quantitative determination of at least some PSTs in contaminated oyster samples. Issues noted by the participants may still need to be resolved including the detection and quantitation of NEO (Laboratory 1), which was found to be affected by chromatographic interferences in the samples, an issue that appears to have been variable. Sensitivity issues at Laboratory 10 prevented the quantitation and reporting of GTX1&4 (and consequently total sample toxicity), further highlighting the well-known issues with analytical sensitivity for PSP toxin analysis using MS detector technologies.

Overall the results have further highlighted the potential for use of this method, but they show the need in some instances for further work and the ongoing requirement for regular QA.

Additional Study Comments

While all samples were shipped to participants within 5 days, one shipment was held up for a total of 10 days prior to being shipped back to CEFAS. Additional analysis of this sample confirmed no sample degradation had taken place, further highlighting the good stability of the materials used. All results were received 4 months after initial sample receipt, with some laboratories delaying analyses because of a number of serious problems. These issues were found to result from performance capabilities of either the instrument or column used for the PCOX-LC-FLD method. Availability of specific PCOX LC columns was a common issue, together with noted and variable issues with LC retention times and toxin identification using the PCOX method. In all cases successful identification was eventually performed, in some instances following additional work involving spiked matrix samples to confirm toxin identification. A few laboratories that reported instrument problems stored extracts in a refrigerator for up to 8 days prior to analysis of GTX/STX toxins by PCOX-LC-FLD (e.g., Laboratories 7 and 8) and C toxins by PCOX-LC-FLD (Laboratory 13). In each instance there was no evidence from any of the data presented by these participants of any adverse effects on results potentially resulting from instability of toxin concentrations over the short to medium term. The slight deterioration in chromatographic performance noted by one participant (Laboratory 16) was not thought by the participant to have affected the quantitative results. This observation seems to be backed up by a comparison of the repeatability results reported by this participant with those reported by other laboratories. Finally, problems with calibration curves were reported by Laboratory 17, but the source of these was not further explained. However, these may be the cause of the high variability of results determined between batches and the questionable results reported for some toxins.

Conclusions

A summary of the toxin concentrations and sample toxicities determined using the three methods showed some differences in results, all of which were statistically significant. Differences between the AOAC **2005.06** Pre-COX method with *N*-hydroxylated toxins quantitated from post-ion-exchange fractions and the AOAC **2011.02** PCOX method were not too large, and although statistically significant they were still within the ranges of values shown by the SDs of the mean results and reported measurement uncertainties. Results obtained by LC/MS showed on the whole a fairly good agreement with the PCOX data, although there were some notable issues with sensitivity and variable effects of matrix interferences that compromised the success of the technique for some of the toxins. Mean results determined by Pre-COX for the *N*-hydroxylated toxins (GTX1&4 and NEO) in the C18-cleaned up extracts were substantially higher than the results determined by the other methods. With the potential for overestimation of toxin concentrations when quantitating C18-cleaned up extracts, there may be the need for laboratories to investigate these issues further in species of local interest.

Intrabatch and interbatch repeatability was generally acceptable for most participants with similar ranges of results reported for both PCOX-LC-FLD and Pre-COX-LC-FLD (quantitation from C18-cleaned up extracts only). Results also provided good evidence for acceptable method repeatability for the PCOX analysis of the C1 and C2 toxins, which were absent from the profiles of samples used in the official method validation study (3). More variable ranges of repeatability were reported for the quantitation of *N*-hydroxylated toxins in post-ion-exchange fractions. The interbatch repeatability was found to be acceptable in five out of the seven laboratories using this approach, while two laboratories returned results showing questionable levels of repeatability. Repeatability data from laboratories using LC/MS showed good levels of variability for most toxins, with

the exceptions being those toxins for which either sensitivity or effects of interferences were an issue.

Overall reproducibility for each method among all participants showed generally acceptable levels for each method. HorRat values were shown to be good for the analysis of most toxins by all methods, with interlaboratory variances being generally similar to or improved as compared with values reported in the OMAs. Again, good evidence was presented for the acceptable reproducibility of the PCOX method for the determination of C1 and C2 toxins in these samples. The higher variability observed in results for *N*-hydroxylated toxins quantitated from fractions has highlighted the need to conduct further assessment into the reproducibility of the method ideally involving future ring trials incorporating a wider range of samples containing *N*-hydroxylated toxins.

Nonstandard protocols and additional data provided by some participants offered some insights into the potential effects of different extraction solvents, matrix-modified standards, and the use of additional cleanup steps. However, with the majority of participants using the stipulated method, further work would be required to determine the effects of these modifications more robustly. Additionally, while use of an additional C18 cleanup step prior to PCOX may have removed some potential problems relating to toxin identification, results do suggest the potential for recovery losses using this approach.

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