EURLMB SOP for the analysis of Paralytic shellfish toxins (PST) by precolumn HPLC-FLD according to OMA AOAC 2005.06

Version 1

June 2020

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Erratum: This erratum corrects EURLMB SOP for the analysis of Paralytic shellfish toxins (PST) by precolumn HPLC-FLD according to OMA AOAC 2005.06, version 1 of June 2020, with the following modifications.

Date modification	Section	Previous text	Replaced or added text
25/01/2021	7.7	300 µl of extract	*900* µl of extract
	7.7	Add 225µL of 1M sodium hydroxide (A.4.1) to the reaction tube, vortex and filter using a 0.45µm membrane filter.	Add 225µL of 1M sodium hydroxide (A.4.1) to the reaction tube, *(3 vol. of extract: 1 vol. of 1M HCl: 1 vol. 1M of NaOH)* vortex and filter using a 0.45µm membrane filter.

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Foreword

This Standard Operating Procedure has been prepared by the technical experts at the EU Reference Laboratory for Marine Biotoxins (EURLMB) with the support of a scientific advisory group composed of James F. Lawrence (CA), Cowan Higgins (UK), Arjen Gerssen (NL) and Conor Duffy (IR).

The SOP has been further reviewed by the experts of the EU-NRLs prior to publication on the EURLMB Website.´

The SOP describes in detail the analytical protocol to perform the official method of analysis (OMA) AOAC 2005.06 for the control of Paralytic Shellfish Toxins (PST), including additional extension of the method to all the PSTs currently included in the EU Legislation.

The first part of the method gives a detailed description of how the method may be applied for the fast screening or *semi* quantitation of PSTs, avoiding the need to perform the full quantitative procedure on samples which are likely to be negative. The second part describes in detail the steps that are necessary where full quantitation of the PSTs involved in the contamination of bivalve molluscs is required.

1. Introduction

Paralytic Shellfish Toxins (PST) and related analogues (Fig. 1) are produced by marine dinoflagellates such as *Gymnodinium* spp, *Alexandrium* spp. etc., and have been detected in marine bivalves and gastropods from around the world. There are more than 21 molecular forms of PST associated toxins produced by those dinoflagellates. These PST were originally classified as paralytic shellfish poisoning (PST) toxins and also called "Saxitoxins" deriving the name from the first evidence of PST intoxication and the natural toxin associated with it (Saxitoxin, *Saxidomus giganteus*).

Clinical symptoms related to PST intoxication are neurological such as ataxia, tachycardia, respiratory depression or failure and heart paralysis, etc. Regulatory limits for PST in shellfish, are laid down in Regulation (EC) No. 853/2004, and has a limit of 800µg STX.2HCl eqv./Kg.

These natural toxins share a common (ring structure) in their chemical make-up, the functional group variation in its structure results in different subgroups according to their chemical and/or toxicological properties (Fig. 1). Many toxin structures with varying toxicities are found in nature (all of them variations on the saxitoxin parent compound).

In addition to the substitution of the R_4 position, substitution of 3 other positions may occur, leading to the numerous structural analogues which occur in this class of compounds. Substitution of these positions with hydrogen, hydroxyl or sulfate groups lead to different relative toxicities (e.g. those with sulfate groups have lower toxicity than those without) and, for example, despite GTX4 having the carbamoyl functional group, similar to STX, the sulfate group in the R_2 position, leads to a lower toxicity relative to NEO, which has a hydrogen replacement.

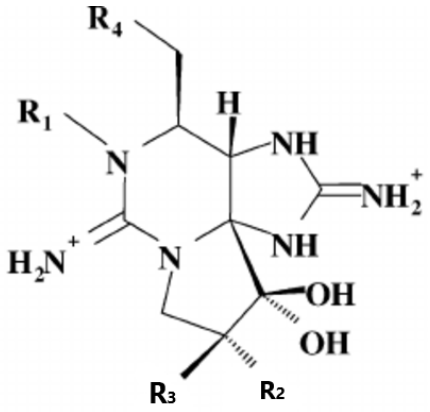


Figure 1: General Chemical structure of PST compounds

Table 1- Physicochemical and toxicological properties of PST compounds

Toxin	R_1	R_2	R_3	R_4	MW (g/mol)	Molecular Formula	Charge	TEF (*)
STX ***(Saxitoxin)***	H	H	H	-$OCONH_2$ (Carbamoyl)	299.3	$C_{10}H_{17}N_7O_4$	+2	1.0
NEO	OH	H	H		315.3	$C_{10}H_{17}N_7O_5$	+2	1.0
GTX1	OH	H	-OSO_3^-		411.4	$C_{10}H_{17}N_7O_9S$	+1	1.0
GTX2	H	-OSO_3^-	H		395.3	$C_{10}H_{16}N_7O_8S$	+1	0.4
GTX3	H	H	-OSO_3^-		395.4	$C_{10}H_{16}N_7O_8S$	+1	0.6
GTX4	OH	OSO_3^-	H		411.3	$C_{10}H_{17}N_7O_9S$	+1	0.7
dcSTX	H	H	H	-OH (Decarbamoyl)	256.3	$C_9H_{16}N_6O_3$	+2	1.0
dcNEO	OH	H	H		276.4	$C_9H_{16}N_6O_4$	+2	0.4

dcGTX2	H	OSO_3^-	H		352.3	$C_9H_{16}N_6O_7S$	+1	0.2
dcGTX3	H	H	OSO_3^-		352.3	$C_9H_{16}N_6O_7S$	+1	0.4
C1 (GTX 8)	H	OSO_3^-	H	-$OCONHSO_3$ N- Sulfocarbamoyl	475.4	$C_{10}H_{17}N_7O_{11}S_2$	0	0.1
C2 (epi GTX8)	H	H	OSO_3^-		475.4	$C_{10}H_{17}N_7O_{11}S_2$	0	0.1
C3	OH	OSO_3^-	H		491.4	$C_{10}H_{17}N_7O_{12}S_2$	0	0.1
C4	OH	H	OSO_3^-		491.4	$C_{10}H_{17}N_7O_{12}S_2$	0	0.1
GTX5 (B1)	H	H	H		379.4	$C_{10}H_{17}N_7O_7S$	+1	0.1

Physicochemical properties

Solubility: Highly soluble in water, acetic acid diluted HCl. Insoluble in hydrophobic solvents (methanol, ethanol, etc.).

Stability: Stable in weak acids solutions, decomposes rapidly in alkaline media.

pKa for most PST analogues are similar to saxitoxin (STX), pKa1= 8.24 and pKa2= 11.60.

X Log P3-AA= -4.6 to -6.0 (Hydrophilic compounds).

(*) TEF: Toxicity Equivalence Factor (proposed by EFSA: The EFSA Journal (2009) 1019, 1-76)

There are 3 main groups of saxitoxin analogues, identified by their substitution at the R_4 position. Saxitoxin is the most potent toxin of the group and, therefore, frequently this class of compounds is referred to as Saxitoxins. Those analogues having a carbamate functional group in this position are known as Carbamoyl saxitoxins, while those with a hydroxyl group are known as Decarbamoyl saxitoxins.

A third class of PST compounds has been identified in which a sulfate group replaces hydrogen at the R_4 position and, is known as N-sulfocarbamoyl saxitoxins. The substitution at the R_4 position affects the relative toxicity of the analogues.

The method described in this operating procedure (and many of the most widely used LC methods employed for the analysis of PST) uses oxidation to produce fluorescent products [3]. Whether based on post- column reaction HPLC (such as many of the earliest chromatographic methods for the PST toxins) [15] or on the precolumn oxidation HPLC-FLD (AOAC 2005.06) [10, 14], the so called Lawrence method [13], the main oxidation products resulting from the PST are purine derivatives, which have the aromatic structure required for fluorescence detection of these compounds. There is a loss of the ring structure near the R_1, R_2, positions.

Two different reagents can be used for the precolumn oxidation: periodate (IO_4^-) and hydrogen peroxide (H_2O_2). The oxidation with hydrogen peroxide does not yield fluorescent derivatives for the hydroxylated compounds (R_1= OH), while the fluorescence intensity for non-hydroxylated compounds (R_1= H) is very high, allowing a high sensitivity for the detection of these compounds. The oxidation yield can be also affected by the matrix (mussels, oysters, etc.) as well as by physicochemical properties such as pH [1].

The peroxide and periodate oxidation reactions yield single products for the non-hydroxylated toxins. The periodate oxidation reaction yields 3 products for the hydroxylated toxins. No products are observed with peroxide for the hydroxylated toxins. The peroxide and periodate oxidation reactions yield 2 major products for the decarbamoyl toxins. The oxidation reaction yields the same products for epimeric pairs (Table 2). The stability of the oxidized compounds is weak and so it is recommended that HPLC-FLD analysis is undertaken promptly following oxidation [1].

Table 2- Summary of the number of chromatographic peaks (oxidation products) observed.

TOXIN		Number of oxidation products	
		Periodate oxidation	Peroxide oxidation
Non N-OH toxins	STX	1	1
	GTX2&3	1	1
	C1&2	1	1
	GTX5 (B1)	1	1
	dcSTX	2	2
	dcGTX2&3	2	2
N-OH toxins	GTX1&4	3	---
	NEO	3	---
	dcNEO	2	---
	GTX6 (B2)	3	---
	C3&4	3	---

2. - Aim and Scope

The aim of this Operating Procedure is to describe the procedure by which the quantitative method, AOAC Official Method 2005.06 [14] can also be applied as a first step to allow rapid routine qualitative screening and semi-quantitation of PST in shellfish without having to perform the full quantitation.

The description of this method is organized in two parts because in most regulatory monitoring programs, the vast majority of samples are either negative for PST or are significantly below the regulatory limit for these toxins. Thus it becomes unnecessary to employ the full quantitative procedure on all samples and therefore a full quantitation is only required when the first screening/*semi* quantitation gives positive results. Although AOAC 2005.06 is fully suitable for rapid screening/semi- quantitation, the method as written does not include specific instructions on how to apply it for these types of rapid analyses. By first carrying out screening and semi- quantitation a significant saving of time and expense can be achieved.

The rapid qualitative screening is used to provide quick “yes or no” results relating to the presence of PST in the samples. This is the same qualitative approach as used in other rapid screening methods.

The semi-quantitative screen can provide an estimate of total toxin concentration in a sample. In this approach, the toxin oxidation product peaks identified in the samples after SPE-C18 solid phase extraction (SPE) cleanup are assumed to be the most toxic of the PST analogues. If only the most toxic analogues were indeed present then this value would be accurate.

However, most positive samples contain a mixture of toxic and less toxic analogues. So under normal circumstances, the semi-quantitative screen will give an over- estimation of true PST toxicity. In a given sample, if the estimated value is below the legal limit then the true value will also be less than the legal limit and no further quantification is necessary. This approach saves the analyst much time and expense since the extra steps involved in the accurate quantification are not needed. Where samples screen positive the extra steps including ion-exchange SPE (SPE-COOH) fractionation and full quantitation need to be carried out.

The *precolumn* HPLC/FLD method as described must be able to detect at least the following toxins: saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 2 and 3 (together: GTX2&3), gonyautoxins 1 and 4 (together: GTX1&4), decarbamoyl saxitoxin (dcSTX), gonyautoxin 5 (B-1 or GTX5), gonyautoxin 6 (B-2 or GTX6), N- sulfocarbamoyl gonyautoxins 2 and 3 (together: C1&2), N- sulfocarbamoyl gonyautoxins 3 and 4 (together: C3&4), decarbamoylgonyautoxins 2 and 3 (together dcGTX2,3) and decarbamoylneosaxitoxin (dcNEO or GTX7) [4, 5, 12].

This method has been initially validated in an interlaboratory study becoming an Official Method of Analysis (OMA) after AOAC evaluation (AOAC 2005.06) [11, 12], the method performance and applicability was further demonstrated in a EURLMB performance test [6-8] quantifying the total toxicity of the sample. The PST toxins not available in the first interlaboratory study, such as dcGTX2&3 and dcNEO were validated in additional interlaboratory studies. Further Information about the levels of validation is also included in bibliography [4-5, 8-9, 19-21].

The quantitative determination of GTX6 [B2] was not included in the first validation but this toxin is detected after fractionation with ion- exchange solid phase extraction (SPE-COOH). The present method is applicable to directly quantify GTX6 depending on the availability of standards, but it is also possible to determine GTX6 indirectly as NEO, after hydrolysis of the SPE-COOH fraction 2 (described in 7.8). This indirect determination of GTX6 was also validated in two additional interlaboratory studies carried out at the EURLMB [6-8]. The quantitative determination of C3&4 was included in the first interlaboratory validation, but this protocol also allows the direct determination of C3&4 when standards become available. If no standards are available, C3&4 can be quantified indirectly as GTX1&4, using the same hydrolysis protocol used for GTX6 and applied in this case to fraction 1 of the SPE-COOH. Implementation of the method for specific species is dependent upon individual laboratories undertaking the necessary validation work. As a minimum, the method must be applicable to the testing of mussels, oysters (Pacific and native), hard clams, razor clams and cockles.

Validation must be in accordance with Article 93 (EC) No 625/2017 and documented in a validation report [16-18]. The method must be shown to meet the minimum performance criteria given in Table 3.

Table3: Minimum Performance Criteria

Criteria	**Minimum Performance**
Chromatographic resolution (Rs)	dcSTX and dcGTX2&3, chromatographic peaks resolution should be ≥1.5
Limit of Detection (LOD)	Individual toxin LOD should be equal or lower than 1:50 of regulatory level, Secondary peaks must be detected for those toxins that have more than one chromatographic peak for their oxidation products.
Limit of Quantitation (LOQ)	Individual toxin LOQ should be equal or lower than 1:20 of regulatory limit.

Calibration range	The lowest concentration point of the calibration curve should be equal or higher than individual toxin LOQ value.	
Linearity	R2≥0.98	
SPE-C18 Recovery (Batch quality control)	Between 80-120%	
SPE-COOH Recovery (Batch quality control)	Between 80-120%	
Recovery	Mussel (51 to 112%) Oyster (51 to 160%) Cockles (90-128%) for individual toxins.	
Selectivity	Absence of chromatographic peaks from co-extractive components from the matrix and from matrix modifier (MM)	
Precision	Intra-batch	Rt (± 0.2 min) Peak Area (RSD ≤ 3.0%) Reproducibility of toxin concentration in positive sample control or CRM material should be ≤ 20%
	Inter-Batch	Reproducibility of toxin concentration in positive sample control or CRM material should be ≤ 25%
Uncertainty	Not applicable	
Ruggedness	Not applicable	

Official control laboratories must have the method accredited and therefore are requested to take part in regular proficiency testing exercises and following the guidelines described in this SOP.

The identification of the presence of PST relies on matching the retention time of any oxidation products in samples with those of the corresponding reference standards.

If PST are identified the sample may be subjected to a semi- quantitative screen analysis. Samples above a specified screen or semi-quantitative threshold are subsequently forwarded for quantitative testing.

