Application Note

Seafood Testing

Determination of Paralytic Shellfish Toxins and Tetrodotoxin in Shellfish using HILIC/MS/MS

Using an Agilent 1290 Infinity LC and an Agilent 6495 LC/MS system

Authors

Andrew Turner, Ph.D.
Centre for Environment, Fisheries and Aquaculture Science, UK

László Tölgyesi, Ph.D.
Agilent Technologies, Inc.

Abstract

Paralytic Shellfish Toxins (PST) are naturally occurring low molecular weight hydrophilic compounds belonging to the saxitoxin family. These potent neurotoxins are produced naturally by phytoplankton, and periodically accumulate in shellfish. This presents the potential of human intoxication, termed Paralytic Shellfish Poisoning (PSP), and the need for regulatory control of shell fishery products. The European Union (EU) reference method is based on precolumn oxidation with liquid chromatography and fluorescence detection. However, the method is multistage and complex, so an alternative approach using tandem mass spectrometric detection is desirable.

Tetrodotoxin (TTX) is another low molecular weight toxin thought to be produced by marine bacteria, which has been found to accumulate in bivalve shellfish.

This Application Note describes a method for analyzing these compounds in shellfish, using hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS). Equipment requirements, sample preparation, and method parameters are described, enabling users to set up the method quickly and easily. Method verification exercises are also provided with validated method performance characteristics. The method was found to provide acceptable levels of sensitivity, accuracy, precision, and reproducibility and to be suitable for routine analysis.

Introduction

PST are potent neurotoxins produced by several species of phytoplankton, and may bio-accumulate in filter-feeding shellfish.[1,2] Human consumption of contaminated shellfish can result in PSP. As a result, monitoring of PST in shellfish is a statutory requirement in many regions of the world, including the EU.[2,3] A regulatory action limit is defined globally as 800 µg saxitoxin equivalents per kg of shellfish tissue (µg STX eq/kg). For decades, the official EU reference method for quantifying PST in bivalve shellfish was the mouse bioassay (MBA).[4,5] However, from 1 January 2019, the reference method became AOAC 2005.06, which uses precolumn oxidation with liquid chromatography and fluorescence detection (LC/FLD).[6] Determination of toxicity in shellfish using chemical detection methods is challenging due to their highly polar hydrophilic nature, the large number of structurally similar congeners, and the differences in toxicity between analogs. Methods employing FLD in particular, are complex, requiring multiple analyses per sample, various clean-up steps, and even then, some PST analogs cannot be detected. Therefore, there is a need to implement simple, rapid one-shot analytical methods, which are more suitable for routine, high-throughput analytical laboratories.

Tetrodotoxin (TTX) is produced by certain bacterial species, and while traditionally associated with pufferfish poisoning in tropical waters, has recently been detected in European bivalve mollusks as well.[7] While TTX is not yet stipulated in the EU regulatory requirements, it is known to be as toxic as some analogs of PST. Therefore, monitoring TTX along with other marine biotoxins in shellfish could further protect public health. However, current methodologies used in control labs (including LC/FLD) are not suitable for its detection, and these labs do not have spare resources to add a further test. A method that can detect all PSTs and TTX together in one analytical method would be advantageous for monitoring the risk from all these hydrophilic marine toxins.

A method for shellfish analysis was developed for 25 PSTs and TTX combined using HILIC/MS/MS analysis following a rapid one-step extraction and simple desalting cleanup procedure.[8] The method was then subjected to full single-laboratory validation.[9,10] The use of UHPLC in HILIC mode coupled to tandem mass spectrometry (HILIC/MS/MS) has the potential to become the gold standard for analyses of these important toxins. This technique provides the necessary sensitivity, accuracy, reproducibility, and ruggedness while providing a rapid, simple, and cost-effective solution in comparison to other analytical detection methods.

Experimental

Instrumentation

Chromatographic separation of PST and TTX analytes for this method was performed using an Agilent 1290 Infinity II LC with a 20 µL injection loop. An Agilent 6495B triple quadrupole LC/MS with the iFunnel and Agilent Jet Stream technology was used as the detector. The analysis was performed using fast polarity switching mode with electrospray ionization. Data acquisition was performed using Agilent MassHunter Acquisition software (Version B.08.00), while data processing using Agilent MassHunter Quantitative and Qualitative Analysis software (Version 10.0).

The Agilent 1290 Infinity II LC comprised the following modules:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with 20 µL loop and 40 µL metering device, standard wash and cooler unit
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)

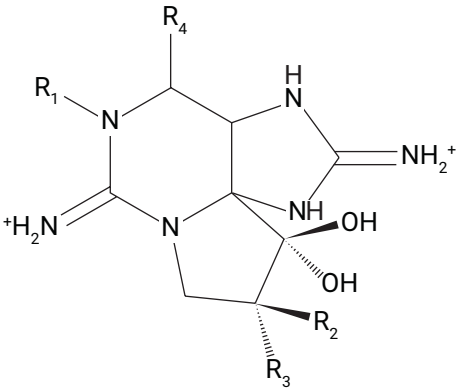
Other Equipment

- Boiling water bath
- Centrifuge, operating at 4,500 × g
- Vortex mixer
- Vacuum manifold for manual Solid Phase Extraction (SPE), if required
- Autopipettes
- Duran bottles
- Volumetric flasks for standards and samples
- 50 mL polypropylene centrifuge tubes
- 15 mL polypropylene centrifuge test tubes
- 1.5 mL polypropylene microcentrifuge tubes (optional)
- Polypropylene 700 µL autosampler vials
- Amide UHPLC column, 130 Å, 1.7 µm, 2.1 × 150 mm
- Amide precolumn, 130 Å, 1.7 µm, 2.1 mm × 5 mm
- Supelco Supelclean ENVI-Carb 250 mg/3 mL or equivalent carbon column
- Gilson Aspec XL-4 SPE liquid handler for automated carbon SPE cleanup processes (Optional for high-throughput labs).

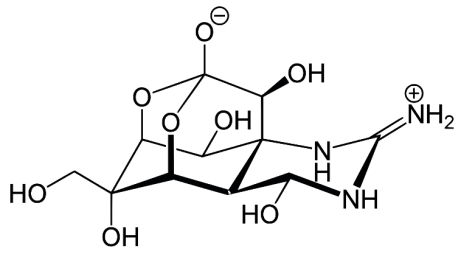
Note: Use of glassware should be avoided to prevent sample loss to surface interactions.

