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NEW METHODS FOR THE DETECTION, AND CONFIRMATION OF THE DETECTION, OF SAXITOXIN IN ENVIRONMENTAL SAMPLES

INTRODUCTION

1. The marine toxin saxitoxin (STX) is a Schedule 1 chemical under the Chemical Weapons Convention that has very high specific toxicity. Because of the high toxicity of STX it can reasonably be expected that environmental samples returned to the OPCW for analysis by the designated laboratory network would contain STX at very low concentration, i.e., less than 1 µg/mL. The current OPCW reporting requirements for chemicals identified in environmental samples require two techniques for the confirmation of a chemical. Whilst the structure of STX lends itself to analysis by LC-MS, the concentration typically prohibits the acquisition of full scan mass spectra for all but the most sensitive of instruments; even then, depending on the instrumental conditions, the mass spectrum may consist of only the molecular ion. As such, the utility of MS/MS, particularly MS/HRMS leads to an increase in the selectivity of the analysis, and hence an increase in the sensitivity of the analysis. In this paper the OPCW Laboratory presents a highly selective and sensitive UPLC-MS/HRMS method using its Orbitrap Q-Exactive Plus mass spectrometer. The limit of detection using this method is in the low pg/mL, which is similar to that of a typical ELISA assay. The method utilises HILIC chromatography, as STX is not optimally retained on reverse phase media.

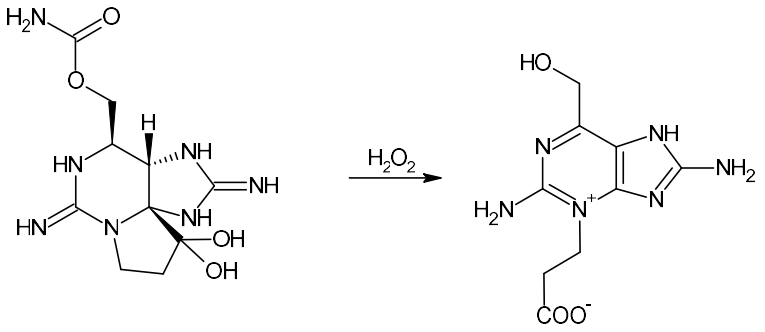
2. For many laboratories the reporting criteria leave very little choice for a confirmatory technique for the detection of STX. OPCW requirements are such that the method must change the basis of the chromatography and that the chemical must be retained on the column. Amide-based stationary phases are very similar to HILIC and do not confer this different basis of separation, and C18 columns do not retain the analyte. Some participating laboratories in the OPCW biotoxin exercises do have ELISA capabilities, but many do not. Similarly, some laboratories have an LC-FLD capability, but many do not. The chemical basis of the oxidation of STX to its fluorescent imino-purine is not well established; the method, however, is well established. Here we present LC-MS/MS and LC-MS/HRMS methods to detect the oxidation product of STX, which will aid the laboratories that do not have a confirmatory technique at present. The chromatography parameters for the separation of the oxidation product are well established and are based on reverse phased columns.

3. The combination of these two methods, the detection of native STX using HILIC LC-MS/MS, and the detection of the oxidation product of STX using reverse phase LC-MS/MS will satisfy the requirements for OPCW proficiency testing for a positive detection of STX. The methods described can be expanded for use to many other marine toxins.

4. Saxitoxin, one of the paralytic shellfish poisoning (PSP) toxins, is highly water soluble, and together with the various PSP toxins can be separated effectively using HILIC chromatography. This separation, coupled to MS/HRMS, creates a highly selective and sensitive method for the analysis of samples containing PSP toxins. Even at low ng/mL concentrations, full scan MS/HRMS spectra can be produced using the data independent scanning mode on the Orbitrap Q-Exactive Plus FTMS. This allows comparison to reference material mass spectra and provides a "data rich" method that is suitable as a primary identification technique for OPCW proficiency tests.