The general procedure of AOAC OMA 2005.06 [14] includes a sample extraction with acetic acid, SPE purification and fractionation and a derivatization step prior to the HPLC injection and detection using a fluorescence detector (FLD). Total PST toxicity is calculated by summing individual toxin concentrations multiplied by the corresponding toxicity equivalence factor (TEF) [22]. The final result must be expressed as saxitoxin equivalents per kilogram of bivalve tissue, which will be compared to the EU regulatory level of 800 µg STX.2HCl eqv. /Kg [23]. Figure 2 shows a flow diagram including all the steps required to perform this method.

3. - Principle of the Method

The method is based on the AOAC Official Method of Analysis (OMA) 2005.06 [14] and applies to samples extracted with acetic acid.

A homogenized sample of bivalve molluscan shellfish is extracted with acetic acid in a boiling water bath, all water-soluble compounds including PST will be extracted and this extract will be further cleaned using solid phase extraction (SPE) C-18 cartridges. A further fractionation by SPE-COOH may be needed before periodate and peroxide oxidation prior to HPLC-FLD analysis for full identification and quantitation of all PST analogues included in the contaminated sample. Some of these steps, in particular fractionation may be omitted depending on the results obtained on the toxin profile, after the preliminary screen.

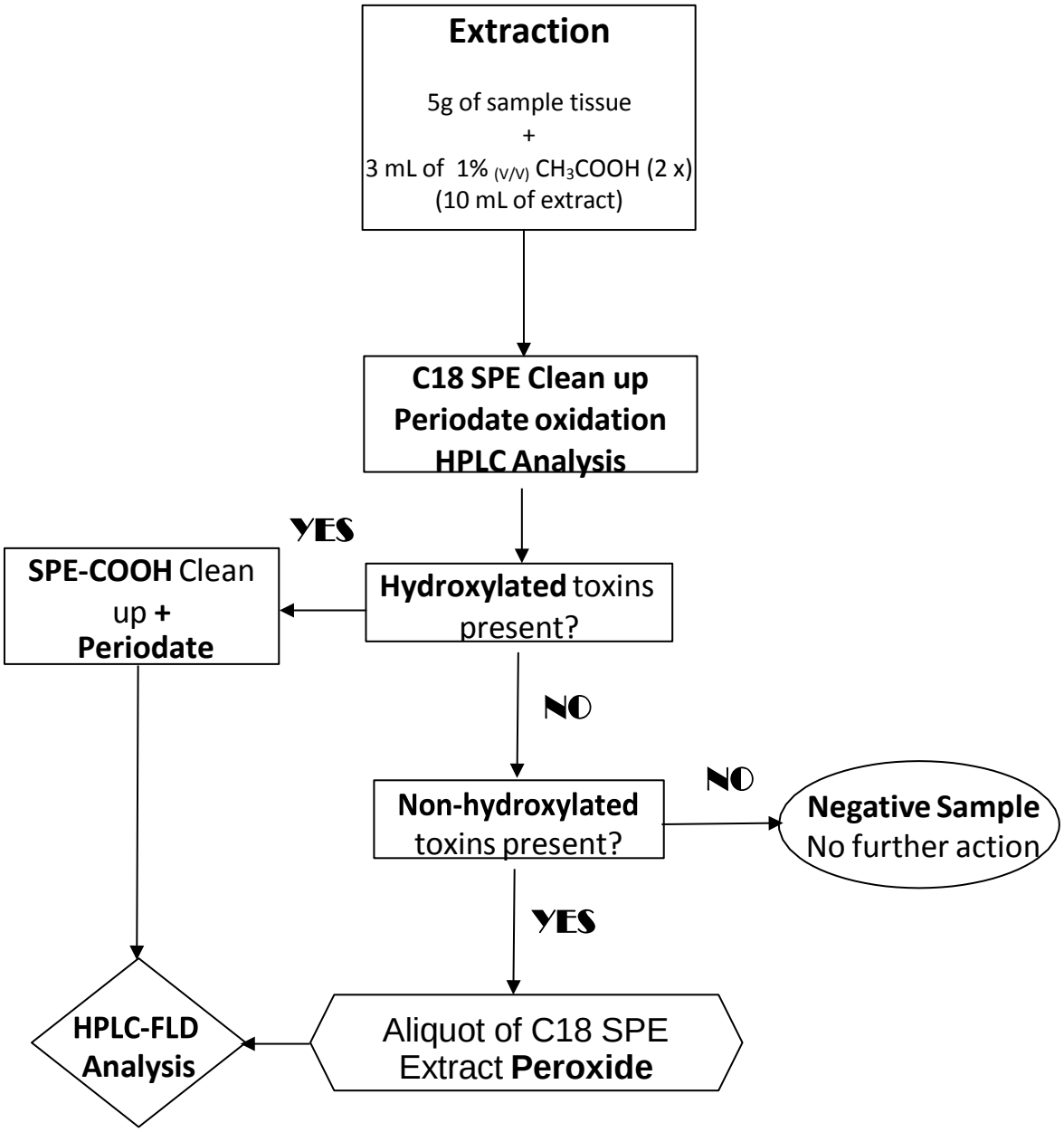


Figure 2: Flow diagram scheme for PST analysis using precolumn oxidation HPLC-FLD method.

4. - Materials

SPE LC-18 cartridges (3 mL, 500mg).

Cationic exchange SPE cartridges, SPE-COOH (3 mL, 500mg).

PVDF membrane filters 0.45µm and 33mm i.d.

PVDF membrane filters 0.45µm and 47mm i.d.

Calibrated volumetric glass material (volumetric flask and pipettes).

15 mL graduate conical test tubes.

Syringes

Plastic centrifuge tubes (polypropylene tubes 15 and 50 mL), with caps.

Ambar vials with caps.

Disposable plastic tips (capacity: 5, 100, 1000, 5000µL).

Disposable glass pipettes

1.5 mL Polypropylene plastic tube with cap

5. - Apparatus

Manifold system for solid phase extraction

Vortex system

Water bath

4000g centrifuge system

Balance (precision ± 0.01g)

Analytical balance (precision ± 0.1mg)

Accurate pH-meter (± 0.01 pH unit)

Orbital shaker

Homogenizer (Ultraturrax®)

Blender

Grinder

Vacuum system

Liquid Chromatography (HPLC) System with fluorescence detector (FLD), with:

- Binary gradient HPLC pump system (or quaternary pump)

- Injector system (up to 100µL loop capacity) preferably a cooled injector
- Fluorescence detector system (FLD)
- Chromatographic data analysis software

Precision micropipettes e. g. with 5-10, 10-20, 20-100, 50-200, 100-1000µL capacity.

6. - Chemicals

6.1- Certified Reference Material (CRM)

Suitable certified reference material (CRM) (standard solutions and matrix reference materials) can be provided by National Research Council Canada, Halifax (Canada). Equivalent products from other suppliers may be used if they can be shown to lead to comparable results.

Further information on suitable calibration solutions is available on the website of the European Reference Laboratory on Marine Biotoxins Standards and reference materials. This information is given for the convenience of the users of this EURLMB SOP and does not constitute an endorsement of any source of supply.

The preparation of standard solution is detailed in annex A.1 to A.3.

C1&2 (GTX8 and epiGTX8) CRM standard solution

GTX1&4 CRM standard solution

GTX2&3 CRM standard solution

GTX5 (B1) CRM standard solution

GTX6 (B2) CRM standard solution

dcGTX2&3 CRM standard solution

STX CRM standard solution

NEO CRM (neoSTX) standard solution

dcSTX CRM standard solution

dcNEO (dc neoSTX) CRM standard solution

6.2- Reagents

All chemicals for chromatographic separation must be of GRADIENT HPLC grade, other Analytical grade (PA), or the purity shall be equivalent according to EN ISO 3696:1995.

Acetonitrile (CH_3CN)

Deionized water

Methanol (CH_3OH)

Sodium hydroxide (NaOH)

Acetic acid (CH_3COOH)

Periodic acid (H_5IO_6)

Hydrogen peroxide (H_2O_2 30% w/v)

Sodium chloride (NaCl)

Ammonium acetate (CH_3COONH_4)

Disodium hydrogenphosphate (Na_2HPO_4)

Hydrochloric acid (HCl, 37% $_{w/v}$)

The preparation of solutions is detailed in annex A.4.

7. - Sample preparation

7.1 Sampling

Where necessary, both the outside and inside of shellfish must be washed with cold water and allowed to drain.

The shellfish tissue must be removed from the shell with a suitable knife, placed in a sieve to drain and then transferred to a blender for homogenization.

Note: Fresh shellfish samples should be immediately homogenized and certainly no longer than 48h after reaching the laboratory. Shellfish tissue homogenates must be stored at ≤ -20 ^{0}C.

7.2 Extraction

Accurately weigh 5.0 ± 0.1 g of tissue into 50 mL polypropylene centrifuge tubes. Add 3.0 mL 1% $_{V/V}$ of acetic acid (A.4.4), and mix thoroughly on a vortex for 2 min. Cap and place in a boiling water bath for 5 min (loosen the lid). Remove from the water bath, and allow to cool to room temperature (tighten the lid). Remix on a vortex for 2 min. Centrifuge at ≥ 3600 g for 10 min. Transfer the supernatant to a 15 mL plastic graduate tube. Add 3.0 mL of 1% $_{V/V}$ acetic acid (A.4.4) and repeat the extraction procedure without the boiling step. Both extraction solvents are combined and the volume adjusted to 10.0 mL with deionized water.

7.3 Purification

Clean-up of the acetic acid extracts must be carried out using SPE C18 cartridges (3 mL, 500mg) as follows (Table 4). These conditions may vary depending on the cartridges SPE-C18 manufactures.

Table 4: SPE-C18 Cleanup procedure for the analysis of PST

Parameter	**Conditions**	**Action**
Conditioning	6 mL of methanol	Discard
	6 mL of ultra-pure water	Discard
Load	1 mL of acetic extract	Collect$_{(a)}$
Wash	2 mL of ultra-pure water	Collect$_{(a)}$

(a) Same graduate tube.

IMPORTANT: Adjust the pH of the cleaned extract to between 6.5 (±0.2) with 0.2M or 0.1M NaOH (A.4.2 and A.4.3) and 0.1M CH_3COOH (A.4.5), and dilute to exactly 4mL with ultra-pure water.

7.4 Fractionation

Cationic exchange SPE cartridges must be used for fractionation (SPE COOH, 3 mL, 500 mg) under the following conditions (Table 5). These conditions may vary depending on the cartridges SPE-COOH manufactures.

Table 5: SPE-COOH fractioning procedure for the analysis of PST compounds

Parameters	Conditions	Action	Final volume[(b)]
Conditioning	10 mL of 0.01M ammonium acetate (A.4.9.)	Discard	
Load	2mL of acetic extract from SPE-C18	Collect tube 1 ([a])	
wash	4mL ultra-pure water	Collect in tube 1 ([a])	6 mL
Elution	4 mL of 0.05M NaCl (A.4.12)	Collect in tube 2	4 mL
	5 mL of 0.3M NaCl (A.4.11)	Collect in tube 3	5 mL

(a) Same graduate tube, (b) adjust final volume with water.

Note: *The purified and fractionated extracts filtrations are recommended. Use a 0.45µm filter membrane. The fractionation step is required to perform the AOAC 2005.06 method for fully quantitative purposes. This step is not necessary when the method is used only for screening or semi-quantitative purposes*.

7.5 Procedural blank (PB)

For the preparation of the procedural blank use the same volume of $1\%_{(v/v)}$ of acetic acid (A.4.4) used in the sample extraction step and taken it through the extraction procedure.

7.6 Preparation of Matrix Modifier (MM)

For the preparation of Matrix Modifier (MM) use uncontaminated oyster tissue homogenized and submitted to sample extraction and purification procedures, as described in 7.2 and 7.3.

Note: *Matrix modifier (MM) is used to increase the efficiency of the periodate oxidation reaction, by increasing the yield of the oxidation products of N-Hydroxylated toxins (GTX6, GTX1&4, NEO) in naturally contaminated samples. Oyster extracts are stable when stored at 4 ^{0}C for ten days or six months in a freezer (-20^{0}C).*

7.7 Hydrolysis

Acidic hydrolysis (Fig. 3) must be carried out for C3&4 and GTX6 quantitation only when CRM standards are not available, following this procedure:

Transfer to 10 mL glass Pyrex® tube, 900µL of extract and 225µL of 1.0M HCl (A.4.7) and vortex, close hermetically the tube as sample loss by evaporation during the hydrolysis reaction must be avoided. Incubate at 90 0C for 20 min. and then cool to room temperature. Add 225µL of 1M sodium hydroxide (A.4.1) to the reaction tube, (3 vol. of extract: 1 vol. of 1M HCl: 1 vol. 1M of NaOH) vortex and filter using a 0.45µm membrane filter. Adjust pH extract to 8.2 with a 1M sodium hydroxide (A.4.1) solution before carrying out the periodate oxidation.

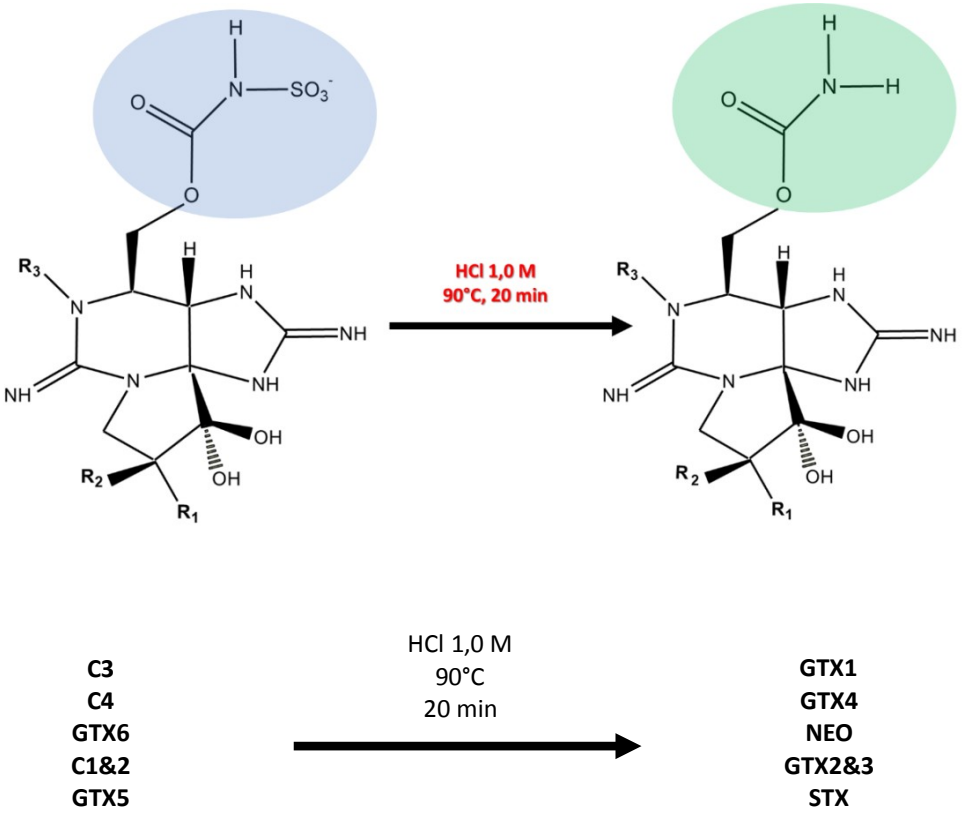


Figure 3: PST sulfocarbamoyl toxins (C3, C4 and GTX6) acidic hydrolysis reaction

7.8 Derivatization reaction: Precolumn oxidation

Derivatization is carried out through an oxidation reaction using periodate or hydrogen peroxide (Fig. 4).

