AOAC 2005.06 Standard Operating Procedure

Simplified Version for the screening and semi-quantitation of PSP toxins

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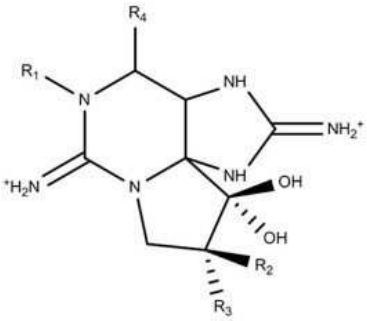
INTRODUCTION:

Paralytic Shellfish Toxins (PST's) and its analogues (Fig. 1) are produced by marine dinoflagellates such as *Gymnodnium 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 and produced by those dinoflagellates. These PST toxins can be called "*Saxitoxins*" deriving the name from the first evidence of PST intoxication and the natural toxin associated (Saxitoxin, *Saxidomus giganteus*).

Clinical symptoms related with PST intoxication are neurological such as ataxia, tachycardia, respiratory depression or failure heart paralysis, etc. The lethal dose (LD_{50}) found for these PST compounds is 800µg STX.2HCl eqv. /Kg of shellfish tissue.

These natural toxins are common part (purine structure) in their chemical structure, the functional group variation in its structure allowed to subdivide in different subgroup according to the chemical or toxicological properties (figure 1). Multiple toxicities, which vary widely according to the many toxin structures 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 occurs, leading to the numerous structural analogues of this class of compound. Substitution of these positions with hydrogen, hydroxyl or sulfate groups can occur and links again to relative toxicity (e.g. those with sulfate groups have lower toxicity than those without) For e.g., despite GTX4 having the carbamoyl functional group, similar to STX, the sulfate group in the R2 position, leads to a lower toxicity relative to NEO, which has a hydrogen replacement.



Toxin	R_1	R_2	R_3	R_4	Toxicity Equivalency Factors	
					Oshima	EFSA
STX	H	H	H		1.00	1.00
GTX2	H	H	OSO_3^-		0.36	0.40
GTX3	H	OSO_3^-	H		0.64	0.60
NEO	OH	H	H		0.92	1.00
GTX1	OH	H	OSO_3^-		0.99	1.00
GTX4	OH	OSO_3^-	H		0.73	0.70
M2	H	OH	H		-	-
M4	H	OH	OH		-	-
GTX5	H	H	H		0.06	0.10
C1	H	H	OSO_3^-		0.01	-
C2	H	OSO_3^-	H		0.10	0.10
C3	OH	H	OSO_3^-		0.01	-
C4	OH	OSO_3^-	H		0.06	0.10
GTX6	OH	H	H		0.06	0.10
M1	H	OH	H		-	-
M3	H	OH	OH		-	-
dcSTX	H	H	H		0.51	1.00
dcGTX2	H	H	OSO_3^-		0.15	0.20
dcGTX3	H	OSO_3^-	H	-OH	0.38	0.40
dcNEO	OH	H	H		-	0.40
dcGTX1	OH	H	OSO_3^-		-	-
dcGTX4	OH	OSO_3^-	H		-	-

Figure 1: Chemical structure of PSP toxins

There are 3 main groups of saxitoxin analogues, identified by their substitution at the R4 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 PSP also has been identified. A sulfate group replaces a hydrogen within the R4 position and, therefore, are known as sulfamate saxitoxins. The substitution at the R4 position affects the relative toxicity of the analogues.

This method (and many of the most widely used LC methods applied to research of these PSP toxins) use oxidation to fluorescent products. Whether based on post-column reaction HPLC (such as many of the earliest chromatographic methods for the PSP toxins) or as in the precolumn oxidation HPLC-FLD (AOAC 2005.06), so called Lawrence method, which is the Reference method for the control of PSP toxins in the EU, the main oxidation products resulting from the PSP toxins are purine derivatives, having the aromatic structure required for fluorescence detection of these compounds. There is a loss of the ring structure near the R1, R_2, positions.

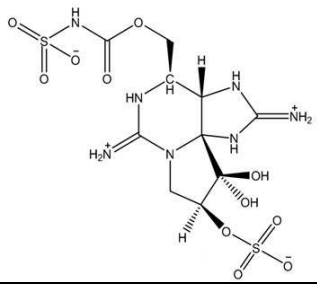
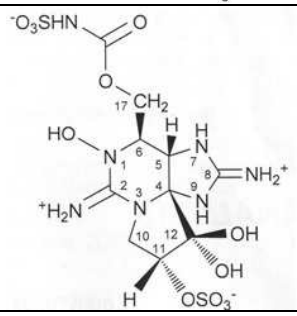
Two different reagents can be used for the precolumn oxidation: peryodate (IO_4^-) and hydrogen peroxide (H_2O_2). The oxidation with Hydrogen peroxide does not yield fluorescent derivatives for the hydroxilated compounds (R_1= OH), while the fluorescence intensity for non-hydroxylated compound (R_1= 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.