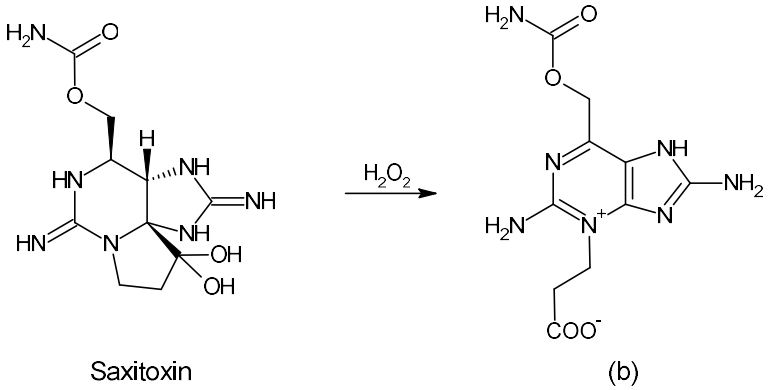
5. The probable structure of the fluorescent product from the oxidation of STX has been reported (Rapoport, 1975) (Quilliam MA, 1993) with the latter publication correcting the earlier. The structure initially was reported as 8-amino-6-hydroxymethyl-2-iminopurine-3(2H)-propionic acid (Figure 1, structure (a) below).

FIGURE 1: STRUCTURE OF THE STX OXIDATION PRODUCT (a) PROPOSED BY RAPOPORT *ET AL* (1)



6. However, subsequent analysis by Quilliaam *et al* (2), using LC-MS techniques in the 1990s, revealed that the initial oxidation product is similar to that reported by Rapoport, except that the carbamoyl group is retained (Figure 2, structure (b)).

FIGURE 2: STRUCTURE OF THE STX PRODUCT (b) PROPOSED BY QUILLIAM AND OBSERVED BY OPCW LABORATORY



7. The second chemical (b) has been observed in the OPCW Laboratory as the major oxidation product of STX using the AOAC method (2005.06) for the oxidation of STX using hydrogen peroxide. Again, using a DIA scanning mode and the same Orbitrap instrument coupled to reverse phased chromatography, the oxidation product can be

isolated and detected at low ng/mL concentrations. We have confirmed this using an inline fluorescence detector placed between the UPLC column and the mass spectrometer.

MATERIALS AND METHODS

8. Saxitoxin was obtained from Cifga Laboratories (Spain) as a certified reference material (CRM); the concentration of STX was 20.3 ± 1.3 µg/mL. Hydrogen peroxide solution (30%) and acetonitrile were obtained from Carl Roth GmbH (Germany). Formic acid, glacial acetic acid, and ammonium formate were obtained from Sigma-Aldrich (the Netherlands).

9. Standard solutions of STX were prepared in 0.1 N HCl solution. A stock solution was prepared by diluting a 200 µL aliquot of the CRM to 25 mL with 0.1 N HCl to make a 132 ng.mL solution (concentration corrected for STX free base), this stock solution was then diluted 1:5, 1:20, 1:50 and 1:100 (26.4, 6.6, 2.6 and 1.3 ng/mL respectively) to prepare a standard curve.

10. The oxidation of STX was carried out according to the AOAC procedure, briefly, 250 µL of a 0.1 N NaOH solution was mixed with 20 µL of a 10% H_2O_2 solution, 100 µL of the analyte was added and the mixture vortexed briefly and allowed to react for two minutes at room temperature before the addition of 20 µL of glacial acetic acid.

11. HILIC chromatography for the separation of native STX was carried out using the conditions listed in Table 1 below.

TABLE 1: HILIC CHROMATOGRAPHY CONDITIONS USED FOR THE ANALYSIS OF STX

Column brand/Phase	Merck SeQuant ZIC-HILIC
Column length x ID x particle size	150 x 1 mm x 200 Å
Column temperature (°C)	30
Eluent A composition	2 mM ammonium formate, 2 mM formic acid (aqueous)
Eluent B composition	95% acetonitrile, 2 mM ammonium formate, 2 mM formic acid
Gradient program	0-2 min 100%B; 2-15 min 100-33%B; 15-16 min 33%B; 16-17 min 33-100%B; 17-22 min 100%B
Flow rate (µL/min)	400
Injection volume (µL)	5

12. Reverse phase chromatography conditions for the separation of the oxidation product of STX are listed in Table 2 below.