IMPORTANT: Extract and oxidizing reagents pH control is necessary and very important, the efficiency of the reaction depends on this factor. It is very important to adjust the extract pH to 6.5 (±0.2) prior to peroxide oxidation.

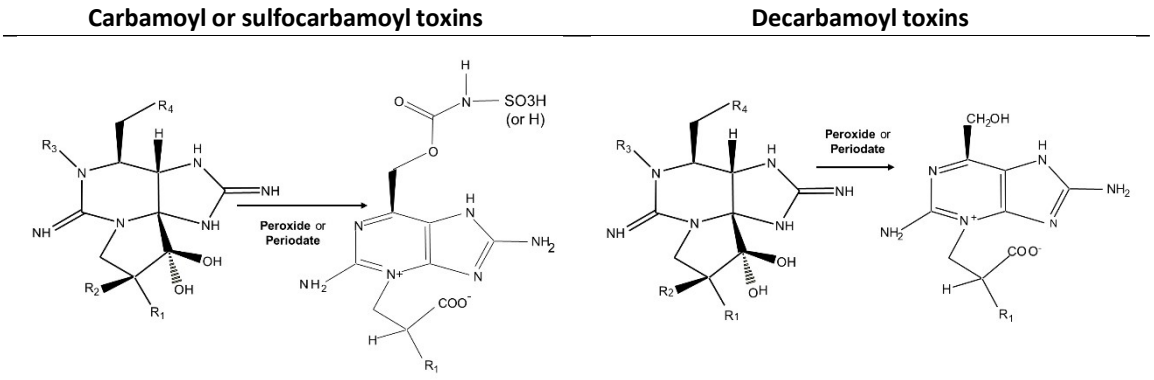


Figure 4: PST products from oxidation reaction

7.8.1 Peroxide oxidation

Transfer to 1.5 mL Eppendorf Tube, as follows: 25µL of 10%$_{V/V}$ hydrogen peroxide (A.4.16), 250µL of 1M sodium hydroxide (A.4.1), vortex and then add 100µL of standard solution or sample extract. Vortex again and allow to react for 2 minutes at room temperature. Add 20µL glacial acetic acid to the oxidation reaction and vortex. Filtration of oxidation solution through 0.45µm membrane filter is recommended, and inject into HPLC-FLD system. The solution is stable for at least 8h at room temperature (Fig. 5).

7.8.2 Periodate oxidation

Transfer to 1.5 mL Eppendorf Tube as follows: 100µL of standard solution or sample extract, 100µL matrix modifier, MM (7.6) and vortex. Add 500µL periodate oxidant reagent (A.4.17), vortex and allow to react for 1 minute at room temperature. To stop the derivatization reaction, add 5µL glacial acetic acid, vortex and allow standing for 10 min. at room temperature. Filtration of oxidation solution through 0.45µm membrane filter is recommended, and inject into HPLC-FLD system. The solution is stable for at least 8h at room temperature (Fig. 5).

NOTE: *The presence of natural fluorescent compounds from the matrices must be checked to avoid misinterpretations (check must be done, running the oxidation protocol with an aliquot of extract without oxidation reagent, using the same volume of deionized water instead of periodate or peroxide). This need not be done with every sample but needs to be done when any regulatory action is to be taken.*

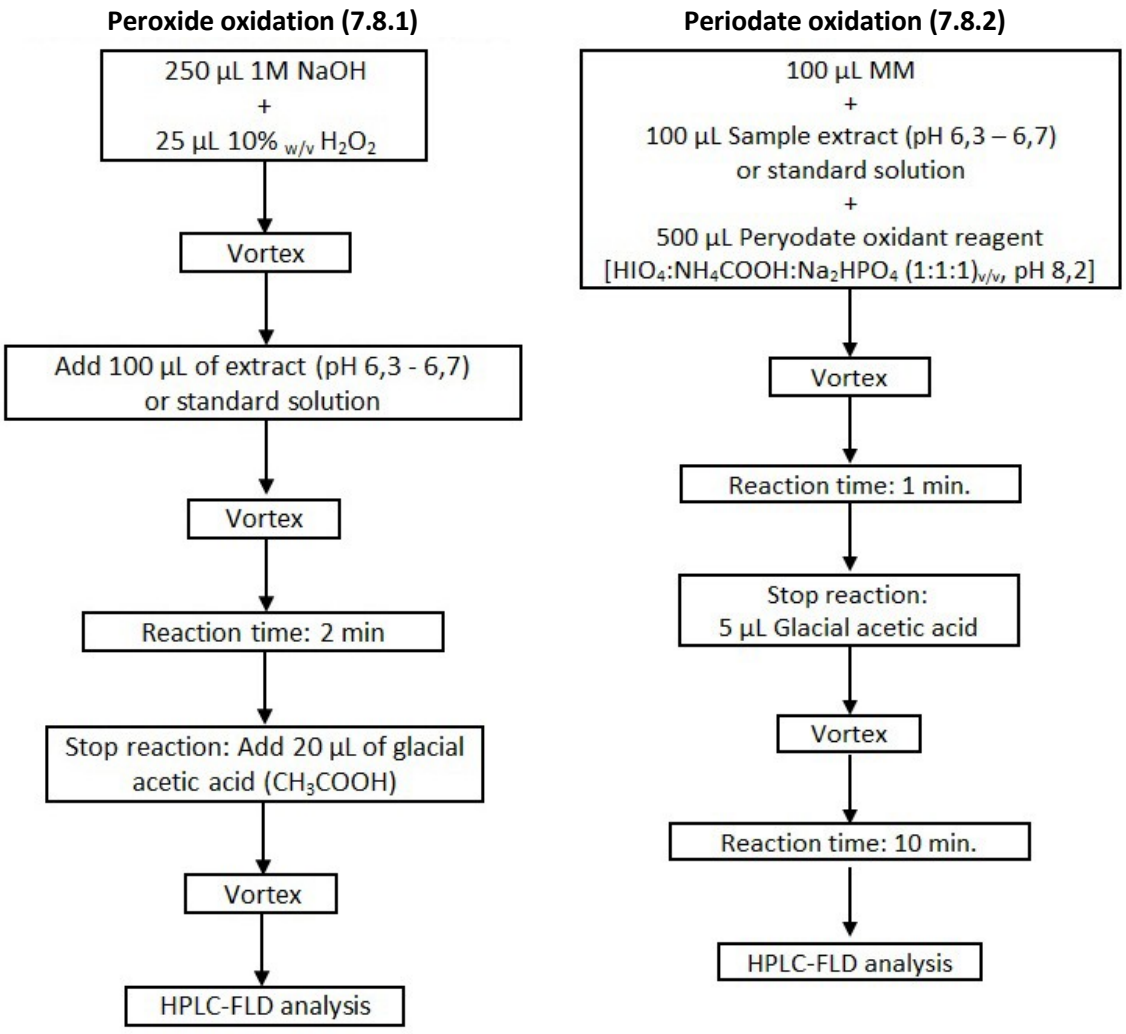


Figure 5: Scheme of PST derivatization reaction

8. - HPLC/FLD determination

Oxidation products from PST analysis are analyzed by high performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD). Chromatographic separation is carried out using a reversed phase RP-C18 stationary phase a gradient elution with the mobile phase (Table 6).

8.1- Preparation of mobile phases

Mobile phase A (A.4.18): 0.1 M ammonium formate in water, pH 6.

Dissolve 6.31 g of ammonium formate salt in water; adjust the pH with 0.1 M acetic acid (A.4.5). Adjust the final volume to 1L with water. Filter with 0.45µm membranes and degas.

Mobile phase B (A.4.19): 0.1 M ammonium formate in water with 5 $\%_{(v/v)}$ in acetonitrile, pH 6.

Dissolve 6.31 g of ammonium formate salt in water, add 50 mL of acetonitrile and adjust the pH with 0.1 M acetic acid (A.4.5). Adjust the final volume to 1 L with water. Filter with 0.45 µm membranes and degas.

8.2- Chromatographic conditions

The chromatographic conditions described in table 6 can and should be adjusted into achieve good chromatographic separation of oxidation products from PST compounds.

Table 6: HPLC-FLD conditions for the analysis of oxidation products of PST toxins

Instrument	Parameter	Description		
HPLC System	***Column***	RP C18-LC column, 150 x 4.6 (i.d.) mm, 5 µm and 100A.		
	Mobile phase A	0.1M ammonium formate, pH 6.0		
	Mobile phase B	0.1M ammonium formate, pH 6.0 with 5$\%_{(v/v)}$ of acetonitrile		
	Flow rate	1mL/min		
	Injection volume	Peroxide	25µL	
		Periodate	100µL	
	Injector temperature	±6 ^{0}C		
	Column temperature	±35 ^{0}C		
	Chromatographic Gradient	***Time (min)***	***A (%)***	***B (%)***
		0	100	0
		5	95	5
		9	30	70
		11	100	0
		15	100	0
FLD Detector	***wavelength***	Excitation	340nm	
		Emission	395nm	
	Run time	15 min.		

For those toxins that procedure two or more chromatographic peaks, it is required that the chromatographic resolution between the peaks should be higher than 1.5.

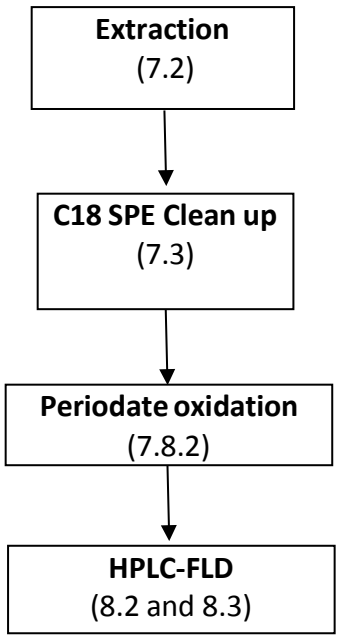
8.3- HPLC/FLD analysis

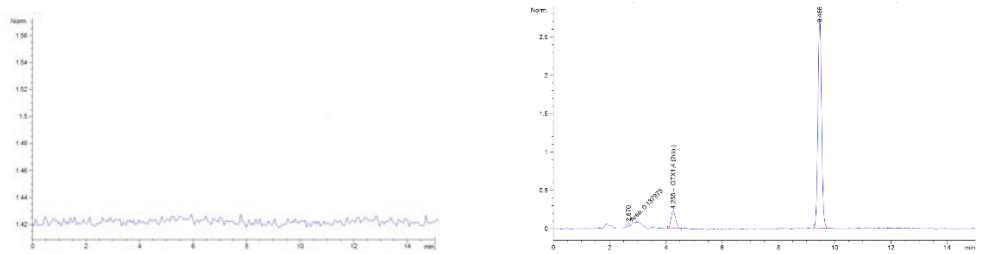
Annex A, describes all standard solutions required for the analysis of PST compounds using AOAC 2005.06 [14].

8.3.1- Screening analysis

This first stage of the method allows a fast screening of the samples in order to remove those that are considered negatives and just focus on those for which further steps are necessary in order to provide a full quantitation of PSTs

Figure 6 shows the steps included in this screening step.





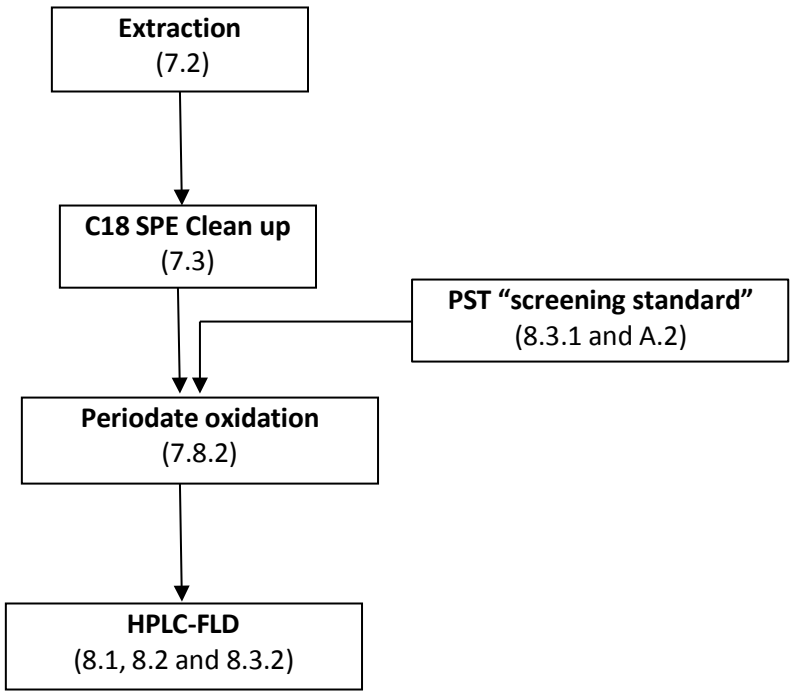
Negative results **Positive results**

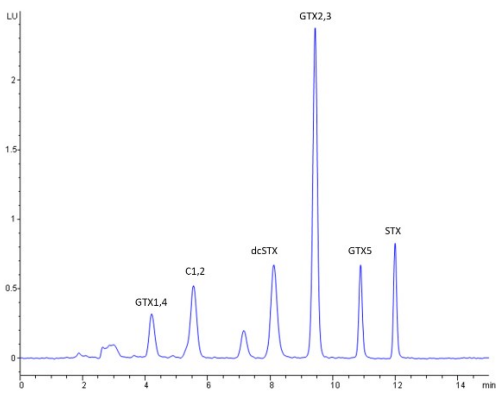
Figure 6: 2005.06 AOAC method in "screening mode"

- ✓ If a **negative result is obtained:** End of analysis (report PST not detected)
- ✓ If a **positive result is obtained:** Proceed with semi quantitative analysis.

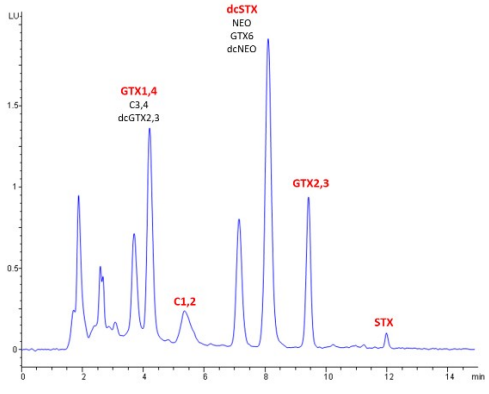
8.3.2- Semi quantitative analysis

The first HPLC-FLD run can give information rapidly, this mode takes results from the screening mode and establishes a *semi* quantitative analysis using a "screening standard" (see annex) for this purpose (Fig. 7).