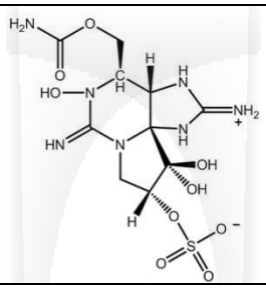
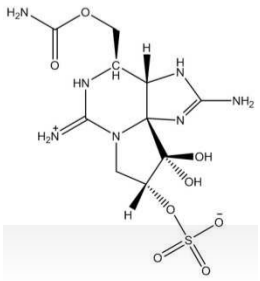
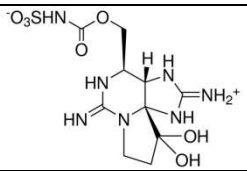
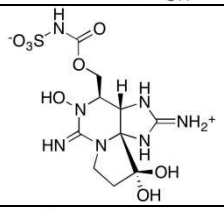
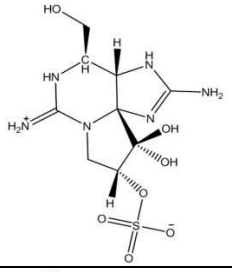
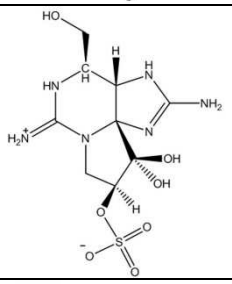
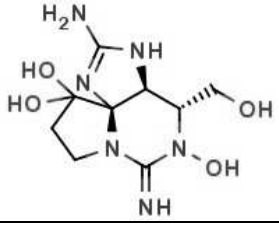
The peroxide and periodate oxidation reactions yield single products for the non-hydroxylated toxins. The periodate oxidation reaction yields 3 products for the hydroxylated toxins. No products observed with peroxide. The peroxide and periodate oxidation reactions yield 2 major products for the decarbamoyl toxins. The oxidation reaction yields the same products for epimeric pairs. The stability of the oxidized compounds is critical being recommended to carry out the HPLC-FLD analysis right after the oxidation.

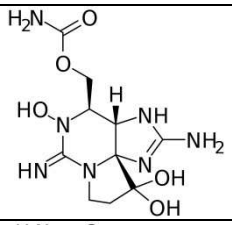
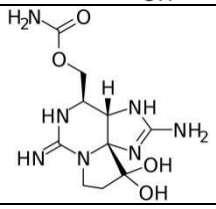
AOAC OMA 2005.06 includes a sample extraction with acetic acid, SPE purification and fractionation and a derivatization step prior to the HPLC injection. Total PSP toxicity is calculated by summing individual toxin concentrations multiplied by the corresponding toxicity equivalence factor (TEF). The final result must be expressed as saxitoxin equivalents per kilogram of bivalve tissue, which will be compared to the EU regulatory level of 80 µg STX eq/100.

PSP - PHYSICO-CHEMICAL PROPERTIES

Table I includes the physico-chemical properties on the PSP toxins included in the scope of the method.

Toxin	Chemical Structure	Molecular formula	Molecular weight (g/mol)	Charge	CAS Numer
C1+C2		$C_{10}H_{17}N_7O_{11}S_2$	475.414	0	80226-62-6
C3+C4		$C_{10}H_{17}N_7O_{12}S_2$	491.4	0	

GTX1+4		$C_{10}H_{17}N_7O_9S$	411.346	+1	60748-39-2
GTX2+3		$C_{10}H_{16}N_7O_8S$	395.349	+1	60508-89-6
GTX5		$C_{10}H_{17}N_7O_7S$	379.352	+1	64296-25-9
GTX6		$C_{10}H_{17}N_7O_8S$	392.35	+1	82810-44-4
dcGTX2+3		$C_9H_{16}N_6O_7S_1$	352.3243	+1	86996-87-4 (dcGTX2) 87038-53-7 (dcGTX3)
dcSTX		$C_9H_{16}N_6O_3$	256.26	+2	58911-04-09
dcNEO		$C_9H_{16}N_6O_4$	272.26	+2	68683-58-9

NEO		$C_{10}H_{17}N_7O_5$	315.286	+2	64296-20-4
STX		$C_{10}H_{17}N_7O_4$	299.291	+2	35523-89-8

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 all most PSP compounds are similar to saxitoxin (STX), pKa1= 8.24 and pKa2= 11.60.