TABLE 2: REVERSE PHASE CHROMATOGRAPHY CONDITIONS USED FOR THE ANALYSIS OF STX

Column brand/Phase	Thermo Vanquish C18+
Column length x ID x particle size	50 x 2.1 mm x 1 µm
Column temperature (°C)	35
Eluent A composition	100 mM ammonium formate adjusted to pH 6 with 0.1 N HOAc
Eluent B composition	100 mM ammonium formate, 5% ACN adjusted to pH 6 with 0.1 N HOAc.
Gradient program	0-5 min 0-5 %B; 5-9 min 5-30%B; 9-11 min 30-70%B; 11-11.5 min 70-0%B; 11.5-15 min 0%B
Flow rate (µL/min)	500
Injection volume (µL)	2

13. The mass spectrometer method parameters are presented in Tables 3, 4, and 5 below.

TABLE 3: MS CONDITIONS USED FOR THE ANALYSIS OF NATIVE STX AND THE OXIDATION PRODUCT OF STX

Ionisation mode	ESI
Polarity	+ve
Acquisition mode	MS/MS - Product ion spectrum
Acquisition parameters (mass range, mass resolution, ions, transitions, etc.)	Scan range 50-750 m/z; resolution 70000
Electrospray/APCI voltage (kV)	3 kV
Type of MS/MS scan	DIA
Scan range	100-above precursor (instrument determined) m/z
Scan time	Instrument set
MS/MS conditions (collision gas, pressures, collision energy (eV) etc.)	See inclusion list
Comments	Inclusion list required for DIA scan mode, see Tables 4 and 5

TABLE 4: INCLUSION LIST FOR DATA INDEPENDENT ANALYSIS OF NATIVE STX

Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	(N)CE	(N)CE type	Comment
300.14148	C10H17N7O4	"+ H"	1	Positive	35	NCE	STX

TABLE 5: INCLUSION LIST FOR DATA INDEPENDENT ANALYSIS OF THE OXIDATION PRODUCT OF STX

Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	(N)CE	(N)CE type	Comment
296.11018	C10H13N7O4	"+ H"	1	Positive	25	NCE	STX

RESULTS

Native STX analysis

14. Native STX is retained well on the ZIC-HILIC column with a retention time well separated from all the other PSP toxins (Neo-STX, dcSTX, GTX1, GTX2, GTX3, GTX4, GTX5 and GTX6 were included in the calibration mix). Extracted ion chromatograms were generated from the MS/HRMS spectrum for the fragment ions m/z 282.13091, 204.08799, and 221.11454 of the m/z 300.14148 precursor ion. The mass spectrum for the peak was combined and background subtracted (before and after the peak). This method was used for the Eurobiotox STX exercise and the results from one of the samples (Sample STX PT2 03, mussel sample) are presented in Figures 3 and 4 below.

FIGURE 3: LC-MS/MS CHROMATOGRAMS SUPPORTING IDENTIFICATION OF SAXITOXIN IN SAMPLE EUROBIOTOX STX PT2. EIC OF m/z 282.13091 GENERATED FROM PRECURSOR m/z 300.14148 FOR REFERENCE (UPPER) AND SAMPLE (LOWER)