"Screening standard" oxidized with periodate (indicated only the reference, see C.1)



Sample extract (with SPE-C18) oxidized with periodate (Reference toxins in red)

Figure 7: 2005.06 AOAC methods in "*semi* quantitative mode"

If total toxicity (µg STX.2HCl eqv./Kg):

- ✓ **< 600µg STX.2HCl eqv./Kg**: End of analysis
- ✓ **>600µg STX.2HCl eqv./Kg**: Proceed with full quantitative analysis

8.3.3 Full quantitative analysis

Complete quantitation is necessary when the toxicity of the samples exceeds the highest limits established in *semi* quantitation mode (Fig. 8).

Full quantitative analysis on C3&4 requires hydrolysis reaction (see 7.8).

If GTX6 standard is not available, proceed with the hydrolysis reaction if required.

A general scheme for the quantitative analysis of PST toxins, according to the AOAC method [14], using precolumn oxidation by HPLC-FLD is presented below (Fig. 9).

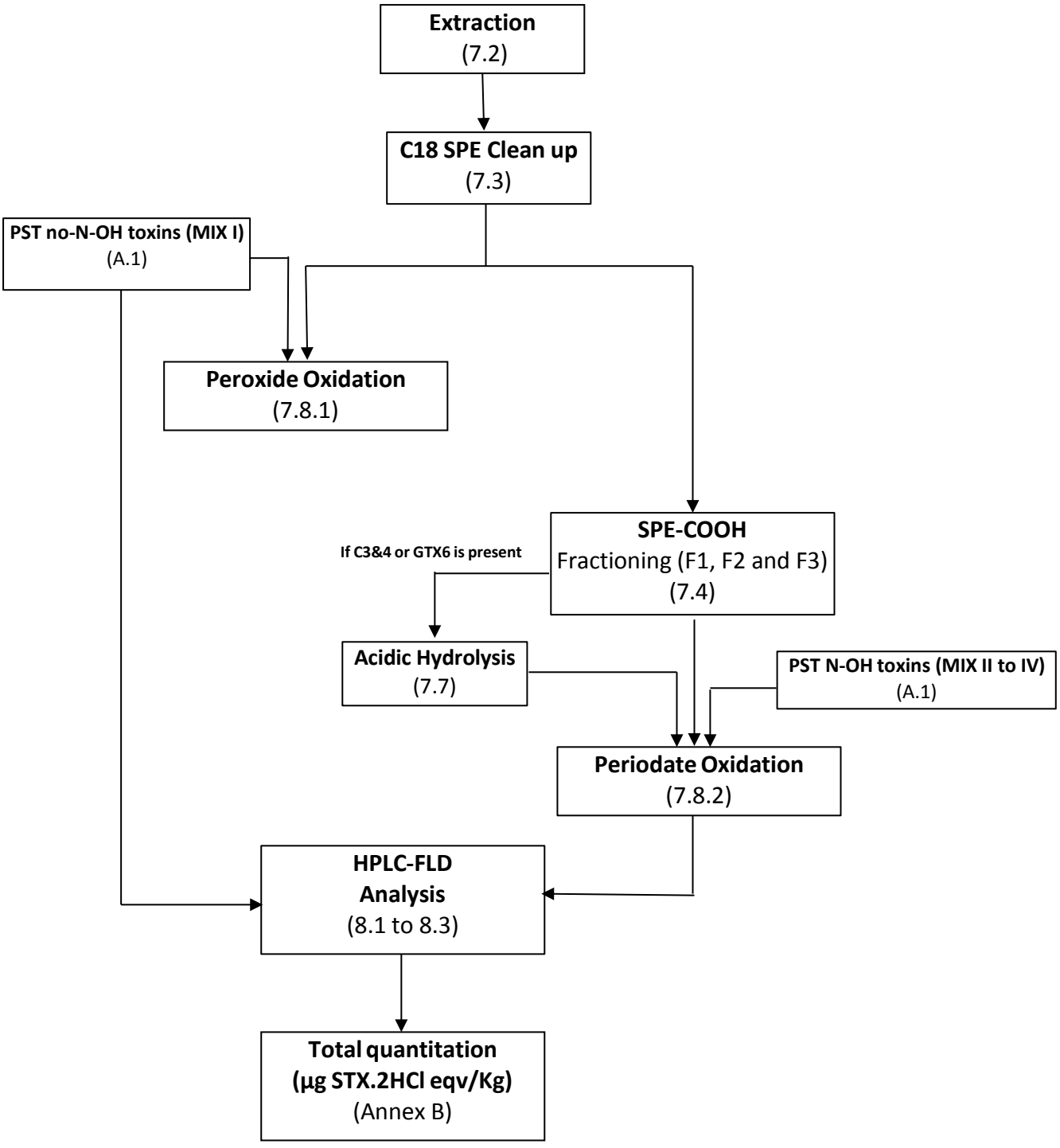
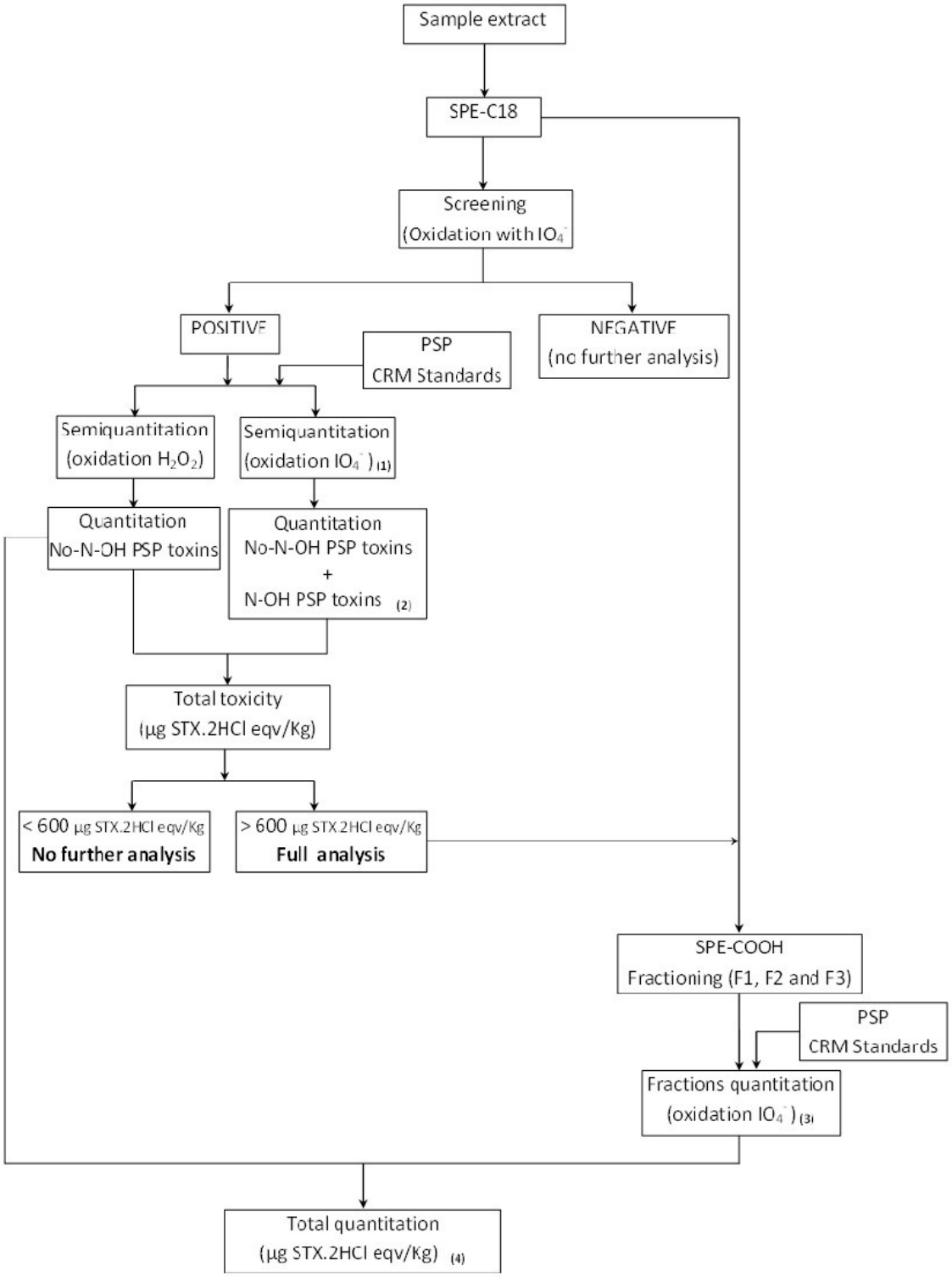


Figure 8- PST toxins quantitation scheme using 2005.06 AOAC method



1) See coelution information data
2) Use quantitative data obtained for each toxin when use PST mix of certified reference standard, which contains GTX1&4, C1&2, dcSTX, GTX2&3, GTX5 and STX and oxidized with periodate.
3) Use quantitative data obtained for each individual non-N-OH PST toxins from semiquantitative determination step
4) For total toxicity use the toxicity equivalence factor (TEF) for each PST compound.

Figure 9- Full 2005.06 AOAC method (screening, *semi* quantitation and quantitation mode) of PST compounds.

9. - Chromatographic results

In the chromatograms from the HPLC-FLD analysis the retention time (Rt) is used for toxin identification, and the peak area for its quantitation, takeing into account the data shown in Table 2 and 3 to ensure the correct qualitative and quantitative analysis.

9.1 Identification

Chromatographic peak identification is carried out by comparing retention times (Rt) between the standard and oxidized sample (variation of retention time should be ≤ ±0.2 min). It is important to identify chromatographic peaks corresponding to the presence or absence of natural fluorescence compounds in samples (by comparing chromatographic results from the sample extract with and without oxidation).

i) Identify the presence or absence of chromatographic peaks corresponding to naturally fluorescent compounds (chromatographic results for the analysis of eluates from SPE-C18 with and without oxidation).

ii) The number of chromatographic peaks resulting from the analysis of the extracts must be equal to the standards established for each toxin. Signal to noise ratio must be ≥3.0

9.2 Calibration curve

Six levels of calibration solutions are prepared for most PST (Mix I to Mix V) using certified reference standards, with concentrations from 0.03 to 2.00 µM used to create the calibration curves.

Calibration curves ($A = m\ C + A_0$) of chromatographic peak area (A) for each toxin against the concentration (C) of the standard solutions are constructed using a least squares method as the mathematical model. The linearity of the analytical method is evaluated by the examination of linearity plot and calibration data: slope (m), intercept (A_0) and correlation factor (R^2), for each toxin. Use interpolation of the peak area of the sample (A_x) on the calibration curve to obtain the final concentration of toxin (C_x) in the sample extract.

9.3 Quantitation

Quantitation can be carried out for each toxin only when the ratio S/N≥10 for the identified toxins (See Annex B.1) and establishing a direct proportionality between peak areas for the standards and the samples. Use calibration data or one-point calibration with a response factor detector (R_f) for each PST toxin (see B.2, Eq. 1).

In case of the lack of standards for C3&4 and GTX6, the quantitation will be carried out indirectly; use the chromatographic data obtained in the analysis of the oxidation products from acidic hydrolysis of extract (7.8). C3&4 will be quantified as GTX1&4 and GTX6 as NEO; however these results will be expressed as C3&4 and GTX6.

Where the HPLC-FLD analysis of oxidation products of PST give more than a single chromatographic peak, the quantitation is carried out with the highest intensity chromatographic peak.

Quantification can be carried out in two different ways:

a) By interpolation in calibration curve: Use the data obtained from calibration (9.2) of the HPLC-FLD system with PST standards (slope, intercept data).
b) Use chromatographic data from a PST standard solution: Calculate the concentration of each toxin in the sample using the response factor (R_f), where $\mathbf{C_x=A_x/R_f}$, **C** is the concentration of the toxin in the sample extract (µM), **A** is the peak area of the toxin in the analyzed sample and $\mathbf{R_f}$ is the response for the standard. This calculation is only possible when the concentration of the standard is within the range of the calibration. The peak area value of the sample should be within the range (if not dilute) of the peak areas obtained in the calibration for each PST standard.
   IMPORTANT: This quantitation method can be used in routine analysis, if the linearity and robustness of response detector is guaranteed.

9.4 Expression of results

The concentration of toxins in extracts should be expressed in micromolar units (µM). Taking into account the sample mass tissue, extract volume, equivalent toxicity factor (TEF) and molecular weight of the STX.2HCl, the molar concentration is converted into microgram of saxitoxin equivalent units per kilogram of shellfish meat (µg STX.2HCl eqv/Kg), see details in Annex B.

The epimeric pairs (dcGTX2&3; GTX1&4; GTX2&3; C1&2; C3&4) after their derivatization reaction produce the same oxidation products, these pairs are quantified together as a single compound, to calculate the equivalent toxicity of

these toxins, use the highest TEF value for the epimeric pair.

The results are expressed in accordance with current legislation (µg STX.2HCl eqv/Kg), calculate the total toxicity for sample as describe in Annex B.

Toxins concentration in samples under the detection limit (LOD) cannot be quantified and should be expressed in any report as not detected (ND) or below the detection limit (<LOD), and the value of the detection limit must be indicated in the report, in the same units as the legislation.

Toxins that can be identified, but whose concentration is under the quantitation limit (LOQ), are not quantified. The report should indicate the concentration is below the quantitation limit (<LOQ), and the value of the quantitation limit must be indicated in the report, in the same units as the legislation.

Toxin concentrations in samples below the LOD and LOQ do not contribute to the determination of total sample toxicity.

10. - Precision

Data precision criteria are defined in this SOP document (Annex C).

11.- Quality control

Use, if available, certified reference materials.

When CRM material is not available, PST standard addition to different uncontaminated shellfish matrices can be used.

Samples from proficiency test with assigned value can be used for this propose.

To evaluate the efficiency of the analytical process determine the recovery value for the quality control samples.

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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 Parliament and of the Council and amending Regulation (EC) No 853/2004 and EC No 854/2004 Official Journal of the European Union L338/27.

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Annex A: Solutions

A1- Standard solutions

Stock PST standard solution mixtures are prepared in 0.01M hydrochloric acid (A.4.8) or 0.03M acetic acid (A.4.6). Some of the certified standards can contain trace levels of other PST toxins. Stock solutions may be stored at -20^{0}C for upto six months, check regularly the mass concentration of each standard solution. The mixture of PST standard stock solution is prepared from individual CRM standard solutions. Further information on suitable calibration solutions is available on the website of the EURLMB (http://www.aecosan.msssi.gob.es/en/CRLMB/web/public_documents/seccion/materiales_referencia.htm). This information is given for the convenience of the users of this EURLMB SOP and does not constitute an endorsement of any source of supply.

Prepare the following standard stock solutions, at a concentration of 2 µM for each toxin:

MIX-I: A mixture of the stock solutions contains STX, dcSTX, dcGTX2&3, C1&2, GTX2&3 and GTX5. No N-hydroxilated compounds.

MIX-II: A mixture of stock solutions containing, GTX1&4 and NEO.

MIX-III: A stock solution containing dcNEO.

MIX-IV: A stock solution containing dcSTX, or use MIX I (for periodate oxidation).

MIX-V: A stock solution containing GTX6.

For calibration curves, prepare six level of calibration solution, for each MIX (I to V) in a range from 0.03 to 2.0 µM in water. The lowest concentration of standard solution for calibration should be equal or higher than the quantitation limit of the method (LOQ).