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

AIM AND SCOPE

The purpose of this Operating Procedure is to describe the procedure by which the quantitative method, ***AOAC Official Method 2005.06*** can be applied to the rapid routine qualitative screening and semi-quantitation of paralytic shellfish toxins (PST) in shellfish. 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. 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 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 saves the analyst much time and expense since the extra steps involved in the accurate quantification are not needed.

The method 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), N-sulfocarbamoyl gonyautoxins 2 and 3 (together: C1, 2), decarbamoylgonyautoxins 2 and 3 (together dcGTX2,3) and decarbamoylneosaxitoxin (dcNEO or GTX7). 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 Annex III of Regulation (EC) No 882/2004 and documented in a validation report. The method must be shown to meet the minimum performance criteria given in Table 1.

Laboratories are expected to take part in regular proficiency testing exercises (where available) and to perform satisfactorily in these tests.

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

Table 1: Minimum Performance Criteria

CRITERIA		Minimum Performance
Chromatographic resolution (Rs)		dcSTX and dc GTX2/3, Chromatographic peaks resolution should be ≥ 1.5
Limit of Detection (LOD)		Individual toxin LOD should be equal or lower than 1:50th of regulatory level, plus presence of applicable secondary peaks.
Limit of Quantitation (LOQ)		Individual toxin LOD should be equal or lower than 1:20th of regulatory level.
Calibration range		Should be equal or higher than individual toxin LOQ value.
Linearity		R^2 ≥0.98
SPE-C18 Recovery (Batch quality control)		Between 80-120%
SPE-COOH (Batch quality control)		Between 80-120%
Recovery		Mussel (51 to 112%) Oyster (51 to 160%) Cockles (90-128%)
Selectivity		Absence of chromatographic peaks from co-extractive components from the matrix and from matrix modifier (MM)
Precision	Intra-Batch	R_t (± 0.2 min) Peak Area (RSD ≤ 3.0%) Reproducibility of toxin peaks in positive sample control or CRM material should be ≤ 20%
	Inter-Batch	Reproducibility of toxin peaks in positive sample control or CRM material should be ≤ 25%
Uncertainity		Not applicable
Ruggedness		Not applicable

MATERIALS

i) REFERENCE MATERIALS (*)

CRM-STX Standard reference material with saxitoxin

CRM-NEO Standard reference material with neosaxitoxin

CRM-dcSTX Standard reference material with decarbamoyl saxitoxin

CRM-dcNEO Standard reference material with decarbamoyl neosaxitoxin

CRM-dcGTX2,3 Standard reference material with mixture of decarbamoyl gonyautoxin 2 and decarbamoyl gonyautoxin 3

CRM-GTX1,4 Standard reference material with mixture of gonyautoxin 1 and gonyautoxin 4

CRM-GTX2,3 Standard reference material with mixture of gonyautoxin 2 and gonyautoxin 3

CRM-GTX5	Standard reference material with gonyautoxin 5
CRM-GTX6	Standard reference material with gonyautoxin 6
CRM-C1,2	Standard reference material with N-sulfocarbamoyl gonyautoxin 2 and N-sulfocarbamoyl gonyautoxin 3

(*) OTHERS:

- When available samples of naturally occurring PSP toxin contaminated materials with assigned values will be used as Laboratory Reference Materials (LRM). These samples should ideally contain a number of both N-Hydroxylated and non N-Hydroxylated toxins.

- PO PST CRM 1101 Reference material raw oyster tissue homogenized sterilized from Pacific (*Crassostrea gigas*) containing: STX, GTX1, GTX2, GTX3, GTX4, NEO, C1 (GTX 8), C2 (*epi*GTX8) and trace of dcGTX2, dcGTX3 and GTX5 is distributed by CEFAS.

IMPORTANT: *Certified Reference Materials and Solutions can be purchased from different sources. This is an example for suitable products available commercially. Other Certified materials can be used if available and if they can be shown to lead to the same results.*

i) CHEMICALS:

Name	Formula	Use for:
Acetic acid (Glacial)	CH_3COOH	Extraction, pH adjust, Stop oxidize reaction
Acetonitrile	CH_3CN	HPLC mobile phase
Ammonium formate	NH_4OOCH	HPLC mobile phase
Ammonium acetate	NH_4COOCH_3	Periodate oxidation
Disodium Hydrogen phosphate anhydrous	Na_2HPO_4	Periodate oxidation
Periodic acid	H_5IO_6	Periodate oxidation
Sodium hydroxide	NaOH	Peroxide oxidation, pH adjustment
Sodium Chloride	NaCl	SPE-COOH fractioning
Methanol	MeOH	SPE-C18 conditioning
Hydrogen peroxide (30%$_{w/v}$)	H_2O_2	Peroxide oxidation
Ultrapure water	H2O	Solution preparation HPLC mobile phase SPE-C18 conditioning