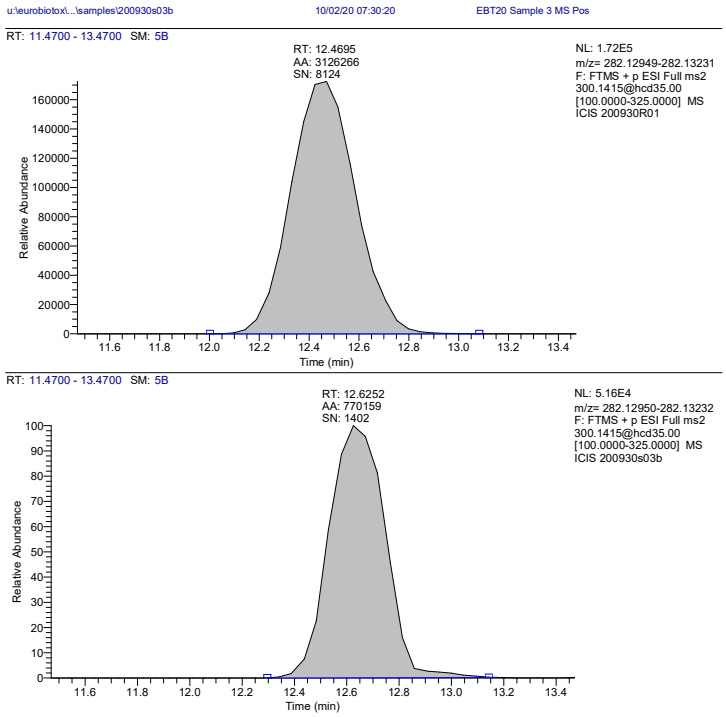
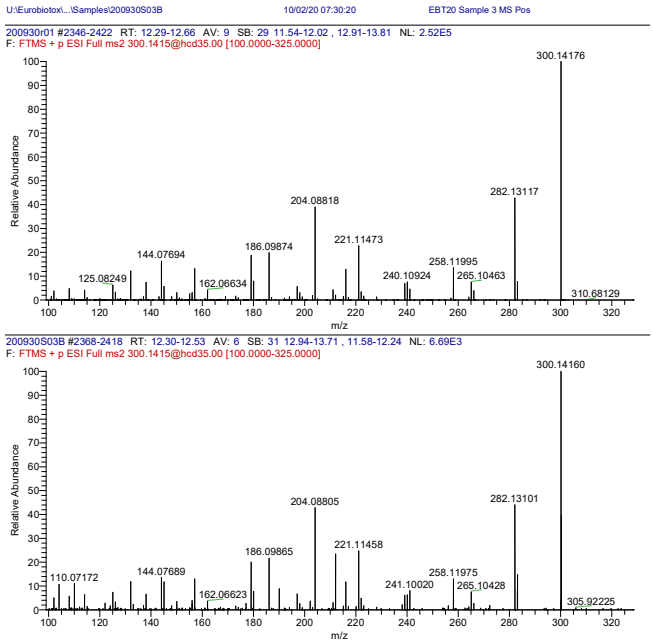
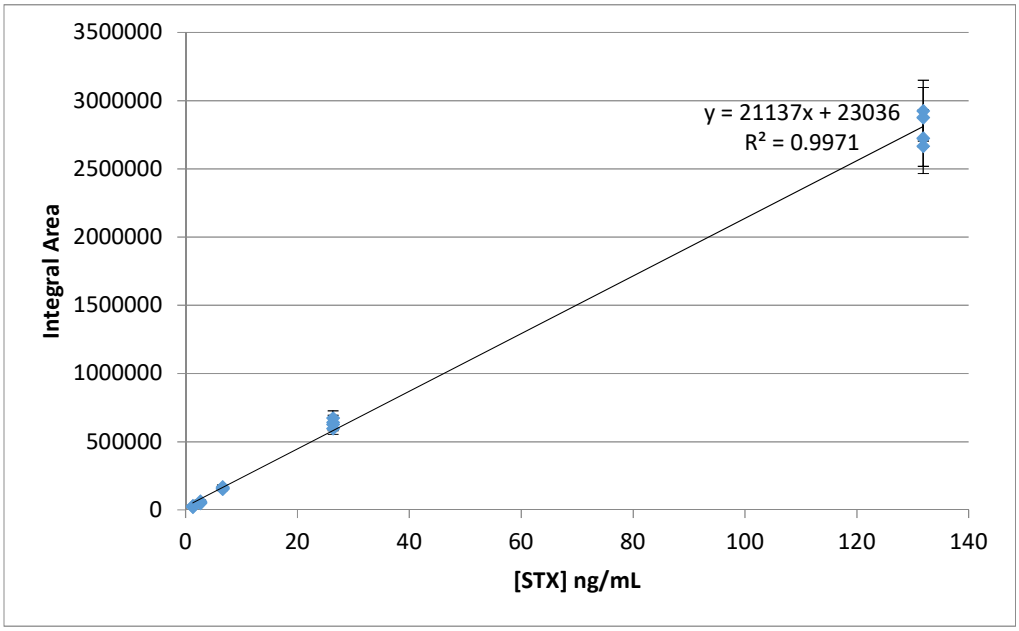