If you use one standard (for example, 1.0µM) for routine analysis, the selected concentration must be included in the range used for the calibration of HPLC-FLD system.

A.2- Screening standard

The stock solution contains 1µM each of GTX1&4, C1&2, dcSTX, GTX2&3, GTX5 and STX (for screening and *semi* quantitation purposes).

A.3- PST standard solution for SPE quality control.

This solution is used when SPE column quality control is required. Prepare a standard of a MIX of all PST solution, with 1µM concentration of each toxin, in 0.6%$_{V/V}$ or 0.1M acetic acid (A.4.5).

A.4- Chemical solution

All aqueous solutions must be prepared with deionized water using appropiate glass volumetric flasks and store adequately. Table-A1 summarizes the conditions for solution preparation, and expiry date.

Table- A1: Chemical solution preparation conditions

Solution	C	V	Preparation	Use	Expire	Code
Sodium hydroxide (NaOH)	1M	100mL	4g of NaOH Dissolve in deionized water	pH adjust H_2O_2 oxidation reaction	1 month	**(A.4.1)**
	0,2M	100mL	20 mL of 1M NaOH (A.4.1)dilute in deionized water	pH adjust	1 week	**(A.4.2)**
	0,1M	100mL	10 mL of 1M NaOH (A.4.1) dilute in deionized water	pH adjust	1 week	**(A.4.3)**
Acetic acid (CH_3COOH)	1% $_{(v/v)}$	100mL	Dilute 1 mL acetic acid in deionized water	Extraction	1 month	**(A.4.4)**
	0,6% $_{(v/v)}$ or 0,1M	100mL	Dilute 572µL of acetic acid in deionized water	pH adjust	1 month	**(A.4.5)**
	0.03M	100mL	Dilute 172µL of acetic acid in deionized water	For standard solution preparation	1 month	**(A.4.6)**
Hydrochloric acid (HCl)	1.0M	100mL	Dilute 8,3mL of hydrochloric acid in deionized water	For PST hydrolysis reaction	1 month	**(A.4.7)**
	0.01M	100mL	Dilute 83µL of hydrochloric acid in deionized water	For standard solution preparation	1 month	**(A.4.8)**
Ammonium acetate (NH_4CH_3COO)	0,01M	100mL	0,077 g of ammonium acetate dissolve in deionized water	SPE-COOH procedure (conditioning step)	1 month	**(A.4.9)**
Sodium chloride (NaCl)	2M	100mL	11,69 g of sodium chloride dissolve in deionized water	To prepare dilute solutions	1 month	**(A.4.10)**
	0,3M	100mL	Dilute 15 mL of 2M NaCl in deionized water (A.4.10)	SPE-COOH procedure (elution step, fraction 2)	1 week	**(A.4.11)**
	0,05M	100mL	Dilute 2,5 mL of 2M NaCl in deionized water (A.4.10)	SPE-COOH procedure (elution step, fraction 3)	1 week	**(A.4.12)**

Peryodic acid (HIO_4)*	0,03M	100mL	Dissolve 0,6838 g of periodic acid (*) in deionized water	Oxidation reagent preparation	1 month 4 ^{0}C ambar flask	**(A.4.13)**
Formiato amónico (NH_4HCO_2)	0,3M	100mL	Dissolve 1.8918 g of ammonium formate in deionized water	Oxidation reagent preparation	1 month	**(A.4.14)**
Disodium hydrogenphosphate (Na_2HPO_4)	0,3M	100mL	Dissolve 4,2588 g of disodium hydrogenphosphate in deionized water	Oxidantion reagent preparation	1 month	**(A.4.15)**
Hydrogen peroxide (H_2O_2)	10% $_{(w/v)}$	9mL	Add 3 mL of 30%$_{(w/v)}$ H_2O_2 30% $_{(p/v)}$ to 6 mL of deionized water	Oxidation reagent	1 day dark	**(A.4.16)**
Peryodate oxidant solution (IO_4^-)	0.01M	15mL	Add 5 mL of 0,03M periodic acid (A.4.13), 5 mL of 0.3M ammonium formate (A.4.14) and 5 mL of 0.3M disodium hydrogenphosphate (A.4.15). Adjust pH to 8.2 with 1M of NaOH	Oxidation reagent	1 day dark	**(A.4.17)**
Mobile phase A(**)	0.1M	1L	6.31 g of ammonium formate dissolve in deionized water. pH 6.0 adjust with acetic acid.	Chromatographic separation	1 week	**(A.4.18)**
Mobile phase B(**)	0.1M with 5%$_{(v/v)}$ acetonitrile	1L	6.31 g of ammonium formate dissolve in deionized water; 50 mL of acetonitrile and pH 6.0 adjust with acetic acid.	Chromatographic separation	1 week	**(A.4.19)**

(*) One mol of commercial available periodic acid is H_5IO_6 and corresponds to 1 mol of HIO_4.

(**) Filter mobile phase through a 0.45µm membrane filter using vacuum.

A.5- Matrix Modifier (MM) extraction

For the preparation of Matrix Modifier (MM), use homogenized, uncontaminated oyster tissue and submit to sample extraction (7.2) and purification (7.3), as described in the SOP document.

Matrix modifier is used to increase the efficiency of the oxidation reaction, by increasing the yield of the oxidation products of N-Hydroxylated toxins (B2, GTX4, NEO) in naturally contaminated samples. Oyster extracts are stable for ten days when stored at 4 ºC and six months in a freezer (< -20 ºC).

Annex B: Quantitation

B1- Identification

PST profile identification (figure 1 and Table 1) is carried out with purified extract from SPE-C18 procedure and oxidized with hydrogen peroxide and/or periodate. Chromatographic peak identification is carried out by comparing the retention times (R_t) of the standard and oxidized sample. The variation cannot be higher than ± 0.2 min. It is important to identify chromatographic peaks corresponding to the presence or absence of natural fluorescence compounds in samples.

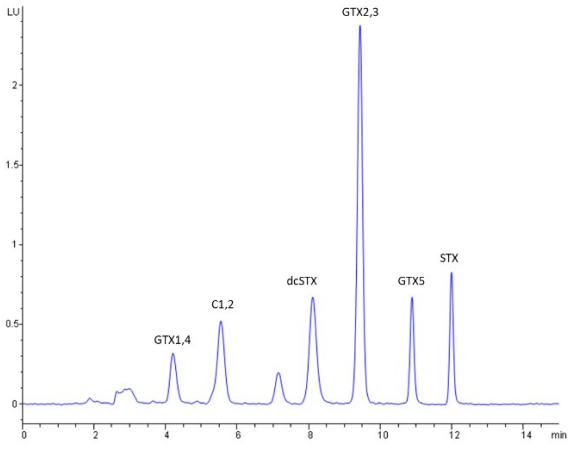

Figure 1a) PST mix standard for screening (periodate oxidation)

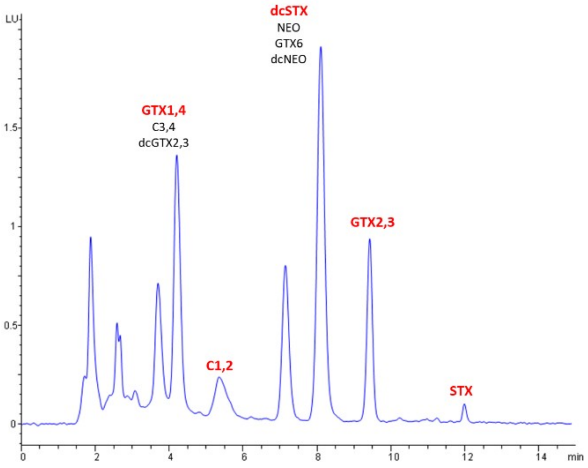

Figure 1b) Contaminated mussel sample with SPE C18 cleanup (periodate oxidation)

Table 1: Number of oxidation products for PST toxins

Toxin	***NUMBER OF OXIDATION PRODUCT***		
	Compound	***IO_4^-***	***H_2O_2***
No-N-OH	C1&2	1	1
	dcGTX2&3	2	2
	dcSTX	2	2

	GTX2&3	1	1
	GTX5	1	1
	STX	1	1
N-OH	C3&4	3	---
	GTX1&4	3	---
	GTX6 (B2)	3	---
	dcNEO	2	---
	NEO	3	---

B2- Semi quantitation

Due to the lack of a fractionation step, the quantitation procedure for chromatographic coeluants is carried out against the individual toxins having the higher toxicity (See Table 2).

Table 2: The main chromatographic peaks of individual toxins with the highest TEFs are used for *semi* quantitation when coelution is observed.

Coelution	Identify/quantitate as:
NEO	NEO
GTX6 (B2)	
C3&4	GTX1&4
GTX1&4	
dcGTX2&3	
dcSTX	dcSTX
dcNEO	

Using the one-point calibration mode calculate the response factor (f_R) for each toxin in the standard (screening standard).

$$R_f = \frac{Peak\ Area\ (Standard)}{C_{\ Standard}(\mu M)} \qquad (\text{Eq. } 1)$$

Toxins are individually quantified using chromatographic data. Each toxin peak identified in sample (A_X) is assessed against the response factor (R_f) and obtain it concentration (C_x).

$$C_X(\mu M) = \frac{A_X}{R_f} \qquad (\text{Eq. } 2)$$

Calculate the total toxicity equivalent to saxitoxin, using:

$$C_{total}\ (\mu M) = \sum TEF_i \times C_{X_i} \qquad (\text{Eq. } 3)$$

Where,

C_{total} is the total toxicity equivalent to saxitoxin, TEF is the toxicity equivalent factor and C_{Xi} the concentration of each toxin identified in extract.

Each toxin is semi-quantified in terms of total toxicity (µg STX.2HCl eqv/Kg),

$$C_{(\mu gSTX\frac{eqv}{Kg})} = C_{total}(\mu M\ STX\ eqv.) \times MW(STX.2HCl) \times \frac{V_E\ (ml)}{m_H(g)} \times D_f \qquad (\text{Eq. } 4)$$

Where,

MW is the molecular weight of saxitoxin.2HCl (372.2 g/mol) (V_E) is the extract volume, (m_H) the weight of homogenized tissue and (D_f) the dilution factor.

Total toxicity (T) *semi* quantified can be obtained as:

$$C_T = \sum_{i=1}^{n} C_{xi}\ (\mu g\ STX.2HCl\ eqv./Kg) \qquad (\text{Eq. } 5)$$

Where,

(C_T) is a total concentration of PST toxins in sample (µg STX. 2HCl eqv./kg) and (C_{Xi}) is the concentration (µg STX.2HCl eqv./kg) of each identified individual PST toxins in sample.

B3- Full quantitation

Quantitation requires the use of calibration parameters (slope and intercept) or the response factor for one point calibration for each PST toxins.

B3.1-non N-OH toxins quantitation

Use for the quantitation of C1&2, dcGTX2&3, dcSTX, GTX2&3, GTX5 and STX (figure 2).

Quantitation using calibration curve parameters with data from peroxide oxidation,

$$C_x(\mu g\ STX.2HCl\ Eqv/Kg) = \left[\left(\frac{A_x - A_0}{m}\right) \times TEF\right](\mu M) \times MW(g/mol) \times \frac{V_E(ml)}{m_H(g)} \times D_f$$

(Eq.6)

Where,

$\mathbf{A_X}$ is the peak area for toxin identified in the sample extract, $\mathbf{A_0}$ the intercept and **m** the slope on calibration curve, TEF the toxicity factor for the toxin recommended by EFSA, **MW** molecular weight for saxitoxin (STX.2HCl; 372.2g/mol), $\mathbf{V_E}$ extract volume (mL), $\mathbf{m_H}$ homogenized tissue extract (g) and $\mathbf{D_f}$ the dilution factor (see table B1).

Use equation 5 to calculate total toxicity in sample.

In routine quantitation, which uses a single standard solution, calculations are performed with response factor and the total toxicity is determined using equations 1 to 5.

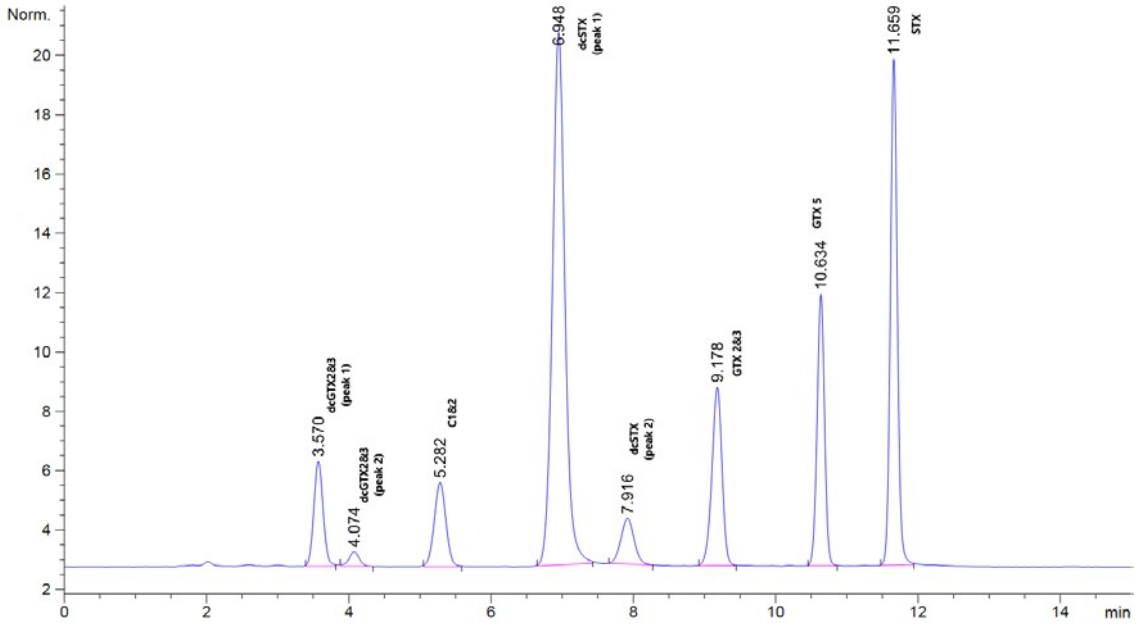

Figure 2: MIX I chromatogram (contains: dcGTX2&3, C1&2, dcSTX, GTX2&3, GTX5 and STX.

B3.2- N-OH toxins quantitation

The quantification of N-hydroxylated toxins (NEO, GTX1&4, GTX6 (B2), C3&4 and dcNEO) is carried out with fractions from the SPE COOH procedure.

IMPORTANT: The contribution of chromatographic peaks corresponding to the matrix modifier (MM) should be subtracted from the standard or sample chromatogram in order to achieve the correct quantification.

B3.2.1- C3&4 quantitation

Due to the lack of certified standard solutions, C3.4 toxins are quantified as GTX1.4, see B.3.2.2. Fraction 1 from SPE-COOH is hydrolyzed with hydrochloric acid and oxidized with periodate.

IMPORTANT: For quantification take into account the dilution factor introduced in SPE-COOH fractioning procedure and acidic hydrolysis dilution factor should be include (see table B1).