Standards

Primary standards were obtained from commercial suppliers at the Institute of Biotoxin Metrology at the National Research Council, Canada and from Cifga, Spain. From Canada, we sourced certified standards of STX di-HCl, NEO, GTX1, GTX4, GTX2, GTX3, GTX5,



PST toxins



Tetrodotoxin (TTX)

Figure 1. The general structure of PST analogs and TTX.

dcSTX, dcNEO, dcGTX2, dcGTX3, C1, C2, and GTX6. From Spain, we sourced a certified standard for TTX (Figure 1). From New Zealand (Cawthron Institute), we were also able to source other noncertified standards including, C3, C4, doSTX, dcGTX1, and dcGTX4. More information is included in references 8 and 9.

Equal volumes of primary toxin standard solutions (400 µL each) were mixed to create a mixed stock solution containing PST and TTX analytes. This stock was used to prepare matrix-modified standards and quality control samples, as described later.

Sample preparation

Shellfish extraction: 5.0 ±0.1 g homogenized shellfish tissue was weighed into a centrifuge tube followed by the addition of 5.0 mL 1% acetic acid. The mixture was vortex mixed for 90 seconds at 2,500 rpm, before being placed into a boiling water bath for five minutes. Samples were cooled for five minutes in cold running water, before further vortex mixing for 90 seconds (2,500 rpm). Samples were next centrifuged at 4,500 rpm for 10 minutes, then a 1.0 mL aliquot of supernatant was pipetted into a 5 mL polypropylene tube and 5 µL 25% ammonia added and vortex-mixed before cleanup.

Extract clean-up: SPE clean-up of acetic acid extracts was performed using Supelclean ENVI-Carb 250 mg/3 mL SPE cartridges. Cartridges were conditioned at 6 mL/min using 3 mL 20% MeCN + 0.25% acetic acid, before the addition of 3 mL 0.025% ammonia. 400 µL of acetic acid extract was loaded onto the cartridge at 3 mL/min, followed by a 3 mL/min wash with 700 µL de-ionized water to elute salts. After each step, an air push (200 to 400 µL) was used to ensure complete elution of each reagent. Sample extracts were eluted and collected with the addition of 2 mL 20% MeCN + 0.25% acetic acid at 3 mL/min. SPE eluants were vortex-mixed before dilution of 100 µL aliquots in 700 µL Verex polypropylene autosampler vials with 300 µL MeCN of LC/MS grade. SPE cartridges were recycled with the addition of 6 mL water followed by 6 mL methanol, both at 6 mL/min (Figure 2). While not essential, the cleanup process can be automated, processing batches of 80 extracts at a time using a standalone liquid handler.

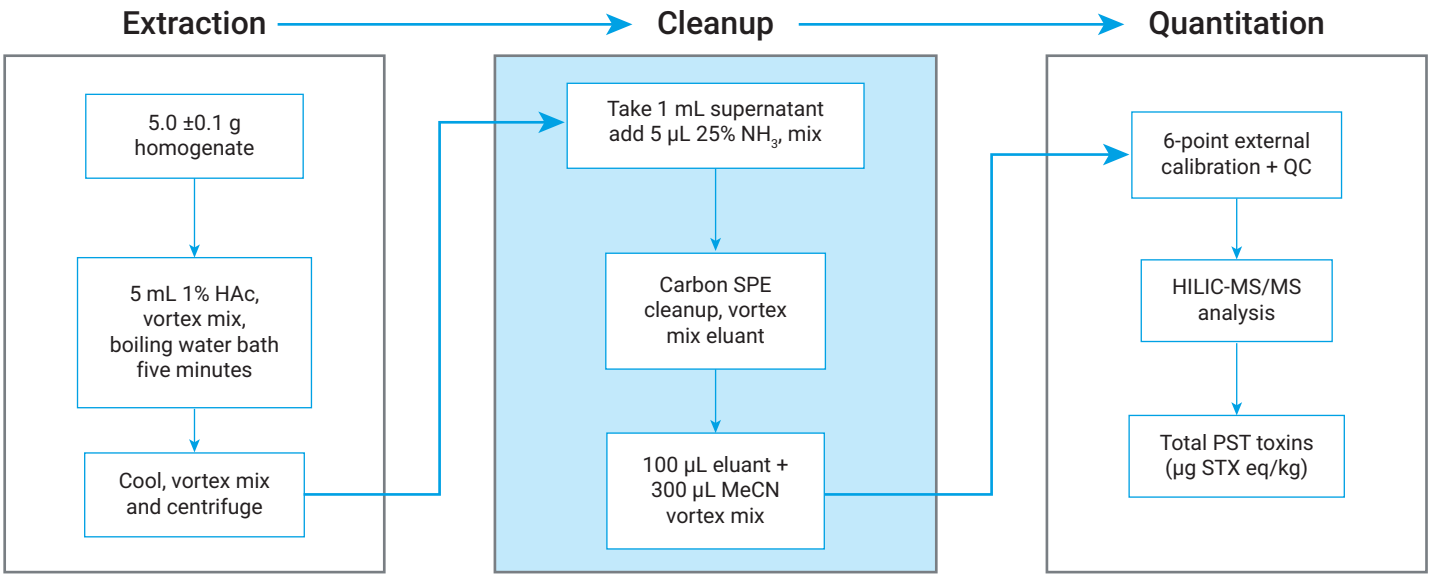


Figure 2. Flowchart illustrating the method procedure.

Table 1. Chemical structures, TEFs, and calibrants applied for quantitation and semiquantitation of PST analogs.