FIGURE 4: FRAGMENT ION MASS SPECTRA OF THE 300.1415 m/z PRECURSOR ION FOR REFERENCE (UPPER) AND SAMPLE (LOWER)



15. A calibration curve was created from the series of standards (Figure 5 below).

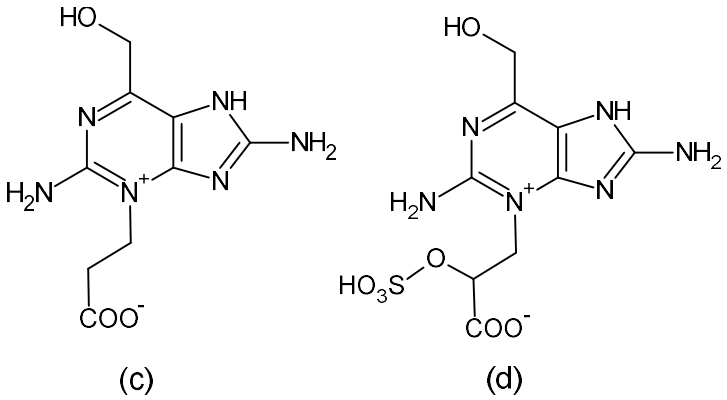
FIGURE 5: CALIBRATION CURVE FOR THE QUANTITATION OF STX



Oxidised STX analysis

16. The oxidised form of STX was retained well on the C18+ column with a retention time separated from the other PSP toxins. Neo-STX and dcSTX form the same oxidation product (c); similarly GTX1, GTX2, GTX3 and GTX4 also form the same oxidation product (d). All were included in the calibration mix.

FIGURE 6: STRUCTURES OF THE OXIDATION PRODUCT OF NEO-STX AND DCSTX (c) AND GTX1, GTX2, GTX3, AND GTX4 (d)



17. Extracted ion chromatograms were generated from the MS/HRMS spectrum for the m/z 235.10437 fragment ion from the m/z 296.11018 precursor ion (Figures 7 and 8 below). The mass spectrum for the peak was combined and background subtracted (before and after the peak) (Figures 9 and 10 below).

FIGURE 7: EIC CHROMATOGRAM OF THE m/z 235.10437 PRODUCT ION FROM THE m/z 296.11018 PRECURSOR ION FOR REFERENCE STX OXIDISED PRODUCT (UPPER) AND FLUORESCENCE CHROMATOGRAM OF SAME SAMPLE