B.3.2.2- GTX1&4, GTX6 (B2) and NEO quantitation

Use slope and intercept calibration curve parameters and also take into account the dilution factor value (D_F) due to the contribution of fractionation and/or the hydrolysis procedure for toxin quantitation (see table B1).

Use the equation 5 and 6 for toxin quantitation and total toxicity determination in the sample.

When a single standard solution is used in routine analysis (figure 3), use the response factor of the detector and equation 1 to 5, or if you use calibration curve data apply equation 6 for toxin quantitation.

GTX1&4 and GTX6 are present in fraction 2 and NEO in fraction 3 from SPE-COOH.

If GTX6 standard is not available, fraction 2 which contain GTX6 can be hydrolyzed and quantified as NEO.

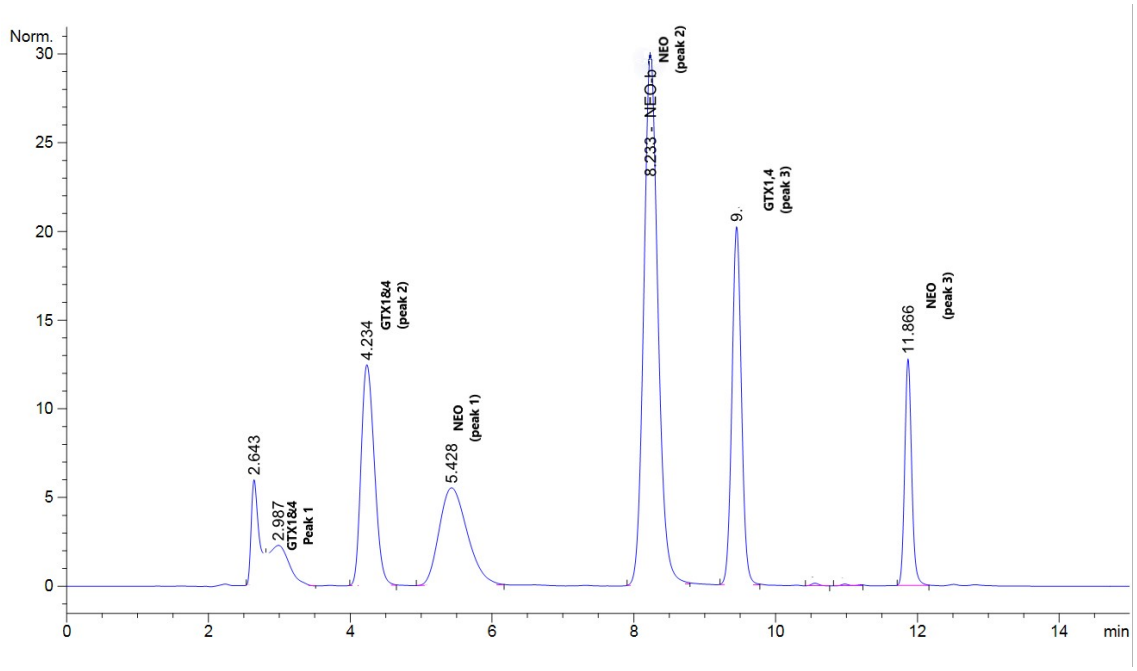

Figure 3: MIX II chromatogram (contains: GTX1&4 and NEO)

B.3.2.3- dcNEO quantitation

The quantification of dcNEO could be affected by the presence of dcSTX due to the chromatographic coelution of its oxidation products (Figure 4), so two quantitation pathways are defined.

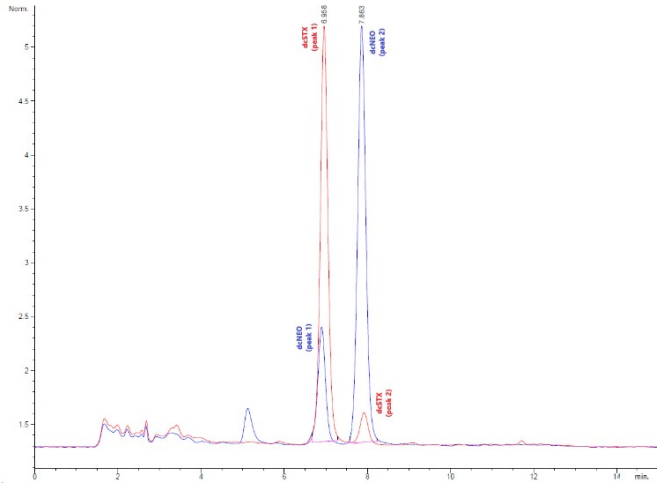

Figure 4: dcNEO (blue) and dcSTX (red) oxidized with periodate (overlapped)

i) <u>In the absence of dcSTX</u>

If dcSTX is not detected in sample extract oxidized with peroxide, and two chromatographic peaks are detected with the same retention times as dcSTX, its means only dcNEO is present. In this case, the quantification of dcNEO is carried out by direct interpolation of the peak area from sample into dcNEO calibration curve (equation 6) or use the response factor for individual standard of dcNEO (equation 1 to 5).

ii) <u>In presence of dcSTX</u>

Evaluate the ratio value of the two chromatographic peaks corresponding to the elution of dcSTX and/or dcNEO in the sample and sample extract when oxidized with periodate (figure 5).

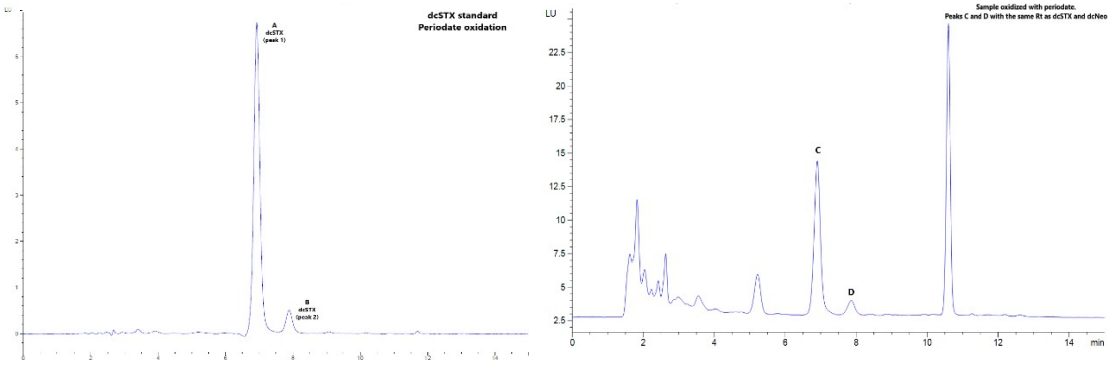

Figure 5: Chromatogram for dc STX standard (a) and sample (b) oxidized with periodate.

If the ratio, A/B ≈ C/D, the sample only contains dcSTX and the quantitation is carried out as described in B-3.1.

If the ratio, A/B ≠ C/D, the sample contains both toxins, dcSTX and dcNEO, for the quantitation of dcNEO proceed as follows:

Using calibration curve data:

a) Use the dcSTX concentration (µM) value in the sample extract when oxidized with peroxide (H_2O_2) and calculate the theoretical peak area corresponding to dcSTX when the extract is oxidized with periodate.

   Use dcSTX calibration curve data for the first peak of dcSTX(1) when oxidized with periodate, input the dcSTX concentration (µM) value from peroxide oxidation in calibration curve equation, and obtain the expected theoretical peak area for dcSTX in sample extract if it is oxidized with periodate.

b) To obtain the dcNEO peak area in sample, subtract the first peak area correspond the coelution of dcSTX and dcNEO in sample when oxidized with periodate to the theoretical peak area calculated in b).

c) The quantification of dcNEO is carried out by direct interpolation of the peak area from sample into dcNEO calibration curve (equation 7).

Summarizes:

$$C_{dcNeo(X)} = \left[\frac{A_x - \left(C_{dcSTX,H_2O_2(X)} \times m_{dcSTX,IO_4}\right) - A^0_{dcSTX,IO_4} - A^0_{dcNEO}}{m_{dcNEO}}\right] \times \frac{V_E(ml)}{m_H(g)} \times f_{dil} \times MW \times TEF \quad (Eq.7)$$

$C_{dcNEO\ (X)}$ = dcNEO concentration in sample (µg equiv STX.2HCl eqv./Kg)

A_X = Chromatographic peak area from the coelution of dcSTX and dcNEO (first peak) in sample extract when oxidized with periodate.

$C_{dcSTX,H2O2\ (m)}$ = dcSTX concentration in simple extract when oxidized with hydrogen peroxide (µM).

$m_{dcSTX,IO4}$= Calibration curve slope for dcSTX when oxidized with periodate.

$A^0_{dcSTX,IO4}$ = Intercept from dcSTX calibration curve when oxidized with periodate.

A^0_{dcNEO}= Intercept from dcNEO calibration curve when oxidized with periodate.

m_{dcNEO} = Slope value from dcNEO calibration curve.

V_E= Extract volume.

m_H= weight of homogenized tissue (g).

f_{dil} = Dilution factor.

MW = STX.diHCl molecular weight (g/mol).

TEF = EFSA toxicity equivalence factor.

Using a single calibration standard solution

a) Determine response factor (R_f) parameters when dcSTX and dcNEO standard solution is oxidized with periodate.
b) Use the dcSTX concentration (µM) value in the sample extract when oxidized with peroxide (H_2O_2) and calculate the theoretical peak area corresponding to dcSTX when the extract is oxidized with periodate. Use dcSTX response factor data, when a dcSTX standard is oxidized with periodate, and equation 1 for this purpose.
c) Subtract the peak area of the sample (dcSTX + dcNEO) to the theoretical peak area of dcSTX determined in b), and obtain the peak area corresponding to dcNEO in the sample.
d) The quantification of dcNEO is carried out by using the response detector factor using the equation 8.

Summarizes:

$$C_{dcNeo(X)} = \frac{C_{dcNEO(std)} \times \left[A_T - \left(\frac{C_{dcSTX,H_2O_2(X)} \times A_{dcSTX,IO_4(std)}}{C_{dcSTX,IO_4(std)}}\right)\right]}{A_{dcNEO(std)}} \times \frac{V_E(ml)}{m_H(g)} \times D_f \times MW \times TEF \quad (Eq.8)$$

A_T = Peak area corresponding to the first chromatographic peak of the coelution of dcSTX and dcNEO in sample extract when oxidized with periodate.
A_{dcNEO} (std) = Peak area corresponding to the first chromatographic peak of dcNEO standard solution when oxidized with periodate.
$A_{dcSTX,IO4}$ (std) = Peak area corresponding to the first chromatographic peak of dcSTX standard solution when oxidized with periodate.
C_{dcNEO} (X) = dcNEO concentration in sample (µg STX.2HCl eqv./Kg)
C_{dcNEO} (std) = dcNEO concentration in individual standard solution (µM).
$C_{dcSTX,H2O2}$ (m) = dcSTX concentration in sample extract when oxidized with peroxide (µM).
$Cd_{cSTX,IO4}$ (std) = dcSTX concentration in individual standard solution when oxidized with periodate (µM).
V_E= volume of extract (mL).
m_H= Weight of homogenized sample used for analysis (g).
D_f = Dilution factor.
MW = Molecular weight of STX diHCl (g/mol).
TEF = EFSA toxicity equivalence factor.

B.3.2.4- NEO quantitation in presence of dcSTX

The chromatographic coelution of the oxidation products from NEO with dcSTX in samples when oxidized with periodate is not very usual, the procedure used for the quantitation of NEO in presence of dcSTX is similar to that described for dcNEO (figure 6).

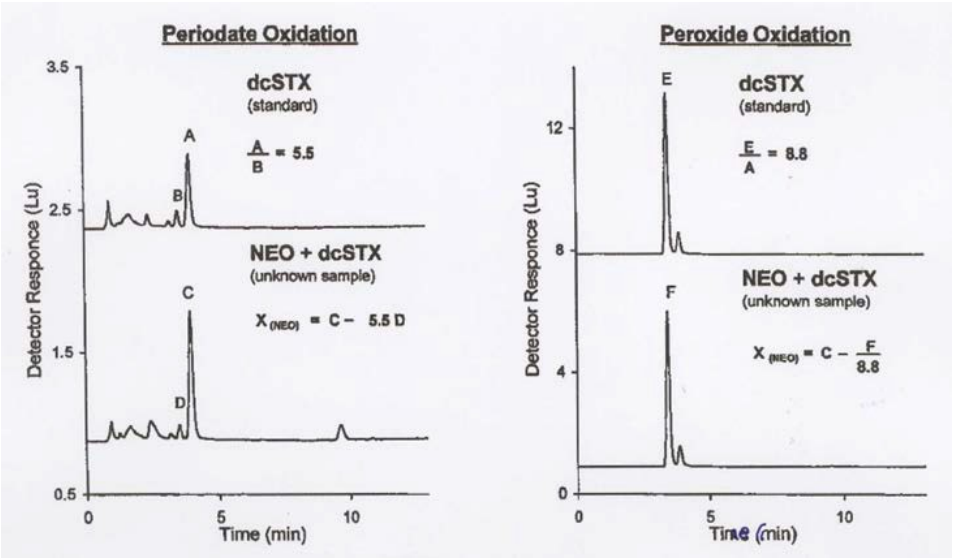

Figure 6: Chromatograms corresponding to the chromatographic coelution of the oxidation products of NEO with dcSTX (peroxide and periodate oxidation, Lawrence *et al* 2005).

Using only data from periodate oxidation.

The selection of the quantitation procedure for NEO, in presence of dcSTX, is based on the signal-to-noise (S/N) ratio, for the lowest chromatographic peak (D) for dcSTX in sample oxidized with periodate (figure 6).

If the intensity of peak D is higher or equal to three times S/N ratio, use the following procedure to carry out NEO quantitation:

i) Take the peak area value for dcSTX in standard solution and sample extract when oxidized with periodate.

ii) Calculate the ratio between two chromatographic peaks for dcSTX for both solution (equation 9 and 10).

$\frac{A_{dcSTX(2)IO_4}}{A_{dcSTX(1)IO_4}} = \frac{A}{B} = R \quad (Eq.9)$	$\frac{A_{dcSTX(2)IO_4}}{A_{dcSTX(1)IO_4}} = \frac{C}{D} \quad (Eq.10)$
For standard solution	For sample extract

R value should be approximately **5.5**.

iii) If A/B = C/D, The sample extract does not contain NEO.

Otherwise, determine the chromatographic peak area for NEO in presence of dcSTX (coelution) using the equation 11 (figure 6):

$$A_{NEO_m} = A_{(NEO+dcSTX)_m} - \left(R \times A_{dcSTX_m}\right) = C - (R \times D) = C - 5.5D \quad (Eq.11)$$

R= Chromatographic peak ratio for dcSTX in standard and sample.

$A_{(NEO+dcSTX)m}$= Total peak area corresponding the coelution of NEO and dcSTX in sample extract oxidized with periodate (C).

$A_{(dcSTXm)}$= Peak area for the first chromatographic peak corresponding to the elution of dcSTX in sample extract oxidized with periodate (D).

iv) The quantitation of NEO in the sample extract oxidized with periodate is carried out as indicated in B.3.2.2, interpolate the peak area value ($A_{NEO(m)}$) in NEO calibration curve data.

Using data from peroxide and periodate oxidation.