ii) WORKING SOLUTIONS

Solution	Preparation	Quantity	Purpose fit:	Expire
1M NaOH	Dissolved 4 g of NaOH in deionized water	100 mL	pH adjust, H_2O_2 Oxidation	1 month
0,2M NaOH	Dilute 1 M of NaOH (20 mL) in deionized water	100 mL	pH adjust	1 week
0,1M NaOH	Dilute 1 M of NaOH (10 mL) in deionized water	100 mL	pH adjust	1 week
1% $_{(v/v)}$ CH_3COOH	Dilute 1 mL of Glacial Acetic acid in deionized water.	100 mL	PSP extraction	1 month
0,6% $_{(v/v)}$ or 0.1M of CH_3COOH	Dilute 572 µL of Glacial Acetic acid in deionized water.	100 mL	pH adjust	1 month
0,01M NH_4OOCCH_3	Dissolved 4 g of 0,077 g of NH_4OOCCH_3 in deionized water	100 mL	SPE-COOH cartridge conditioning	1 month
2M NaCl	Dissolved 11.7 g of NaCl in deionized water	100 mL	Stock solution	1 month
0,3M NaCl	Dilute 15 mL of 2M NaCl in deionized water	100 mL	SPE-COOH fractioning procedure	1 week
0,05M NaCl	Dilute 2,5 mL of 2M NaCl in deionized water	100 mL	SPE-COOH fractioning procedure	1 week
0,03M H_5IO_6 (HIO_4)*	Dissolved 0.6838 g of H_5IO_6 in deionized water	100 mL	Oxidizing reagent (periodate)	1 month (4 ^{0}C)
0,3M $HCOONH_4$	Dissolved 1.8918 g of $HCOONH_4$ in deionized water	100 mL	Oxidizing reagent (periodate)	1 month
0,3M Na_2HPO_4	Dissolved 1.8918 g of Na_2HPO_4 in deionized water	100 mL	Oxidizing reagent (periodate)	1 month
10% $_{(w/v)}$ of H_2O_2	Dilute 3 mL of 30% $_{(w/v)}$ H_2O_2 into 6 mL of deionized water.	9 mL	Oxidizing reagent (peroxide)	1 day (store in the dark)
Oxidizing solution (IO_4^-)	Add: 5 mL of 0.03M HIO_4 , 5 mL of 0,3M NH_4COOH and 5 mL of 0.3M Na_2HPO_4 (Adjust pH to 8,2 with 1M NaOH)	15 mL	Oxidizing Reaction (periodate)	1 day
0,1M NH_4COOH	Dissolve 6,31 g of NH_4COOH into 800 mL of deionized water (adjust pH to 6.0 with 0.1M of CH_3COOH and/or 0,1M NaOH)	1000 mL	Mobile phase A (for HPLC)	1 week
0,1M NH_4COOH (with 5% CH_3CN)	Dissolve 3,16 g of NH_4COOH into 400 mL of deionized water add 25 mL of CH3CN (adjust pH to 6.0 with 0.1M of CH_3COOH and/or 0,1M NaOH)	500 mL	Mobile phase B (for HPLC)	1 week

iii) CONSUMABLES AND EQUIPMENTS

Type	Item
Consumable	General glassware
	SPE-C18 cartridges
	SPE-COOH cartridges
	1 mL syringe
	0,45µm membrane filter
	Glass vials (1 mL capacity)
	Centrifuge tubes
	15 mL plastic graduate centrifuge tubes
	Plastic Eppendorf vials
	Plastic and glass Pasteur pipettes
	Tip plastic pipette
Equipment	Analytical balance
	Pipettes (5-20, 20-100, 100-1000 and 1000-5000 µL)
	HPLC System and FLD detector
	Water bath
	pH meter
	Vortex system
	Ultraturrax homogenizer
	Centrifuge (1000 to 10000rpm)
	Manifold system
	Vacuum pump

HPLC-FLD METHOD

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	1 mL/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			

NOTE: (*) Chromatographic conditions can be adjusted and optimized. Mobile phases must be filtered using 0.45µm filter membrane and degas.

REQUIREMENTS FOR SEQUENCE ANALYSIS

The table 2 shows briefly and general solutions it is necessary for PSP toxins using HPLC-FLD method.

Table 2: General requeriments for PSP analysis using HPLC-FLD with pre-oxidation derivatization

Parameter	**H_2O_2 oxidation**	**IO_4^- oxidation**
Procedural blank	√	√
Uncontaminated sample extract	√	√
Contaminated sample extract	√	√
Matrix modifier (MM)	X	√
Blank of oxidation	√	√
No N-OH PSP standards solution	√	X
N-OH PSP standards solution	X	√
dcSTX standard solution	√	√

ANALYTICAL PROCEDURE

1.- Preparation of samples

The outside of shellfish must be washed with cold water if necessary. The inside of shellfish must be also washed with cold water if necessary and further drain.