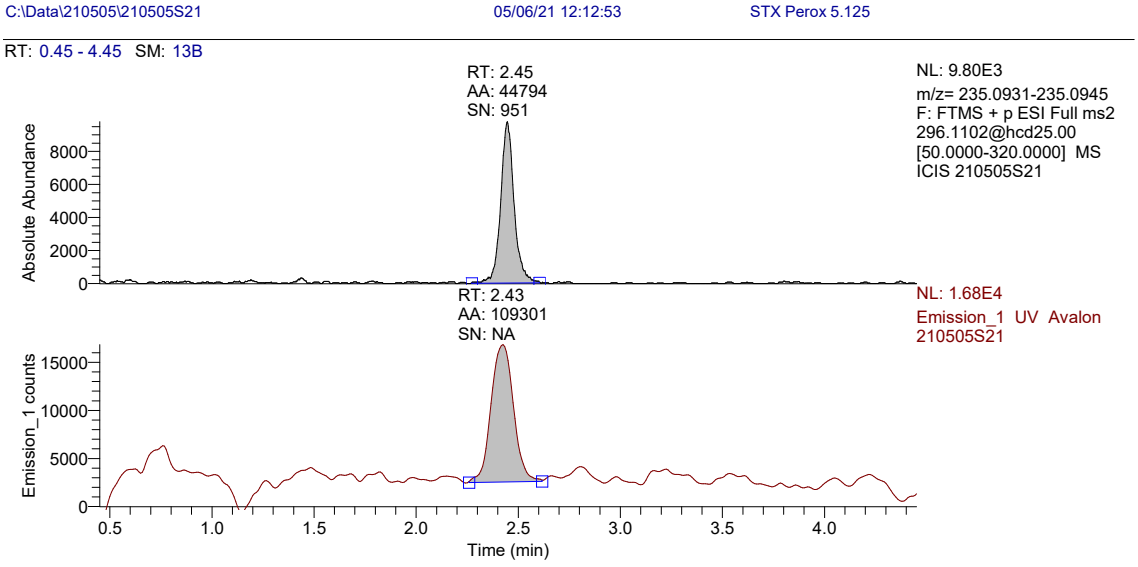


FIGURE 8: EIC CHROMATOGRAM OF THE m/z 235.10437 PRODUCT ION FROM THE m/z 296.11018 PRECURSOR ION FOR SAMPLE BT20/PL05 FROM THE 2020 OPCW BIOTOXIN EXERCISE OXIDISED PRODUCT (UPPER) AND FLUORESCENCE CHROMATOGRAM OF SAME SAMPLE