Group (Charge State)	Analog	R1	R2	R3	R4	Chemical Name	TEF	Calibrant
C toxins (0)	C1	H	H	OSO_3^-	$OCONHSO_3^-$	N21-sulfocarbamoyl-11α-hydroxysulfate-saxitoxin	0.01	C1
	C2	H	OSO_3^-	H	$OCONHSO_3^-$	N21-sulfocarbamoyl-11 β -hydroxysulfate-saxitoxin	0.1	C2
	C3	OH	H	OSO_3^-	$OCONHSO_3^-$	N21-sulfocarbamoyl-11α-hydroxysulfate-neosaxitoxin	0.02	C1
	C4	OH	OSO_3^-	H	OSO_3^-	N21-sulfocarbamoyl-11β-hydroxysulfate-neosaxitoxin	0.1	C2
GTXs (1)	dcGTX3	H	H	OSO_3^-	OH	Decarbamoyl-11α-hydroxysulfate-saxitoxin	0.2	dcGTX3
	dcGTX2	H	OSO_3^-	H	OH	Decarbamoyl-11β-hydroxysulfate-saxitoxin	0.4	dcGTX2
	dcGTX1	OH	H	OSO_3^-	OH	Decarbamoyl-11α-hydroxysulfate-neosaxitoxin	0.5[a]	dcGTX2
	dcGTX4	OH	OSO_3^-	H	OH	Decarbamoyl-11β-hydroxysulfate-neosaxitoxin	0.5[a]	dcGTX3
	GTX2	H	H	OSO_3^-	$OCONH_2$	11α-hydroxysulfate-saxitoxin	0.4	GTX2
	GTX3	H	OSO_3^-	H	$OCONH_2$	11β-hydroxysulfate-saxitoxin	0.6	GTX3
	GTX1	OH	H	OSO_3^-	$OCONH_2$	11α-hydroxysulfate-neosaxitoxin	1	GTX1
	GTX4	OH	OSO_3^-	H	$OCONH_2$	11β-hydroxysulfate-neosaxitoxin	0.7	GTX4
	GTX5	H	H	H	$OCONHSO_3^-$	N-sulfocarbamoyl-saxitoxin	0.1	GTX5
	GTX6	OH	H	H	$OCONHSO_3^-$	N-sulfocarbamoyl-neosaxitoxin	0.1	GTX6
STXs (2)	doSTX	H	H	H	H	Deoxydecarbamoyl-saxitoxin	0.05[b]	doSTX
	dcSTX	H	H	H	OH	Decarbamoyl-saxitoxin	1	dcSTX
	dcNEO	OH	H	H	OH	Decarbamoyl-neosaxitoxin	0.4	dcNEO
	STX	H	H	H	$OCONH_2$	Saxitoxin	1	STX
	NEO	OH	H	H	$OCONH_2$	N1-hydroxy-saxitoxin (Neosaxitoxin)	1	NEO

[a] dcGTX1 and dcGTX4 based on assumed toxicity equivalency factors[11]

[b] doSTX toxicity equivalency factor[12]

Calibration standard and quality control samples

Instrumental calibrations were prepared with successive dilutions of the mixed stock solution. The matrix used for calibrant preparation was an extract of a toxin-free crude mussel, which was extracted and cleaned up according to the sample preparation section. A six-point calibration from working standards was used with each sample run for PST and TTX quantitation. Working standards were stored for a maximum of one week under refrigerated conditions (4 °C), because evaporation can otherwise take place out of the vial as well as degradation in the targets of interest. For this reason, standards and samples also needed to be kept cool while waiting in the autosampler; thus, the cooler unit provided with the 1290 Infinity II was essential to successful analysis.

Instrument conditions

Reagents:

- Acetonitrile (MeCN), LC/MS grade
- Methanol (MeOH), LC/MS grade
- Water, de-ionized 18.2 MΩ.cm (Milli-Q), or LC/MS grade
- Formic acid 98 to 100%
- Glacial acetic acid (HAc)
- Ammonium hydroxide LC/MS additive (25% as NH_3)

Mobile phases:

- **Mobile Phase A1:** 500 mL water + 75 µL formic acid + 300 µL ammonium hydroxide. Mix well between and after formic acid and ammonium hydroxide additions. Store at room temperature, shelf life 24 hours.
- **Mobile Phase B1:** 700 mL acetonitrile + 300 mL water + 100 µL formic acid. Mix well. Store at room temperature for three months.
- **Mobile Phase A2:** 200 mL water + 1 mL formic acid. Mix well, store at room temperature for one week.
- **Mobile Phase B2:** Methanol. Store at room temperature for one month.
- **Seal Wash:** 5% isopropyl alcohol + 95% water. Mix well, store at room temperature for one month.

LC methods: New columns require preconditioning before their first use; this is achieved through an automated sequence of methods. The first requirement is to flush the column for approximately 60 column volumes with mobile phase A1 while heating to 60 °C to minimize solvent viscosity. Flow rates were kept low to ensure that the column and system pressures were not exceeded (Table 2). This is followed immediately by the shutdown (Table 3), then start up method (Table 4), before proceeding with LC analysis (Table 5) of blanks, standards, and samples. Finally, the shutdown method is applied once more. For established columns, an automated method sequence can be started directly from the start up method.

MS method: MS/MS acquisition methods were set-up using the source conditions and specific MRM transitions as summarized in Tables 6 and 7. Positive mode (ESI+) transitions were used exclusively for STX, NEO, dcSTX, dcNEO, doSTX, and TTX. Negative mode (ESI-) transitions were used exclusively for GTX1, GTX2, dcGTX2, dcGTX1, and C1 (α-epimers). For the remaining analogs (GTX3, GTX4, GTX5, GTX6, dcGTX3, dcGTX4, C2, C3, and C4) a mix of positive and negative MRMs were used. Primary (quantitative) MRMs were those indicated in bold in Table 7. Sodium formate clusters were monitored using the selected ESI- transitions, to provide an excellent indication of chromatographic separation of salts from the early eluting C toxins.

Table 2. Column conditioning method (column at 60 °C).

Time (min)	Flow Rate (mL/min)*	% A1	% B1
Initial	0.100	100.0	0.0
1.50	0.200	100.0	0.0
3.00	0.300	100.0	0.0
4.00	0.350	100.0	0.0
30.00	0.350	100.0	0.0

*If the column has already been conditioned, then flow rate can be set to 0.35 mL/min throughout the run.

Table 3. UHPLC shutdown LC method.

Time (min)	Flow Rate (mL/min)	% A2	% B2
Initial	0.300	100.0	0.0
4.00	0.300	100.0	0.0
8.00	0.300	0.0	100.0
9.00	0.300	0.0	100.0
11.00	0.600	0.0	100.0
15.00	0.600	0.0	100.0

Table 4. UHPLC start-up LC method (column at 60 °C).