If the signal-to-noise (S/N) ratio, discussed above, is less than 3.0 an alternative procedure for the quantification of NEO in the presence of dcSTX is required, this procedure use chromatographic data from the sample extract when oxidized by peroxide and periodate.

i) Take the peak area value, for the first chromatographic peak (E), corresponding to the elution of dcSTX standard solution when oxidized with peroxide (figure 6).

ii) Take the peak area value, for the chromatographic peak (F), corresponding to the elution of oxidation products of sample extract, with the same retention time as the first chromatographic peak of dcSTX, when oxidized with peroxide (figure 6).

iii) Determine the ratio (R) between E and A (figure 6) in the dcSTX standard when oxidized with peroxide and periodate (equation 12).

$$\frac{A_{dcSTX(1)H_2O_2}}{A_{dcSTX(2)IO_4}} = \frac{E}{A} = R \quad (Eq.12)$$

iv) Determine the same ratio between F and C in sample when oxidized by peroxide and periodate, use (equation 13).

$$\frac{A_{dcSTX(1)H_2O_2}}{A_{dcSTX(2)IO_4}} = \frac{F}{C} \quad (Eq.13)$$

v) If F/C ratio value is similar to E/A ratio, coelution between NEO and DCSTX is not observed. If not, use equation 14 to calculate the peak area of NEO in sample.

$$A_{NEO_m} = A_T^{IO_4} - \frac{A_{dcSTX(1)}^{H_2O_2}}{R} = C = \frac{F}{R} \quad (Eq.14)$$

A_{NEOm}= NEO peak area for oxidized sample extract (2nd peak).
A_T^{IO4} =Total peak area for sample oxidized with periodate (2 peak)
$A_{dcSTX(1)}^{(H2O2)}$ = dcSTX peak area for sample oxidized with peroxide (1st peak)
R= Ratio between peak areas for dcSTX in sample oxidized with peroxide (1st peak) and periodate (2nd peak)

vi) The quantitation of NEO in sample extract oxidized with periodate is carried out as indicated in B.3.2.2, interpolate the peak area value ($A_{NEO(m)}$) in NEO calibration curve data.

Table 3. Identification of chromatographic peaks for the coelution of oxidation products from dcSTX with NEO.

Chromatographic peaks	Oxidation	Solution	Peak position
A	IO_4^-	dcSTX standard	2nd
B	IO_4^-	dcSTX standard	1st
C	IO_4^-	Sample	2nd
D	IO_4^-	Sample	1st
E	H_2O_2	dcSTX standard	1st
F	H_2O_2	sample	1st

B.3.2.5- GTX1&4 quantitation with dcGTX2&3 chromatographic coelution.

There is no evidence of the simultaneous presence of these toxins in molluscs. This situation occurs when recovery studies is carried out by external standard addition to matrix.

Quantitative chromatographic peak from the oxidation products of GTX1&4 (2nd peak) overlaps with the second from the dcGTX2&3, when periodate oxidation is used for the analysis.

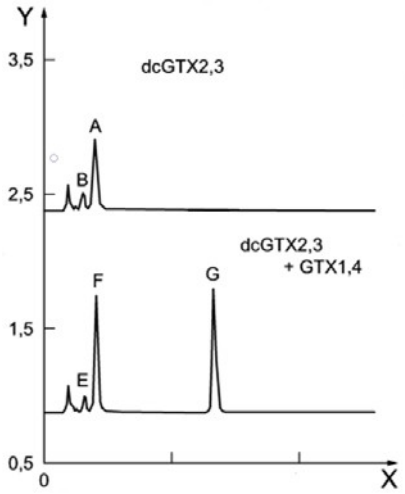

Figure 7: Chromatograms corresponding to the separation of the oxidation products from dcGTX2,3 and GTX1,4 both oxidized with periodate, rom CEN 14526 [9].

The quantification of GTX1&4 in this coelution [9] could carry out following the same procedure described for the dcSTX in presence of dcNEO.

i) Take into account the concentration of dcGTX2&3 determined in peroxide oxidation. ($C_{(dcGTX2\&3\text{-}H2O2)}$)

ii) Determine the response factor for dcGTX2&3 (Mix I or individual standard) when oxidized with periodate ($f_{R(dcGTX2\&3\text{-}IO4)}$)

iii) Determine the theoretical peak area for dcGTX2&3 ($A_{X\text{-}IO4}$) in the sample that should be obtained (equation 15) if it is oxidized with periodate, as described in B.3.2.3.

$$A_{X_{dcGTX2\&3,IO_4}} = C_{X_{dcGTX2\&3,H_2O_2}} \times f_{R_{dcGTX2\&3,IO_4}} \quad (Eq.15)$$

iv) Take the peak area of the second chromatographic peak of GTX1,&4 in the sample when oxidized with periodate and subtract to the area value determined in iii) for dcGTX2&3. The final results correspond to GTX1&4 peak area contribution.

v) Follow the quantitation procedure described in B3.2.2 for GTX1&4.

B.4- Additional information

Table B1: Dilution factor (D_f) for each SPE procedure.

Toxins	SPE Procedure	Parameter	Dilution factor
C1,2 GTX2,3 dcGTX2,3 GTX5 dcSTX STX	RP-C18 (Load+washing)	Extract vol. = 10 mL Sample weight = 5 g Load vol. = 1 mL Eluate vol. = 4 mL	**4.0**
C3,4	RP-C18 (Load+washing) Fraction nº1 SPE-COOH	Extract vol. = 10 mL Sample weight = 5 g Load vol. $_{(SPE\ C18)}$ = 1 mL Eluate vol. $_{(SPE\ C18)}$ = 4 mL Load vol. $_{(SPE\ COOH)}$ = 2 mL Eluate vol. $_{(SPE\ COOH)}$ = 6 mL	**12.0**
GTX1,4 GTX6	RP-C18 (Load+Washing) Fraction nº2 SPE-COOH	Extract vol. = 10 mL Sample weight = 5 g Load Vol. $_{(SPE\ C18)}$ = 1 mL Eluate vol. $_{(SPE\ C18)}$ = 4 mL Load vol. $_{(SPE\ COOH)}$ = 2 mL Eluate vol. $_{(SPE\ COOH)}$ = 4 mL	**8.0**

NEO dcNEO	RP-C18 (Load+washing) Fraction nº3 SPE-COOH	Extract vol. = 10 mL Sample weight = 5 g Load vol. $_{(SPE\ C18)}$ = 1 mL Eluate vol. $_{(SPE\ C18)}$ = 4 mL Load vol. $_{(SPE\ COOH)}$ = 2 mL Eluate vol. $_{(SPE\ COOH)}$ = 5 mL	**10.0**

B.5- Standard solutions and extracts recommend for analysis

1- Chemical blank (oxidize acetic acid 1 $\%_{(v/v)}$ solution with peroxide and periodate, for check the HPLC-FLD system is ready to run the analysis).
2- Matrix modifier extract (periodate oxidation, for identification of possible interferences)
3- Sample extract without oxidation following the oxidation conditions described for periodate and peroxide (identification of natural fluorescence products).
4- MIX I oxidized with peroxide
5- MIX II oxidized with periodate
6- dcNEO Standards solutions oxidized with periodate
7- dcSTX standard oxidized with periodate
8- dcGTX2&3 oxidized with periodate

Annex C: Laboratory results evaluation

C.1- Semi quantitative data analysis (Screening method)

To evaluate the over estimation of PST levels in samples, with coelution of oxidation products from some toxins groups, a mixture of some PST were prepared and analyzed, following the conditions described in this SOP, and quantified against PST screening standard solutions. The results are shown in table C.1.

Table C.1- Semiquantitation of different mixtures of PST compounds.

Added C (µM)	Peak overlap	Reference toxin for quantitation	Calculated C (µM)	Observation
3.0	C3&4 dcGTX2&3 GTX1&4	***GTX1&4***	4.2	Over estimation
1.0	C1&2 GTX6 (B2), 1st peak NEO (1st peak)	***C1&2***	16.3	Over estimation
4.0	NEO GTX6 (B2) dcNEO dcSTX	***dcSTX***	10.3	Over estimation
1.0	GTX2&3 GTX1&4 (3rd peak) C3&4 (3rd peak)	***GTX2&3***	1.9	Over estimation
1.0	NEO GTX6 (B2) dcNEO	***STX***	2.4	Over estimation

Different concentrations of PST mixtures are added to uncontaminated mussel tissue and quantified against screening standard as described in this SOP. The results are shown in Table C.2.

Table C.2- Semiquantitation of PST in mussel samples using external standard addition.

Peak overlap	C (µM)	C (µM eqv.)	Level added (µg STX.2HCl	*Semi* quantitation (µg STX.2HCl eqv/Kg)	Ratio
dcSTX dcNEO NEO GTX6 (B2) GTX1&4 dcGTX2&3	**0.01**	***0.04***	116	679	5.9
	0.05	***0.20***	581	2680	4.6
	0.10	***0.40***	1161	5626	4.8
	0.20	***0.80***	2323	10035	4.3
	0.50	***2.00***	5806	24381	4.2

Ratio > 1 (Over estimation), Ratio = 1 (No changes), Ratio < 1 (Under estimation)

Identified toxins using screening standard: GTX1&4 and NEO.
dcGTX2&3 coelute with GTX1&4. The dcNEO, GTX6 and NEO coeluted with dcSTX.

Example of *semi* quantitation:

$$C(\mu M\ equv.) = \sum C \times TEF = 0.01 \times 1 + 0.01 \times 4 + 0.01 \times 1 + 0.01 \times 1 + 0.01 \times 1 + 0.01 \times 0.4$$

$$= 0.039 \approx 0.04 \mu M\ eqv.$$

$$C_{(\mu gSTX\frac{eqv}{Kg})} = C_{total}(\mu M\ STX\ eqv.) \times MW(STX.2HCl) \times \frac{V_E\ (ml)}{m_H(g)} \times D_f \qquad (\text{Eq. } 4)$$

Dilution factor for SPE-C18 procedure is 4.0 (see Table-B1)

$$C_{(\mu gSTX\frac{eqv}{Kg})} = 0.04\ \mu M\ STX\ eqv. \times 372.2 \frac{\mu g}{\mu mol} \times \frac{10ml}{5g} \times 4\ = 116.0$$

$$Ratio = \frac{C.semiquantitation}{Level\ added} = \frac{679}{116} = 5.9 \qquad (Overestimation)$$

PST *semi* quantitation carried out in uncontaminated mussel samples (blank) with external standard addition of PST with low TEF (toxicity equivalence factor) value and quantified against screening standard solution as described in this SOP. The results are shown in Table C.3.

Table C.3- *Semi* quantitation of PST in mussel samples using external standard addition of PST with low TEF value.

Peak overlap	**C (µM)**	**C (µM eqv.**	**Level added (µg STX.2HCl**	***Semi* quantitation (µg STX.2HCl eqv/Kg)**	**Ratio**
C3,4, GTX6, C1,2, dcGTX2,3, dcNEO, GTX5	**0.01**	***0.01***	36	497	13.8
	0.05	***0.06***	179	1499	8.4
	0.10	***0.12***	357	2847	8.0
	0.20	***0.24***	715	5362	7.5
	0.50	***1.00***	1787	12613	7.1

Ratio > 1 (Over estimation), Ratio = 1 (No changes), Ratio < 1 (Under estimation)

Identified toxins using screening standard: GTX1&4, C1&2, GTX5, dcSTX

C3&4 coelute with GTX1&4. The dcNEO and GTX6 coeluted with dcSTX.

An example of *semi* quantitation:

$$C(\mu M\ equv.) = \sum C \times TEF$$

$$= 0.20 \times 0.1 + 0.20 \times 0.1 + 0.20 \times 0.1 + 0.20 \times 0.4 + 0.20 \times 0.4 + 0.20 \times 0.1$$

$$= 0.24 \mu M\ eqv.$$

$$C_{(\mu gSTX\frac{eqv}{Kg})} = C_{total}(\mu M\ STX\ eqv.) \times MW(STX.2HCl) \times \frac{V_E\ (ml)}{m_H(g)} \times D_f \qquad \text{(Eq. 4)}$$

Dilution factor for SPE-C18 procedure is 4.0 (see Table-B1)

$$C_{(\mu gSTX\frac{eqv}{Kg})} = 0.24\ \mu M\ STX\ eqv. \times 372.2 \frac{\mu g}{\mu mol} \times \frac{10ml}{5g} \times 4\ = 714.6 \approx 715$$

PST *semi* quantitation carried out in uncontaminated mussel samples (blank) with external standard addition of PST with high TEF (toxicity equivalence factor) value and quantified against screening standard solution as described in this SOP. The results are shown in Table C.4.

Table C.4- *Semi* quantitation of PST in mussel samples using external standard addition of PST compounds with high TEF value.

Peak overlap	C (µM)	C (µM eqv.)	Level added (µg STX.2HCl eqv/Kg)	*Semi* quantitation (µg STX.2HCl eqv/Kg)	Ratio
dcSTX NEO GTX1&4 STX GTX2&3	**0.01**	***0.05***	149	581	3.9
	0.05	***0.25***	744	1690	2.3
	0.10	***0.50***	1488	3109	2.1
	0.20	***1.00***	2978	6208	2.1
	0.50	***3.00***	7444	15052	2.0

Ratio > 1 (Over estimation), Ratio = 1 (No changes), Ratio < 1 (Under estimation)

Identified toxins using screening standard: GTX1&4, STX, GTX2&3, dcSTX, NEO.

PST *semi* quantitation carried out in uncontaminated mussel samples (blank) with external standard addition of all PST compounds, with low and high TEF (toxicity equivalence factor) value and quantified against screening standard solution as described in this SOP. The results are shown in Table C.5.

Table C.5- *Semi* quantitation of PST in mussel samples using external standard addition of PST with low and high TEF value.

Peak overlap	C (µM)	C (µM eqv.)	Level added (µg STX.2HCl eqv/Kg)	*Semi* quantitation (µg STX.2HCl eqv/Kg)	Ratio
C1&2 **C3&4 dcGTX2&3** **GTX1&4** **GTX2&3** **GTX5** **GTX6** **dcSTX** **dcNEO** **NEO** **STX**	**0.01**	***0.06***	173	738	4.3
	0.05	***0.29***	864	2689	3.1
	0.10	***0.58***	1727	5041	2.9
	0.20	***1.16***	3454	10169	2.9
	0.50	***3.00***	8635	24339	2.8

Ratio > 1 (Over estimation), Ratio = 1 (No changes), Ratio < 1 (Under estimation)

Identified toxins using screening standard: GTX1&4, C1&2, GTX5, GTX2&3, STX and NEO. C3&4 and dcGTX2&3 quantified as GTX1&4. The dcNEO, dcSTX and GTX6 quantified as NEO.

PST *semi* quantitation carried out with different matrices of shellfish contaminated with PST compounds. These samples have different toxin profiles with assigned values of total toxicity. The *semi* quantitation was carried out against the screening standard as described in this SOP. The results are shown in Table C.6.

Table C.6- *Semi* quantitation of PST in naturally contaminated shellfish samples.