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

Note: *Fresh shellfish samples should be immediately homogenized no longer then 48h after reaching laboratory.* Shellfish tissue homogenates must be stored at ≤ -12 ^{0}C.

2.- Preparation of extracts:

Accurately weigh 5,0 ± 0,1 g of tissue into 50 mL polypropylene centrifuge tubes. Add 3,0 mL 1% $_{V/V}$ CH_3COOH, and mix thoroughly on a vortex for 2 min. Cap and place in a boiling water bath for 5 min. Remove from water bath, and cool until achieve room temperature. Remix on a vortex for 2 min. Centrifuge at ≥ 4000 g for 10 min. Transfer the supernatant to a 15 mL plastic graduate tube. Add 3,0 mL of 1% $_{V/V}$ CH_3COOH and repeat the extraction procedure without

boiling step. Both extraction solvents were combined and the volume was adjusted to 10.0 mL with deionised water.

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

For the preparation of ***Matrix Modifier (MM)*** use uncontaminated oyster tissue homogenized and submits to sample extraction and SPE-C18 purification.

Note: *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*

Acetic acid shellfish extracts and purified SPE-C18 extracts may be stored at 2-9 0C for maximum of 10 days.

3.- Purification of extracts:

Clean-up of the acidic acid extracts must be carried out using SPE C18 cartridges (3 mL, 500 mg) as follows:

Parameter	Conditions	Action
Conditioning	6 mL of methanol	Discharge
	6 mL of water	Discharge
Load	1 mL of acetic extract	Collect (*)
Wash	2 mL of water	Collect(*)

(*) same graduate tube. Adjust pH of cleaned extract between 6.3 -6.7 with 0.2M or 0.1M NaOH

4.- Fractionation : Cationic exchange SPE cartridges must be used for fractionation (SPE COOH, 3 mL, 500 mg) under the following conditions:

Parameter	Conditions	Graduate tube	Action
Conditioning	10 mL of 0.01M ammonium acetate		Discharge
Load	2mL of acetic extract from SPE-C18	1	Collect (*)
Wash	4mL desionised water	1	Collect(*)
Elution	4 mL of 0.05M NaCl	2	Collect
	5 mL of 0.3M NaCl	3	Collect

(*) Same graduate tube. Purified extracts must be filtered through 0.45µm filter membrane

Note: 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.

5.- Derivatization reaction:

Derivatization is carried out through an oxidation reaction using periodate or hydrogen peroxide.

Matrix Modifier (MM)		5g oyster tissue, extraction with 1%$_{(V/V)}$ acetic acid follow SPE-C18 purification (*see extraction and purification section*).
Oxidation reagent	***Peroxide***	Hydrogen peroxide (10%$_{V/V}$)
	Peryodate	Peryodic acid: Ammonium formate: Sodium Hydrogen phosphate (1:1:1)$_{V/V}$, pH 8.2 (adjust with NaOH)
Stop Reaction		Glacial acetic acid

i)**Periodate oxidation**: Transfer to 1.5 mL Eppendorf Tube as follows:

a) 100µL of standard solution (or sample extract)
b) 100µL matrix modifier (MM) and vortex
c) 500µL periodate oxidant reagent, vortex, and let react 1 minute at room temperature.
d) 5µL glacial acetic acid, vortex, and let stand 10 minutes at room temperature
e) Filter the reaction solution through 0.45µm membrane filter, and inject into HPLC-FLD.

ii)**Peroxide oxidation**: Transfer to 1.5 mL Eppendorf Tube, as follows:

a) 25µL of hydrogen peroxide (H_2O_2, 10%$_{V/V}$), 250µL of 1M NaOH and vortex.
b) 100µL of standard solution or sample extract, vortex and let react 2 minutes at room temperature.
c) 20µL glacial acetic acid and vortex
d) Filter the reaction solution through 0.45µm membrane filter, and inject into HPLC-FLD.

IMPORTANT: *Oxidation reagents should be prepared freshly and right before the oxidation reaction. Periodate oxidation needs matrix modifier from oyster tissue (matrix modifier is prepared following the conditions described in preparation of extract section). Oxidised PSP standards and extracts are stable a maximum of 12h.*

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 deionised water instead periodate or peroxide). This need not be done with every sample. It needs to be done when any regulatory action is to be taken.

6. Acidic hydrolysis

Acidic hydrolysis must be carried out for C3 / C4 and GTX6 quantitation only when CRM standards are not available.