Time (min)	Flow Rate (mL/min)	% A1	% B1
Initial	0.300	50.0	50.0
4.00	0.300	50.0	50.0
6.00	0.500	50.0	50.0
15.00	0.500	50.0	50.0
16.00	0.500	2.0	98.0
17.00	0.400	2.0	98.0
17.5	0.400	2.0	98.0

Table 5. UHPLC LC method (column at 60 °C).

Time (min)	Flow Rate (mL/min)	% A1	% B1
Initial	0.400	2.0	98.0
5.00	0.400	2.0	98.0
7.50	0.400	50.0	50.0
9.00	0.500	50.0	50.0
9.50	0.500	2.0	98.0
10.0	0.800	2.0	98.0
10.60	0.800	2.0	98.0
10.61	0.400	2.0	98.0
11.00	0.400	2.0	98.0

For full quantitative acquisition of PST/TTX analogs using dynamic MRM, Table 7 summarizes the MRM transitions and associated collision energies for each target analyte.

Preparing for analysis

With HILIC chromatography, it is good practice to perform a scouting run at the beginning of each day or batch to verify that the retention times are well established. Thus, a simplified dynamic MRM method was used incorporating a lower number of MRM transitions with acquisition windows running from one to nine minutes for all compounds (RT = 5.0 minutes, RT window = 8.0 minutes, Table 8).

Once RTs were established, the dynamic MRM method, containing all compound transitions, was used for calibration standard and sample analysis (Table 7).

Table 6. Optimized MS source conditions.

Parameter	Value
Detection	MS/MS
Ionization	Agilent Jet Stream Technology, electrospray, positive and negative ionization
Source Settings	
Drying Gas Temperature	150 °C
Drying Gas Flow	15 L/min
Nebulizer Pressure	50 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	2,500 V (positive), 2,500 V (negative)
Nozzle Voltage	0 V (positive), 0 V (negative)
High/Low Pressure Funnel RF (V)	150/80 V (positive), 150/80 V (negative)
Acquisition Settings	
Acquisition Mode	Dynamic MRM
Cycle Time	400 ms
Time Filter	0.04 minutes
ΔEMV	0 V (positive) | 0 V (negative)

Table 7. MRM transitions for dynamic MRM acquisition method. Transitions used for quantification (primary) are indicated in bold. MS1 Res = Unit, MS2 Res = Unit, cycle time = 400 ms, retention time (RT) window = one minute, cell accelerator voltage = 5 V.

Analog	ESI+ Transition (MRM1 and 2)	CE (MRM1, 2)	Approximate RT (min)	ESI- Transition (MRM1 and 2)	CE (MRM1, 2)
STX	**300.1 → 204.1**, 138.0	31, 24	7.8		
NEO	**316.1 → 126.1**, 298.1, 220.1	30, 20	7.8		
dcSTX	**257.1 → 126.1**, 222.0	20, 20	7.7		
dcNEO	**273.1 → 126.1**, 225.1	24, 18	7.7		
doSTX	**241.1 → 60.0**, 206.1	25, 20	7.5		
TTX	**320.1 → 302.1**, 162.1	28, 44	6.6		
GTX2			5.1	**394.1 → 351.1**, 333.1	20, 18
GTX3	396.1 → 298.1	16–20	5.9	**394.1 → 333.1**	22
GTX1			5.4	**410.1 → 367.1**, 349.1	15, 20
GTX4	412.1 → 314.1	18	6.4	**410.1 → 367.1**	15
GTX5	**380.1 → 300.1**	15	7.0	378.1 → 122.0	22
GTX6	**396.1 → 316.1**	12	7.3	394.1 → 122.0	24
dcGTX2			5.4	**351.1 → 164.0**, 333.1	22, 12
dcGTX3	**353.1 → 255.1**	15	6.4	351.1 → 333.1	18
dcGTX1			5.6	**367.1 → 274.1**, 349.1	20, 17
dcGTX4	**369.1 → 271.1**	20	6.6	367.1 → 349.1	16
C1			3.2	**474.1 → 122.0**, 351.1	38, 30
C2	**396.1 → 298.1**	15	3.7	474.1 → 122.0	38
C3	412.1 → 332.1	12	3.6	**490.1 → 410.1**	16
C4	**412.1 → 314.1**	14	4.3	490.1 → 392.1	20
Sodium*			2.9	452.7 → 133.0, 588.6 → 316.8	26

* Monitored as sodium formate clusters. For some CE voltages, a range of values is given. These are taken from the values obtained following optimization on three separate systems in three different laboratories.

Method verification

Matrix-modified standards and naturally incurred shellfish tissue sample extracts were used to verify the method performance.

Low-level standards prepared in matrix according to the sample preparation section were used to calculate limits of detection (LOD) and quantitation (LOQ), as well as assessing linearity and calibration range for each analyte.

Sample extracts containing each analyte were used to generate within-batch and between-batch repeatability. Naturally incurred shellfish tissue was used for the long-term (one year) assessment of within-laboratory reproducibility. A certified matrix reference material was used for the long-term (one year) assessment of trueness/accuracy. Figure 2 shows an example chromatogram from this study.

Table 8. MRM transitions for scouting dynamic MRM sample acquisition method used to determine RTs.

Analogs	ESI+ Transitions	CE	ESI- Transition	CE
STX, dcSTX, dcNEO	300.1 → 204.1	31		
NEO	316.1 → 126.1	30		
C1 and 2			474.1 → 122.0	38
C3 and 4			490.1 → 410.1	16
doSTX	241.1 → 60.0, 206.1	20–25, 20		
TTX	320.1 → 302.1	28		
GTX2 and 3			394.1 → 333.1	20
GTX1 and 4			410.1 → 367.1	15
dcGTX2 and 3			351.1 → 164.1	15
GTX5	380.1 → 300.1	15		
GTX6	396.1 → 316.1	12		
Sodium			588.6 → 316.8	26