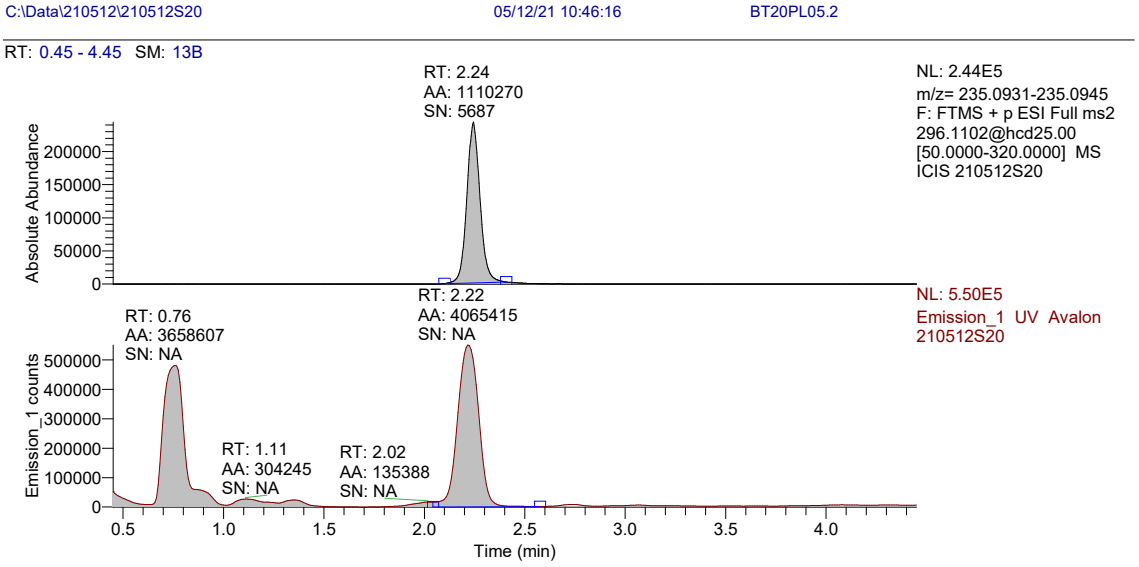


FIGURE 9: MS/HRMS OF THE m/z 296.11018 PRECURSOR ION FOR THE REFERENCE SAMPLE

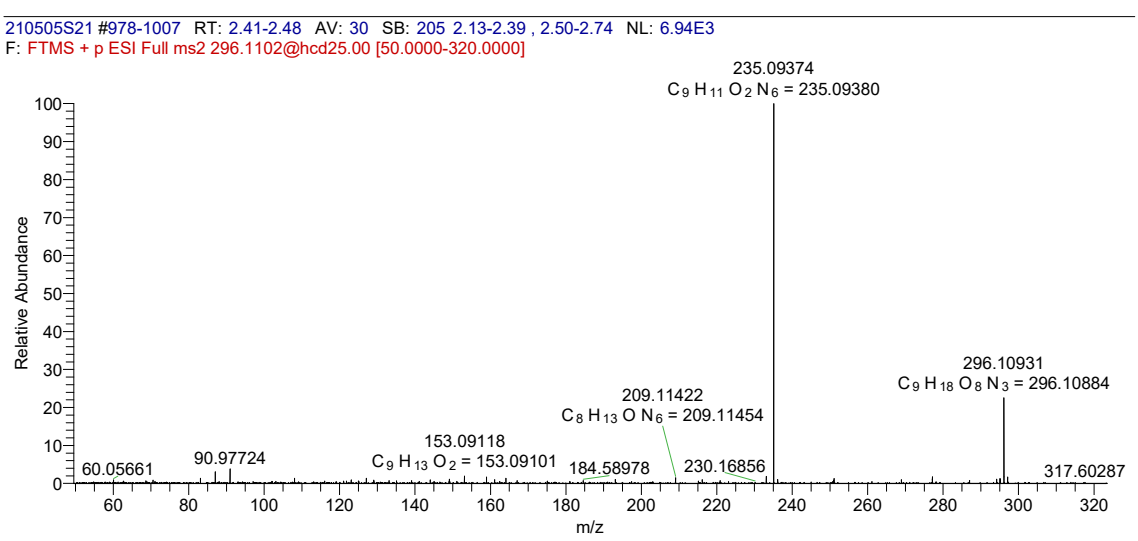
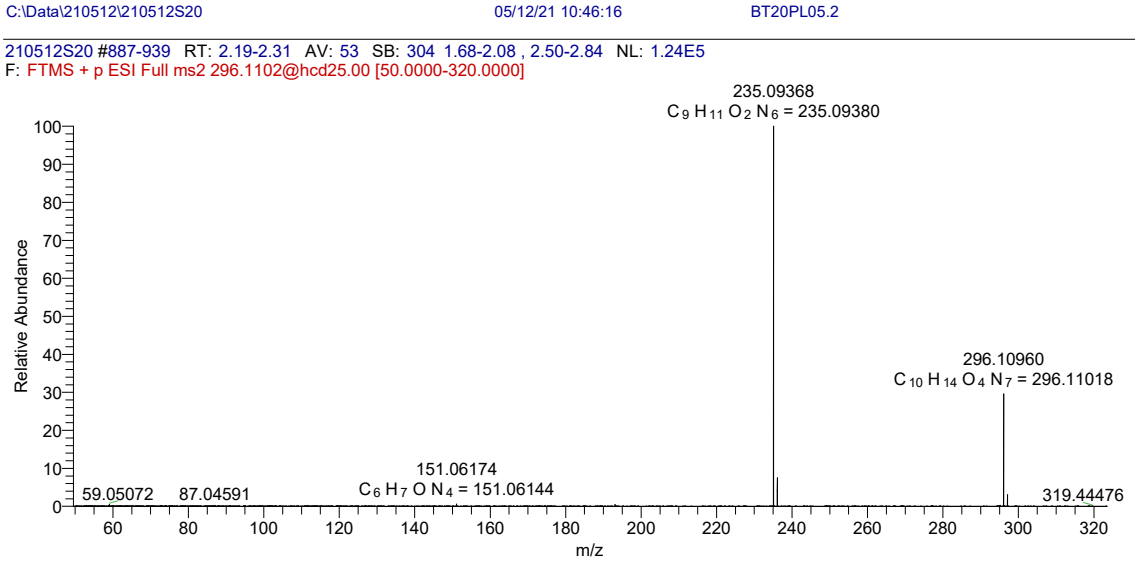


FIGURE 10: MS/HRMS OF THE M/Z 296.11018 PRECURSOR ION FOR THE 2020 BIOTOXIN EXERCISE SAMPLE



DISCUSSION AND CONCLUSION

18. The application of the HILIC method utilising DIA as an acquisition mode provides laboratories with another method for the detection of PSP toxins. The OPCW Laboratory has also performed the analysis with several other PSP toxins (see comment column in Table 6), many of which cannot be uniquely distinguished using LC-FLD. Shown in Table 6 is the acquisition inclusion list for both positive and negative ion polarity for the PSP toxins. Shown in Table 7 are the EIC parameters that were used to detect and quantify these toxins.

19. The HILIC-MS/HRMS method described in this Note will fulfil the reporting criteria as a primary technique for the native PSP toxins. The C18-MS/HRMS method will fulfil the reporting criteria as a secondary method for STX; further experiments are needed before this secondary method is accepted for the other PSP toxins.