The toxin quantitation is carried out indirectly as the product from the hydrolysis reaction.

Toxins (Sulfamate)	Reaction conditions	Product of reaction
C3	**0.1M HCl**	***GTX1***
C4	90 ^{0}C	***GTX4***
GTX6	20min.	***NEO***

Carry out the reaction as follows:

Transfer to 10 mL glass Pyrex® tube,

a) 900µL of extract and 225µL of 0.1M HCl, vortex, close hermetically the tube (sample lost during the hydrolysis reaction must be avoided).
b) Incubate at 90^{0}C for 20 minutes and then cool at room temperature
c) 225µL of 1M sodium hydroxide (NaOH), vortex and filter using 0.45µm membrane filter
d) Hydrolyzed extract can be used for oxidation purposes.

QUALITY ASSURANCE

Parameter	Acceptance criteria
Retention times drift	≤ ± 2,0%
Toxins identification	The toxin peaks are identified in LRM or samples by comparing the match of the retention times with those expected of the standard reference material.
Shewhart chart	Record on suitable Shewhart chart area response for peak of interest in LRM material and Standards.
Procedural blank	Any signal (S/N ≤ 3.0)

Oxidation performance	Toxins give more then than 1 oxidation product, the ratio between peak areas should be controlled. dcSTX (H_2O_2), ratio= ± 8.0, dcSTX (IO_4^-)=± 4.0.
LRM* (Laboratory Reference Material)	RSD (%) for RT (n=3)≤ ± 2,0% RSD(%) for peak area (n=3) ≤ ±25%

(*) Laboratory Reference Material: Certified shellfish tissue reference material for PSP if commercially available, shellfish samples with assigned values e.g. contaminated materials from PT exercises or uncontaminated materials spiked with PSP toxins pure standard solutions (matrix matched standards).

DATA ANALYSIS:

Figure 2 shows the scheme of the different steps of ***AOAC OMA 2005.06***

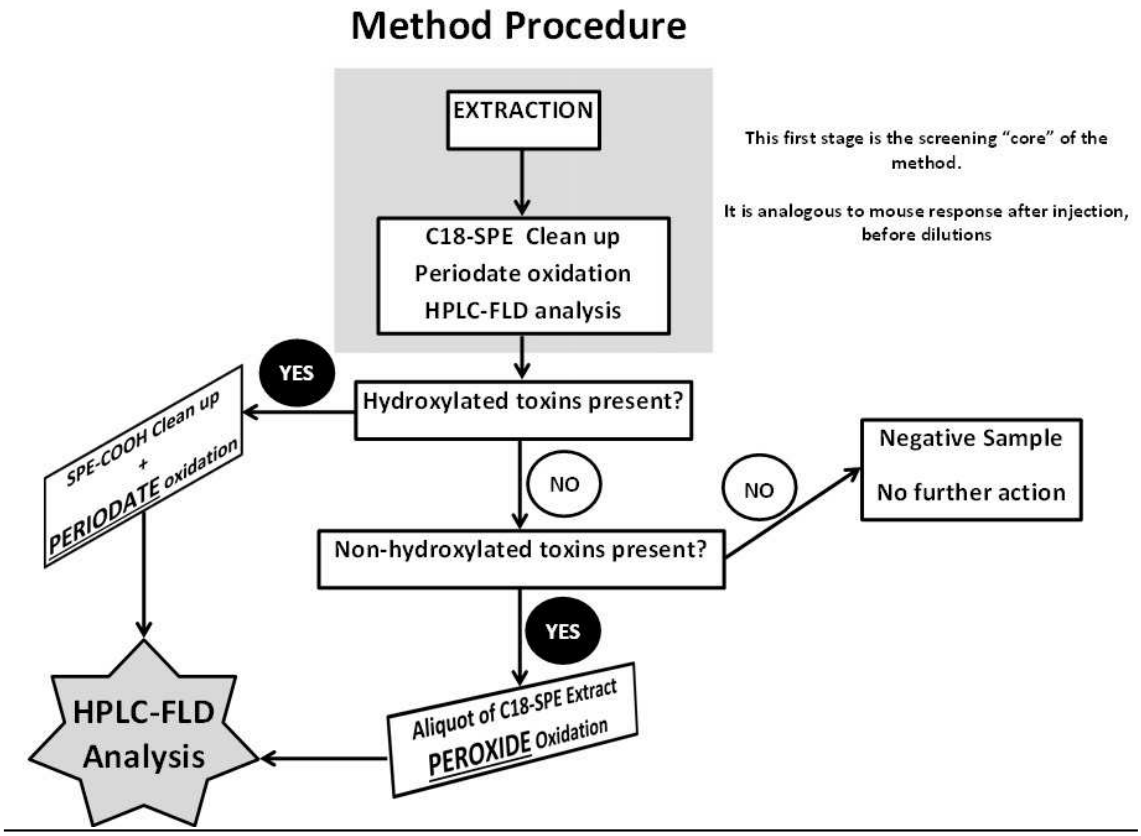


Figure 2: Scheme of PSP toxin analysis by HPLC-FLD using Prechromatografic oxidation

a) Interpretation of chromatographic data

Providing that the HPLC run has been acceptable, the following procedures are applied to determine the PSP toxins profile.