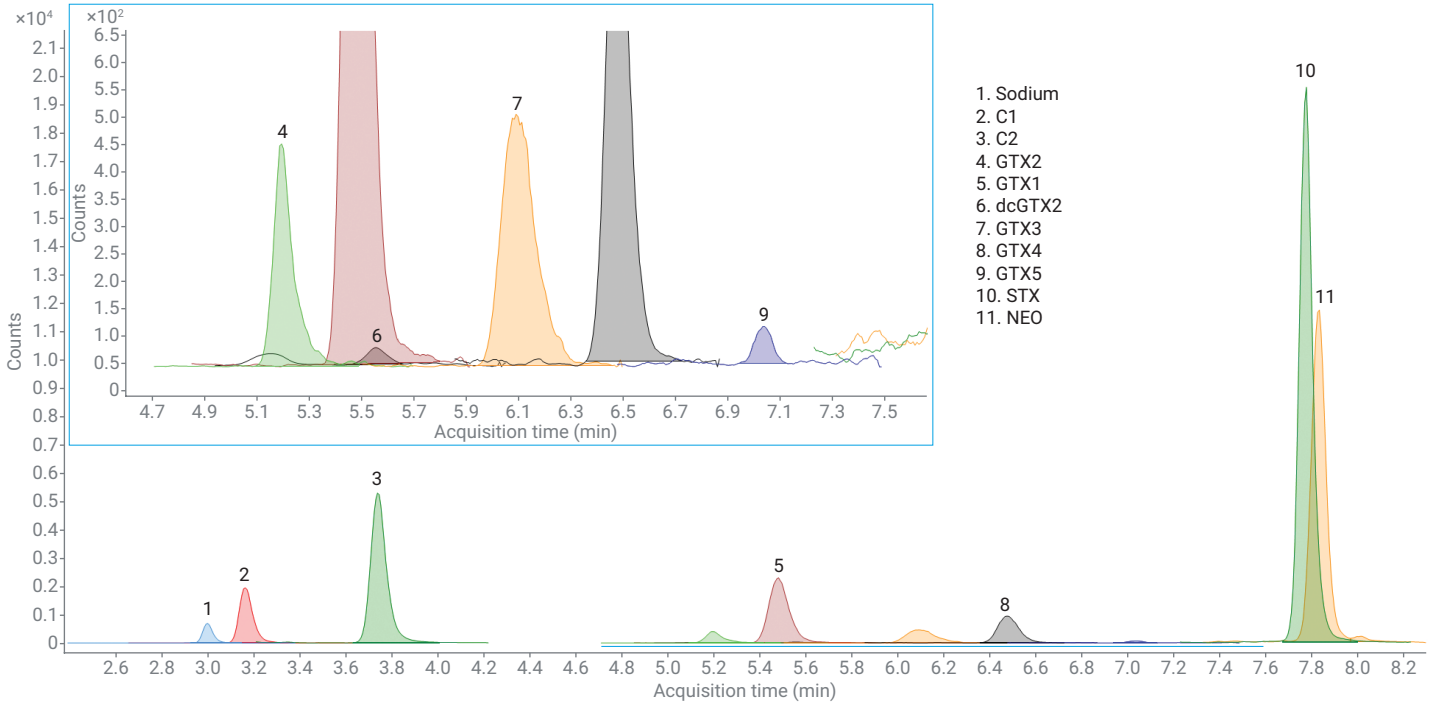


Figure 2. Representative quantifier MRM chromatograms obtained for a CRM sample (overlaid EIC representation without scaling).

Results and discussion

Compound selection and optimization

The analytes were selected for this method based on those assessed and validated previously[7,9] and available commercially as CRMs. MassHunter software was used in compound optimization mode to determine compound-specific parameters including precursor and product ions, together with optimum collision energies for each MRM transition to maximize compound-specific sensitivity. Initially, a minimum of three transitions were selected where possible. The number was then reduced to two once an assessment of relative intensity and potential matrix interferences had been conducted using shellfish matrix.

Optimization of the LC/MS method

There are two key chromatographic challenges that must be met when analyzing PSTs. First, there are several epimer pairs whose identical masses require chromatographic resolution. It is important that separation methods can resolve between epimeric pairs: C1 from C2, C3 from C4, GTX1 from GTX4, GTX2 from GTX3, dcGTX1 from dcGTX4, and dcGTX2 from dcGTX3 (Figure 3). Second, in-source fragmentation is known to occur on most commercially available MS systems when running in electrospray mode. This can lead to unknown PST analogs sharing MRM transitions with some of the targets of the method and so it is necessary to check for chromatographic resolution from such potential interferences when they are typical for a certain sample type.

Gaussian peak shapes were achievable for most toxins following proper column conditioning and start-up procedures. No additional prerun or postrun equilibration times were used. Figure 2 illustrates a representative extracted ion chromatogram obtained for a CRM sample.