TABLE 6: ACQUISITION PARAMETERS FOR THE INCLUSION LIST FOR PSP TOXINS USING A DIA METHOD

Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	(N)CE	(N)CE type	Comment
300.14148	C10H17N7O4	+ H	1	Positive	35	NCE	STX
396.09321	C10H17N7O8S	+ H	1	Positive	25	NCE	GTX3 & GTX6
412.08812	C10H17N7O9S	+ H	1	Positive	25	NCE	GTX4
257.13566	C9H16N6O3	+ H	1	Positive	40	NCE	dcSTX
316.13639	C10H17N7O5	+ H	1	Positive	40	NCE	NEO & GTX2
380.09829	C10H17N7O7S	+ H	1	Positive	25	NCE	GTX5
332.13131	C10H17N7O6	+ H	1	Positive	40	NCE	GTX1
273.13058	C9H16N6O4	+ H	1	Positive	35	NCE	dcNEO

Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	(N)CE	(N)CE type	Comment
394.07865	C10H17N7O8S	-H	1	Negative	25	NCE	GTX2, GTX3 & GTX6
410.07357	C10H17N7O9S	-H	1	Negative	25	NCE	GTX4 & GTX1
378.08374	C10H17N7O7S	-H	1	Negative	25	NCE	GTX5

TABLE 7: EIC PARAMETERS FOR DETECTION AND QUANTITATION OF PSP TOXINS

Positive Ion					
	RT	**MH+**	**Quant**	**Qual**	**Qual2**
STX	**12.42**	300.14148	282.13091	204.08799	221.11454
NEO	**12.37**	316.13639	298.12583	225.10945	126.06619
dcSTX	**12.74**	257.12566	126.06619	239.12510	222.09855
dcNEO	**12.58**	273.13058	255.12001	225.10945	126.06653
GTX2	**9.50**	316.13639	220.08290	148.07167	298.12583
GTX3	**10.87**	396.09320	298.12583	316.13639	378.08264
GTX4	**10.66**	412.08810	314.12086	332.13131	394.07756
GTX5	**11.62**	380.09830	300.14148	282.13091	221.11454
GTX6	**11.88**	396.09320	316.13639	298.12583	187.08257
Negative Ion					
	RT	**M-H-**	**Quant**	**Qual**	**Qual2**
GTX1	**9.40**	410.07360	349.05609	367.06666	269.10037
GTX2	**9.49**	394.07860	351.07174	333.06118	376.06699
GTX3	**10.84**	394.07860	333.06118	351.07174	376.06699
GTX4	**10.65**	410.07360	367.06666	349.05609	274.02541
GTX5	**11.63**	378.08370	121.95425	360.07315	280.11526
GTX6	**11.88**	394.07860	121.95425	376.06699	196.98628

REFERENCES

Quilliam MA, J. M. (1993, June). Characterization of the oxidation products of paralytic shellfish poisoning toxins by liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom, 7*(6). doi:10.1002/rcm.1290070616

Rapoport, H. A. (1975). Chemical assay for saxitoxin, the paralytic shellfish poison. *J. Agric. Food Chem, 23*(2), 237-239.

Annex: List of Acronyms

Annex

LIST OF ACRONYMS

AOAC	Association of Official Analytical Chemists
APCI	atmospheric pressure chemical ionisation
CRM	certified reference material
dcSTX	decarbamoyl saxitoxin
DIA	data-independent acquisition
EIC	extracted ion chromatogram
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
FTMS	Fourier transform mass spectrometer
GTX	gonyautoxin
HILIC	hydrophobic interaction liquid chromatography
HOAc	acetic acid
LC-FLD	liquid chromatography fluorescence detection
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
m/z	mass to charge ratio
MS/HRMS	mass spectrometry/high resolution mass spectrometry
MS/MS	tandem mass spectrometry
Neo-STX	neo saxitoxin
OPCW	Organisation for the Prohibition of Chemical Weapons
PSP	paralytic shellfish poisoning
STX	saxitoxin
UPLC	ultra performance liquid chromatography

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