Sample	Matrix	Assigned profile	Screening profile	Asigned value (µg STX.2HCl eqv/Kg)	Toxicity (µg STX.2HCl eqv/Kg)	Ratio	Species Profile
16/P/01	Mussel	GTX2&3, STX	dcGTX2&3, GTX2&3, STX, GTX1&4	848	948	1,1	*Alexandrium*
16/P/02	Mussel	-----	-----	Blank	---	-------	---
16/P/03	Mussel	dcGTX2&3, C1&2, C3&4, dcSTX, GTX5, GTX6	dcGTX2&3, dcSTX, GTX2&3, GTX5, STX, GTX1&4, NEO	1084	4715	4,3	*Gymndodinium*
17/P/01	Mussel	C1&2, C3&4, dcGTX2&3, dcNEO, dcSTX, GTX5, GTX6	dcGTX2&3, dcSTX, GTX5, STX, GTX1&4, NEO	1190	10753	9.0	*Gymndodinium*
17/P/02	Mussel	GTX2&3, STX	dcGTX2&3, GTX2&3, STX, GTX1&4	1989	1998	1,0	*Alexandrium*
17/P/03	Cockle	GTX2&3, STX, GTX1&4	dcGTX2&3, GTX2&3, STX, GTX1&4	825	1139	1,4	*Alexandrium*
18/P/01	Mussel	GTX2&3, STX	dcGTX2&3, GTX2&3, STX, GTX1&4	1674	1788	1.1	*Alexandrium*
18/P/02	Cockle	GTX2&3, STX, GTX1&4	dcGTX2&3, GTX2&3, STX, GTX1&4	497	973	2.0	*Alexandrium*
18/P/03	Clam	dcGTX2&3, dcSTX, dcNEO, GTX5, GTX6	dcGTX2&3, dcSTX, STX, GTX1&4, NEO	3549	4557	1.3	*Gymndodinium*

Ratio > 1 (Over estimation), Ratio = 1 (No changes), Ratio < 1 (Under estimation)

In all cases, the *semi* quantification gives an over estimation of the total toxicity in different matrices of shellfish samples.

Shellfish samples with a simple PST profile, such as *Alexandrium spp.*, give a total toxicity value in agreement with the assigned value for the sample.

Semi quantitative procedure was successfully applied to the screening of naturally contaminated shellfish samples for monitoring purposes.

C.2- Quantitative data analysis (Full method)

The quantitative data analysis was obtained from the performance method evaluation organized by the European Reference Laboratory for Marine Biotoxins (EURLMB), in accordance with the OMA AOAC 2005.06 method [14].

The following data were obtained in an interlaboratory study organized by Health Canada institution, Food Research Division, Ottawa, Canada in accordance with the AOAC® Official Methods Validation Program [13] in 2001. For this interlaboratory study, PST standard solutions from NRC Canada were used. Also, samples of shellfish, both blank and naturally contaminated, were mixed and homogenized to provide different PST toxin profiles at different concentration levels. The shellfish samples used in this study were clams, oysters, and scallops. 21 samples in total were sent to 21 collaborators but 3 laboratories withdrew after receiving the test materials. Results were obtained from 18 laboratories from different countries. Data from two laboratories were not included in the report, because insufficient information was provided to verify results [6-8].

Table C.7 shows the characteristics of the samples, and Table C.8 shows precision data for the blind duplicates after SPE C18 clean-up and C.9 after SPE-COOH cleanup, obtained by the interlaboratory study organized by Heath Canada.

Tabla C.7- Matrices used on the interlaboratory study organized by Heath Canada

Sample/Duplicate	Matrix	Observation
1	Scallop tissue	Naturally contaminated
2	Clams tissue	Naturally contaminated
3	Oysters tissue	Blank
4	Mussel tissue	Blank
5/5D	Clams tissue	Blank
6	Clams tissue	Naturally contaminated
7	Mussel tissue	Naturally contaminated
8/8D	Mussel tissue	Naturally contaminated
9	Mussel tissue	Naturally contaminated
10/10D	Oyster tissue	Naturally contaminated
11	Oyster tissue	Naturally contaminated
12	Mussel tissue	Naturally contaminated

Table C.8- Precision data for the blind duplicates after SPE C18 clean-up obtained by the interlaboratory study organized by Heath Canada

PST Toxin	STX			NEO	dcSTX	GTX1&4		GTX2&3			GTX5 (B1)			C1&2		
Sample/Dup.	5 /5D	8/8D	10/10D	8/8D	5/5D	5/5D	8/8D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D
Nº of Lab.	15	15	15	13	12	9	11	16	16	15	13	15	12	15	8	11
Accepted results	15	15	15	13	12	9	11	16	16	15	13	15	12	15	8	11
Average (µg/kg)	520	313	140	280	7.46	64.6	601	117	785	347	41.7	331	38.6	241	101	169
STD. Dev. (µg/Kg)	31.4	67.8	25.0	43.1	0.68	12.9	120	15.8	136	66.6	5.26	27.9	8.35	37.5	28.6	53.5
RSD (%)	6.03	21.7	17.8	15.4	9.08	19.9	20.0	13.5	17.3	19.2	12.6	8.42	21.6	15.5	28.3	31.7
HorRat	0.78	1.23	1.46	1.55	0.81	1.55	1.50	0.90	1.69	1.25	0.73	0.79	1.16	1.12	1.68	1.52

Table C.9- Precision data for the blind duplicates after SPE COOH clean-up obtained by the interlaboratory study organized by Heath Canada

PST Toxin	STX			NEO		dcSTX	GTX1&4		GTX2&3			GTX5 (B1)			C1&2		
Sample/Dup.	5 /5D	8/8D	10/10D	8/8D	5/5D	5/5D	5/5D	8/8D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D	5/5D	8/8D	10/10D
Nº of Lab.	15	13	14	13	10	9	12	15	15	16	14	11	14	10	14	8	14
Accepted results	15	13	14	13	10	9	12	15	15	16	14	11	14	10	14	8	14
Average (µg/kg)	492	273	132	263	40.5	6.89	73.9	660	124	775	357	40.7	280	35.3	230	101	203
STD. Dev. (µg/Kg)	44.0	36.2	32.9	67.7	7.82	0.88	11.1	85.8	19.9	91.1	84.3	5.04	40.6	8.49	46.1	20.0	37.1
RSD (%)	8.94	13.2	24.9	25.8	19.3	12.8	15.0	13.0	16.0	11.8	23.6	12.4	14.5	24.1	20.0	19.8	18.3
HorRat	0.98	1.13	1.32	1.75	1.51	1.35	1.11	1.20	1.03	1.69	1.26	0.98	0.89	1.02	1.35	1.78	1.28

PST contaminated fresh shellfish samples (Table C.10) were used to evaluate the performance of National Reference Laboratory network, organized by European Reference Laboratory for Marine Biotoxins (EURLMB) [6-8], to apply the Lawrence HPLC-FLD method for the Official Control of PST toxins (AOAC 2005.06).

Different shellfish matrices, naturally contaminated with PST, were analyzed following the conditions described in this SOP based on AOAC Official Method (AOAC 2005.06) for the determination of PST using precolumn HPLC-FLD method (Table C.11 to C.14).

Table C.10- Matrices used on the interlaboratory study organized by EURLMB from European Union (UE).

sample No	matrix	characteristics
CRL/06/P01	mussel, naturally contaminated	blind duplicate of CRL/06/P/05
CRL/06/P02	*Acanthocardia tuberculata*, giant cockle, naturally contaminated	
CRL/06/P03	clams, naturally contaminated	
CRL/06/P04	scallop, naturally contaminated	
CRL/06/P05	mussel, naturally contaminated	blind duplicate of CRL/06/P/01
CRL/06/P06	mussel, naturally contaminated	
CRL/07/HYDR01	Mussels (*Mytilus galloprovincialis*), Galicia-Spain	naturally contaminated
CRL/07/HYDR02	Mussels (*Mytilus galloprovincialis*), Galicia-Spain	naturally contaminated
CRL/08/P01 and CRL/08/P07	Clam, *Venuerupis Pullarstra*, Obidos Lagoon (Portugal), Galicia (Spain)	Blank sample (Galicia) mixed with naturally contaminated sample (Portugal)
CRL/08/P02	Mussel, Mytilus galloprovincialis, Galicia (Spain)	Blank sample, acquired at retail market
CRL/08/P03 and CRL/08/P08	Mussel, Mytilus galloprovincialis, Galicia (Spain)	Blind duplicates, blank material mixed with naturally contaminated sample.
CRL/08/P04	Clam, *Spisula solida*, Sines (Portugal)	Naturally contaminated
CRL/08/P05	Mussel, *Mytilus galloprovincialis*, Galicia (Spain)	Sample CRL/08P02 spiked with NRC dcGTX2&3b at 0,807 µmol/kg.
CRL/08/P06	Clam, *Meretryx lyrata*, Vietnam	Sample CRL/08P10 spiked with NRC dcGTX2&3b at 0,773 µmol/kg.
CRL/08/P09	Mussel, *Mytilus galloprovincialis*, Galicia (Spain)	Sample CRL/08/P02 spiked with NRC dcGTX2&3b at 1,84 µmol/kg.
CRL/08/P10	Clam, *Meretryx lyrata*, Vietnam	Blank sample
CRL/08/P11	Clam, *Meretryx lyrata*, Vietnam	Sample CRL/08/P10 spiked with NRC dcGTX2&3-b at 1,80 µmol/kg.

Table C.11- Precision data for the Total toxicity in raw shellfish samples obtained by the interlaboratory study organized by EURLMB.

Sample	CRL/06/P01	CRL/06/P02	CRL/06/P03	CRL/06/P04	CRL/06/P05	CRL/06/P06
Nº LAB	18	18	16	17	18	13
Nº Outliers	2	1	1	2	2	2
Total toxicity (μg STX2HCl eq/kg)	1708,3	1033,9	2829,4	319,8	1524,9	105,4
Std. Dev. (μg STX2HCl eq/kg)	469,1	277,8	610,0	82,6	418,7	34,1
RSDD (%)	27,89	24,10	19,48	17,92	22,35	24,80
HorRat	1,88	1,51	1,42	0,95	1,49	1,10

Table C.12- Precision data for the Total toxicity in raw shellfish samples obtained by the interlaboratory study from 2007 and 2008 organized by EURLMB.

Sample	CRL/07/HYDR01	CRL/07/HYDR02	CRL/08/P01	CRL/08/P03	CRL/08/P04	CRL/08/P07	CRL/08/P08
Nº LAB	8	7	10	10	10	10	10
Nº Outliers	1	0	1	2	1	1	2
Total toxicity (μg STX2HCl eq/kg)	260	1261	3277	3030	1436	3322	3078
Std. Dev. (μg STX2HCl eq/kg)	90,9	441,3	983	908	430	996	923
RSDD (%)	16,7	33,4	39,5	21,2	21,2	27,0	28,3
HorRat	0,85	2,16	2,95	1,57	1,39	2,02	2,09

Table C.13- Precision data for individual PST in raw shellfish samples obtained from interlaboratory study in 2007 and 2008 organized by EURLMB.

Sample	dcGTX2&3	C1&2	dcSTX	GTX5 (B1)	GTX6 (B2)	dcNEO
	Level (µmol/kg) Std. Dev. RSD (%) ***HorRat***	Level (µmol/kg) Std. Dev. RSD (%) ***HorRat***	Level (µmol/kg) Std. Dev. RSD (%) ***HorRat***	Level (µmol/kg) Std. Dev. RSD (%) ***HorRat***	Level (µmol/kg) Std. Dev. RSD (%) ***HorRat***	Level (µmol/kg) Std. Dev. RSD (%) ***HorRat***
CRL/07/HYDR01		1.96 0.65 44.4 ***2.74***	0.33 0.11 26.1 ***1.12***	1.26 0.20 11.6 ***0.65***	2.64 0.92 20.9 ***1.31***	
CRL/07/HYDR02	1.19 0.42 46.1 ***2.53***	7.93 1.15 42.4 ***3.24***	2.18 0.47 35.6 ***2.04***	5.42 1.07 7.27 ***0.51***	11.7 4.08 45.6 ***3.59***	
CRL/08/P01	2.07 0.62 35.5 ***2.12***	15.3 2.23 19.4 ***1.63***	4.23 0.45 15.6 ***0.99***	13.7 2.71 16.8 ***1.25***	12.8 3.85 37.7 ***3.01***	4.61 1.38 72.4 ***4.68***
CRL/08/P03	2.48 0.75 34.7 ***2.13***	19.1 2.78 18.7 ***1.63***	3.97 0.42 16.8 ***1.05***	9.50 1.88 22.9 1.74	18.8 5.65 39.2 ***3.31***	4.67 1.40 70.4 ***4.57***
CRL/08/P04	3.84 1.15 28.02 ***1.85***		3.44 0.36 16.4 ***1.01***			2.18 0.65 56.4 ***3.26***
CRL/08/P05	0.43 0.13 30.3 ***1.57***					
CRL/08/P06	0.45 0.13 29.9 ***1.54***					
CRL/08/P07	2.45 0.74 40.4 ***2.48***	15.9 2.32 19.7 ***1.67***	4.31 0.45 32.1 ***2.03***	14.30 2.82 17.5 ***1.41***	13.3 3.98 30.9 ***2.48***	4.78 1.43 53.9 ***3.51***
CRL/08/P08	2.62 0.79 45.7 ***2.82***	20.8 2.72 26.7 ***2.36***	3.88 0.41 16.9 ***1.06***	9.50 1.88 19.5 ***1.48***	17.4 5.22 34.2 ***2.86***	5.22 1.57 48.7 ***3.21***
CRL/08/P09	0.99 0.29 29.1 ***1.71***					
CRL/08/P11	0.96 0.38 39.6 ***2.31***					

Table C.14- Precision data for individual PST compounds and total of toxicity in duplicate of raw shellfish samples from interlaboratory study in 2008 organized by EURLMB.

	dcGTX2&3		C1&2		dcSTX		GTX5 (B1)		GTX6 (B2)		dcNEO		Total toxicity	
Sample	*CRL/O8/ P1 and 7*	*CRL/O8/ P3 and 8*	*CRL/O8/ P1 and 7*	*CRL/O8/ P3 and 8*	*CRL/O8/ P1 and 7*	*CRL/O8/ P3 and 8*	*CRL/O8/ P1 and 7*	*CRL/O8/ P3 and 8*	*CRL/O8/ P1 and 7*	*CRL/O8/ P3 and 8*	*CRL/O8/ P1 and 7*	*CRL/O8/ P3 and 8*	*CRL/O8/P 1 and 7*	*CRL/O8/P 3 and 8*
Nº LAB	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Nº Outliers	1	3	2	1	2	2	1	1	3	4	3	3	1	1
Nº Lab no outliers	10	8	9	10	10	9	10	10	8	7	8	8	10	10
Total toxicity (μg STX2HCl eq/kg)	2,3	2,3	15,2	20,2	4,1	3,9	14,0	9,5	4,0	4,1	13,0	18,1	2976,8	3120,6
Std. Dev. (μg STX2HCl eq/kg)	0,70	0,36	3,25	2,98	0,61	0,31	1,46	0,65	0,93	1,36	1,36	2,74	395,4	631,2
RSD (%)	30,9	15,7	21,4	14,9	15,0	7,99	10,5	6,82	23,5	32,9	10,4	15,1	13,3	20,2