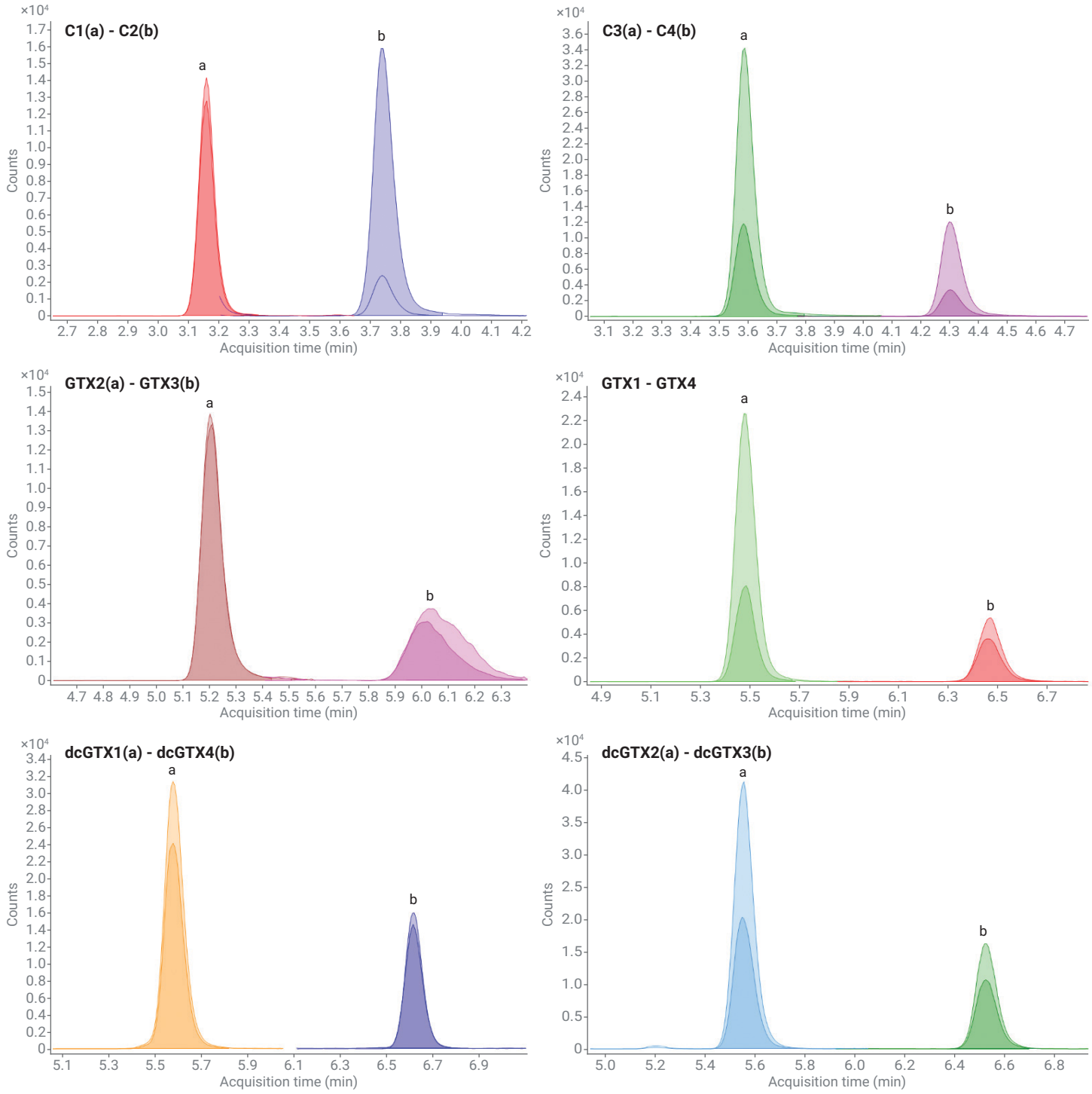


Figure 3. Separation between epimers.

Calibration

Matrix-modified calibration standards were analyzed to confirm linearity over a range of concentrations. The calibration ranges vary between analogs due to the differences in concentration of toxins provided in primary standards and differing responses in the MS source. In all instances, linearity was acceptable over the full calibration range (Table 9). Figure 4 shows some example calibration curves from the list of the studied toxin analytes.

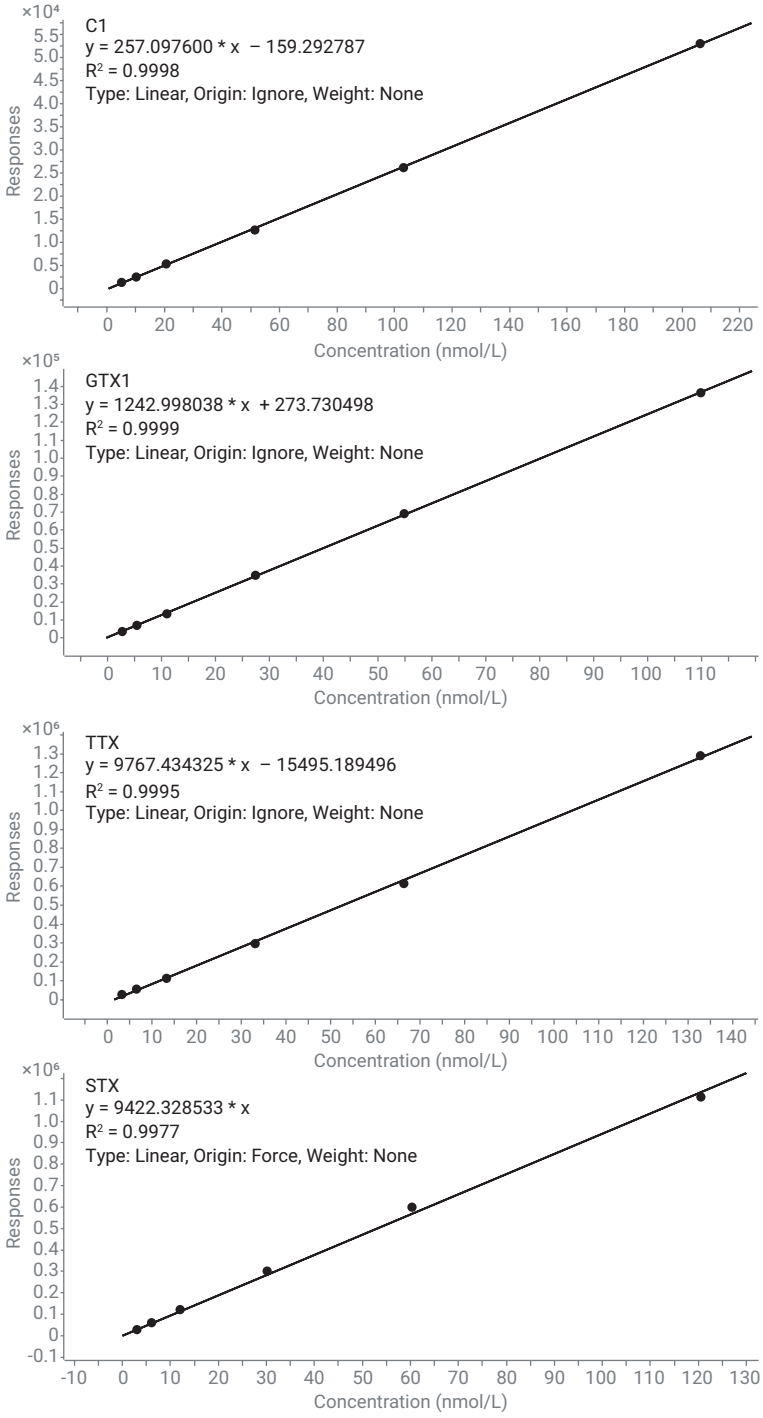


Figure 4. Example calibration curves for C1 (ESI- mode), GTX1 (ESI- mode), TTX (ESI+ mode), and STX (ESI+ mode).

Quantitation of PST

Equation 1 shows the calculation of analyte levels in samples, corrected for saxitoxin equivalent toxicity.