The chromatographic result of C18 purified extracts and standard used for screening purpose and both oxidized with periodate, the following decisions must be made:

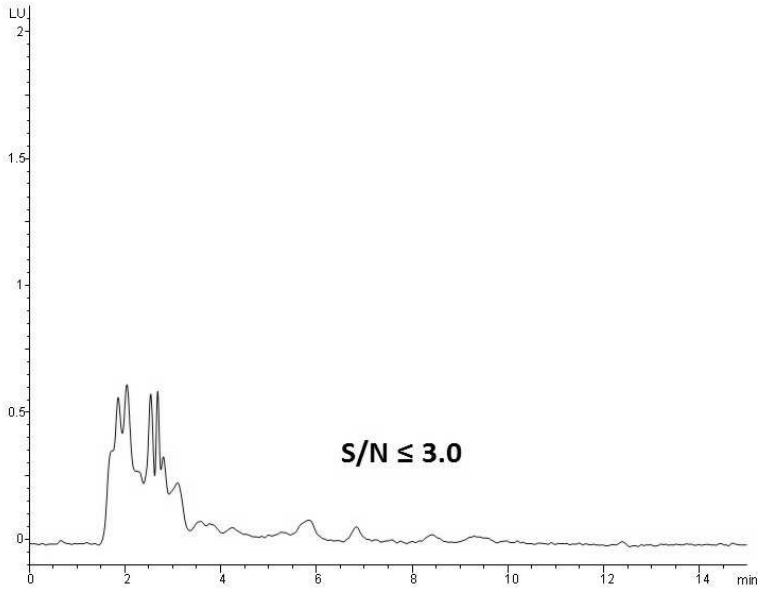


Figure 3: Uncontaminated mussel sample oxidized with periodate (Blank)

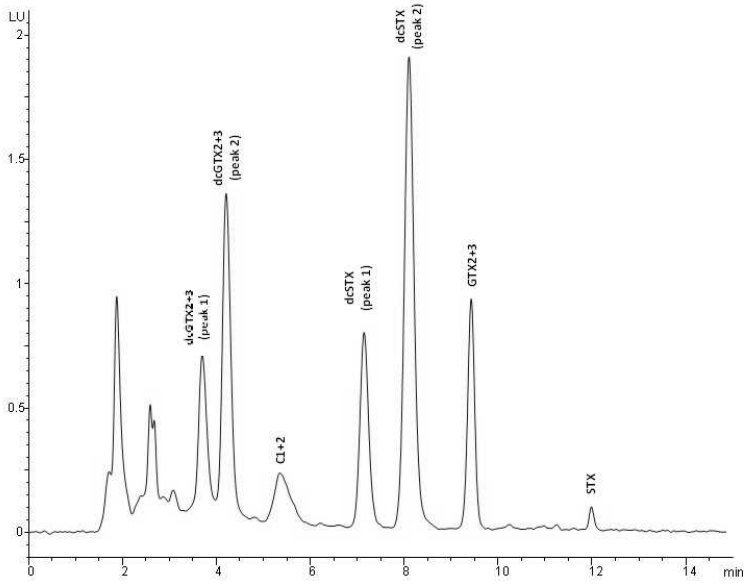


Figure 4, Contaminated mussel sample oxidized with periodate

If no toxin oxidation product peaks are detected (fig. 3) then the sample is considered negative and no further analysis or calculation is required for that sample.

Use a PSP mix of certified reference standards, which contains GTX1/4, C1/2, dcSTX, GTX2/3, GTX5 and STX, for Identification and semiquantitation purposes in contaminated samples (Fig.4).

Figure 5 shows the scheme to perform AOAC 2005.06 for screening, semiquantitation and quantitation.