Quantitation can be conducted using this equation for all toxins available as CRMs. For new analogs or those unavailable as CRMs, quantitation should be conducted using the calibration generated from the most relevant analog(s). For example, in the absence of calibration standards for C3 and C4, quantitation for these analogs is recommended using C1 and C2 respectively. Toxicity equivalency factors (TEF) were taken from Turner *et al.* 2015, in turn, based primarily on those recommended by EFSA, 2009.[5]

$$Conc_{\mu g\ STX.2HCl\ eq/kg} = \frac{Conc_{nmol/L} \times M_{STX} \times DF \times TEF}{1000}$$

Equation 1.

Where:

$Conc_{nmol/L}$	Measured concentration of sample extract in nmol/L
$Conc_{\mu g\ STX.2HCl\ eq/kg}$	Saxitoxin dihydrochloride equivalents in sample homogenate in µg STX.2HCl eq/kg
M_{STX}	Molecular weight of saxitoxin dihydrochloride salt (372.4)
DF	Dilution factor of shellfish homogenate to diluted SPE eluent for injection (40)
TEF	Molar toxicity equivalency factor

Specificity

Injections of PST-free SPE-cleaned and acetonitrile-diluted shellfish extract were performed in triplicate for a range of bivalve shellfish species. No MRM transition peaks were observed at the expected retention times for any of the PST analogs.

LOD/LOQ

Repeat analysis (n = 6) of matrix-matched standards enabled the measurement of signal-to-noise ratios (S/N) of primary MRM peaks and the subsequent calculation of LOD and LOQ by extrapolation. LODs for each analyte were calculated based on the concentration of toxin (nmol/L) giving rise to an MRM chromatographic peak with S/N = 3.0. The LOQ was calculated based on the concentration of toxin (nmol/L) giving rise to an MRM chromatographic peak with S/N = 10.0. LODs ranged from 0.02 to 0.37 nmol/L depending on the analyte, with LOQs ranging from 0.08 to 1.22 nmol/L (Table 8).

When calculated in terms of saxitoxin equivalence, these equate to a LOD of 0.005 to 3.4 µg STX eq/kg (mean 0.85) and a LOQ of 0.02 to 11.4 µg STX eq/kg (mean = 2.85), thereby providing a highly sensitive detection method for each of the PST analogs.

Precision

The within-batch repeatability of the method was assessed with the repeat analysis (n = 42) of a quality control sample containing each toxin analog in one analytical sequence. Relative standard deviations (RSD) calculated from data ranged from 4 to 17% (mean = 9%) showing good precision for the method in matrix over a long analytical sequence. The higher RSD values related to analogs where concentrations were low, such as dcGTX4, GTX6, and dcSTX).

Between-batch repeatability was assessed over one week, with the repeat analysis of the same quality control sample (n = 20). Repeatability varied from 8 to 23% (mean = 13%) showing that the repeatability of method between batches over one week was acceptable.

Full within-laboratory method reproducibility was assessed with the repeated extraction, clean-up, and analysis of a PST-positive quality control shellfish sample over a period of one year (n = 31). The naturally incurred sample contained only those PST congeners present naturally within the shellfish tissue. Reproducibility varied between 16 and 27% (mean = 21%), with the higher variabilities associated with analogs present at low concentrations (for example GTX5). Overall, the data highlights good reproducibility of the method over the long term.

Trueness/accuracy

A commercial certified oyster tissue reference material supplied by Cefas consisting of known concentrations of C1 and C2, GTX1-4, NEO, and STX in Pacific oyster tissue was used for the assessment of method trueness. The tissue reference material was extracted, cleaned-up, and analyzed 33 times over a one-year period. The accuracy of the PST concentrations generated for each of the toxins varied from 86 to 116% (mean 101%) (Table 9). The mean total sample toxicity determined from the repeat analysis was 645 µg STX eq/kg, which compared well with the certified total sample toxicity of 668 µg STX eq/kg, demonstrating excellent accuracy of the method for the analysis of the material, over time.

Quality control

HILIC is a sensitive form of chromatography, particularly in comparison to reversed-phase LC, and chromatographic separation must be approached in a systematic manner. Key factors that enabled successful chromatography included keeping the sequence running continually. It was important not to allow the system to pump the mobile phase at starting conditions. If analysis had already started, and samples were not ready on the instrument, repeated blanks, or QC mix solutions were analyzed to keep the system running properly. New columns must be conditioned properly before use following the specified protocol.

Some variability in RT is evident between sequences run on different days. Therefore, after running start-up LC gradients and blanks, a QC mix containing all relevant analytes was analyzed several times using the scouting MRM method with wide RT windows to enable the determination of analyte RTs. These were then used to update RTs within the full dynamic MRM acquisition method for that sequence. During the sequence, RT drift was minimal, but with some occasional slight variability noted, particularly if running different shellfish matrices. The EURL SOP for determination of lipophilic toxins in shellfish using reverse-phase LC/MS/MS detection recommends an allowable retention time drift of 3%.[12]

Table 9. Summary of method performance data including LOD/LOQ and calibration range (PST analog concentrations in nmol/L), percentage accuracy (certified PST analog concentrations in µg STX di-HCl eq/kg) and precision, repeatability, and reproducibility (relative standard deviation) compounds with TEF of 1 are marked with an asterisk.

Analog	LOD	LOQ	R^2	Cal Range	Accuracy % (n = 33)	Within Batch Repeatability % (1 Batch; n = 42)	Between Batch Repeatability % (1 Week; n = 20)	Reproducibility % (1 Year; n = 31)
C1	0.07	0.24	0.999	4.58 to 419	116 (2.5)	6	10	24
C2	0.05	0.17	0.997	1.38 to 126	86 (27.5)	6	11	19
C3	0.28	0.92	1.000	5.45 to 515	–	7	9	–
C4	0.33	1.09	0.998	2.3 to 217	–	9	11	–
dcGTX1	0.08	0.27	0.995	4.01 to 284	–	8	12	–
dcGTX2	0.24	0.78	0.998	4.04 to 273	–	8	12	–
dcGTX3	0.04	0.14	0.994	1.19 to 81	–	9	9	–
dcGTX4	0.05	0.15	0.994	1.44 to 102	–	17	18	–
dcNEO	0.18	0.59	1.000	1.22 to 64	–	14	8	–
dcSTX*	0.15	0.49	1.000	2.67 to 132	–	15	12	–
doSTX	0.05	0.16	0.998	0.22 to 10	–	12	11	–
GTX1*	0.02	0.08	1.000	2.29 to 181	96 (152.6)	6	10	22
GTX2	0.13	0.43	1.000	4.1 to 312	107 (29.8)	5	12	23
GTX3	0.30	0.99	0.994	1.74 to 132	112 (51.4)	8	16	24
GTX4	0.29	0.72	0.993	0.72 to 57	89 (86.0)	4	14	21
GTX5	0.37	1.22	0.999	2.34 to 171	96 (1.7)	7	23	27
GTX6	0.15	0.50	0.997	0.5 to 38	–	15	20	–
NEO*	0.06	0.19	1.000	2.6 to 63	96 (238.2)	7	10	16
STX*	0.02	0.08	1.000	2.68 to 62	115 (81.9)	7	12	17
TTX	0.04	0.15	1.000	2.92 to 179	–	11	15	–

Note: The identical pattern of congener occurrence between the accuracy and reproducibility tests is purely coincidental, and relates to the natural toxin profiles present in the source algae for both materials.

Given the notable greater level of sensitivity in HILIC in comparison to RPLC, a recommended allowable drift of 5% in total was used here across the sequence.

Any problems with chromatography were usually resolved with either a repeat shutdown/startup sequence, or with a repeat conditioning of the column before shutdown/startup and QC sample analysis to redefine retention times. Analyte peaks eluting too late or too early are usually indicative of incorrectly prepared mobile phases or the need to recondition the HILIC column. Acquisition of the sodium formate cluster ions (eluting around 2.89 minutes) is monitored to evidence the reduction in salt content within processed samples and matrix-based standards (data not shown). With a failure to perform effective clean-up, higher levels of salt can seriously affect chromatographic and mass spectrometric performance.

Calibrations should be linear across the concentrations of standards. An initial calibration and end of sequence calibration is recommended (bracketing calibration), and there should be minimal drift between the two calibrations so that the correlation for both calibrations combined is ≥ 0.98. The sensitivity of the system should allow detection of the low-level standard, using both the primary and secondary MRM. If the low-level standard cannot be detected in this way, then the LOD of the method should be adjusted appropriately to a higher concentration. A drift in calibration gradients was sometimes observed. Typically, this was found to occur when using a newly cleaned system or a new column. If significant drift occurs, usually repeating the batch helps to improve the situation. Normally, once the response has settled, the drifting effect disappeared.

Conclusion

This Application Note describes the analysis of PST analogs and TTX in shellfish tissues using the Agilent 1290 Infinity II LC coupled to an Agilent 6495B Triple Quadrupole LC/MS in 11 minutes. For all analyses, the sensitivity of the detection method was more than adequate, with LOQs at levels to facilitate detection and quantitation, which for each congener was at least as low as 16 µg STX eq/kg (regulatory action limit for the total of all PST congeners is 800 µg STX eq/kg). The method is specific, with the use of two interference-free MRM transitions for each compound, repeatable, and reproducible. The excellent quantitative performance was achieved with good linearity for all compounds over the entire linear range using matrix-modified calibration curves. Overall, the method is simple, rapid, and cost-effective in terms of analyst time, consumables, and the absence of expensive internal standards.

References

1. Hallegraef, G.M. Harmful Algal Blooms: A Global Overview. In *Manual on Harmful Marine Microalgae*; Hallegraef, G.M., Anderson, D.M., Cembella, A.D., Eds.; UNESCO: Paris, France, **2003**; 25–49.
2. Llewellyn, L. *et al.* Paralytic shellfish toxins in tropical oceans, *Toxin Reviews* **2006**, *Vol. 25, No. 2*, 159–196.
3. Anon. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29th April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union* **2004**, *L139*, 206–320.
4. Anon. (**2005**) AOAC Official Method 959.08. Paralytic shellfish poison. Biological method. Final action. In *Truckses MW (ed) AOAC official methods for analysis*, 18th Edition, Chapter 49: Natural toxins. AOAC International, Gaithersburg, 79–80.
5. Anon. 2005b. COMMISSION REGULATION (EC) No 2074/2005 of 5th December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organization of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. *Official Journal of the European Union* **2005**, *L338*, 27–59.
6. Anon. Commission Regulation (EC) No 1664/2006 of 6th November 2006 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. *Official Journal of the European Union* **2006**, *L320*, 13–45.
7. Turner, A.D. *et al.* Detection of the pufferfish toxin Tetrodotoxin in European bivalves, England, 2013–2014. *Eurosurveillance* **2015**, *Vol. 20*, *No. 2*, ArticleId=21009.
8. Boundy, M. *et al.* Development of a sensitive and selective HILIC-UPLC-MS/MS method for high throughput analysis of paralytic shellfish toxins using graphitic carbon solid phase extraction. *Journal of Chromatography A* **2015**, *1387*, 1–12.
9. Turner, A.D. *et al.* Single-Laboratory Validation of a Multitoxin Ultra-Performance LC-Hydrophilic Interaction LC-MS/MS Method for Quantitation of Paralytic Shellfish Toxins in Bivalve Shellfish. *Journal of AOAC International* **2015**, *Vol. 98 No. 3*, 609–621.
10. Turner, A.D. *et al.* Single laboratory validation of a LC-MS/MS method for quantitation of Tetrodotoxins in mussels and oysters. *Journal of AOAC International* **2017**, *Vol. 100, No. 5*, 1–14.
11. Sullivan, J. *et al.* Enzymatic transformation of PSP toxins in the littleneck clam. *Biochemical and Biophysical Research Communications* **1983**, *Vol. 114*, 465–472.
12. Harwood, D.T. *et al.* Paralytic shellfish toxins, including deoxydecarbamoyl-STX, in wild-caught Tasmanian abalone (*Haliotis rubra*). *Toxicon* **2014**, *Vol. 90*, 213–225.
13. EURL (**2015**) EU-harmonised standard operating procedure for determination of lipophilic marine toxins in molluscs by LC-MS/MS. http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/laboratorios/LNRBM/ARCHIVO2EU-Harmonised-SOP-LIPO-LCMSMS_Version5.pdf

www.agilent.com/chem

Printed in the USA, July 9, 2019
5994-0967EN

Agilent
Trusted Answers