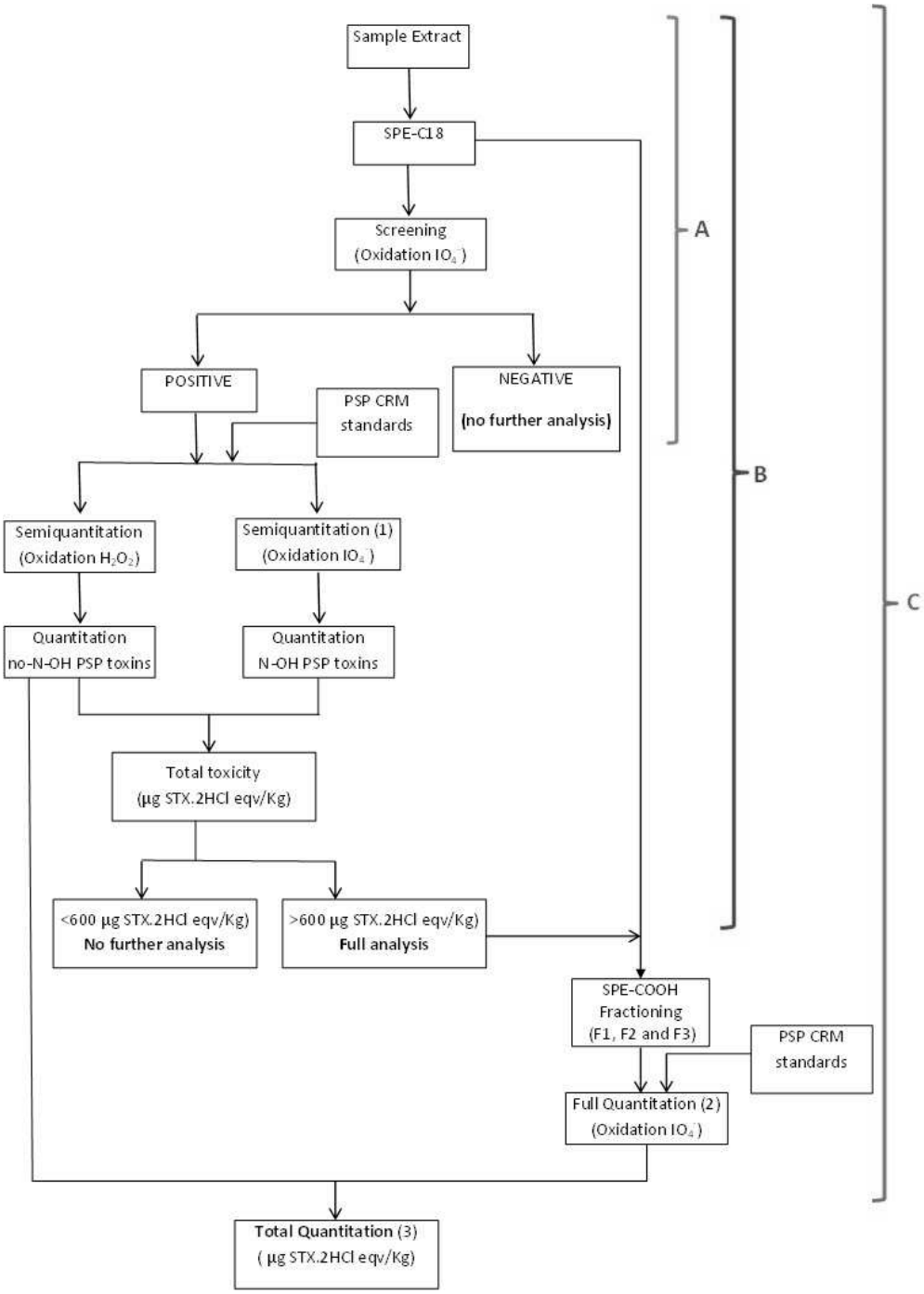


Figure 5: scheme to perform AOAC 2005.06 method for the analysis of PSP toxins

1) See coelution information data (Table 4)
2) Use quantitative data obtained for each individual no-N-OH PSP toxins from semiquantitative determination step (1).
3) Use quantitative data obtained for each individual no-N-OH PSP toxins from semiquantitative determination step (1) and toxicity equivalence factor for each PSP toxin.

Note: the first screening (Fig. 5, section A) provides very quick information about negative samples for which no further analyses are required. Samples considered positive due to the presence of chromatographic peaks corresponding to the ones obtained for PSP standards are submitted to semi quantitation (Fig. 5, Section B), and the results obtained are compared with those established on the semi-quantitative range between 400 to 600 µg STX.2HCl eqv./Kg. The complete quantitation (Fig. 5, Section C) is necessary when the toxicity of the samples exceeds the highest limits established in this range.

a) ***Identification***:

PSP profile screening (Figure 5, section A) is carried out with purified extract from SPE-C18 procedure and periodate oxidation. Chromatographic peak identification is carried out comparing retention times (R_t) between standard and oxidized sample (figure 6 and 7, table 3). It is important to identify chromatographic peaks corresponding to the presence or absence of natural fluorescence compounds in samples.

Table 3: Number of oxidation products for PSP toxins

TOXIN	**NUMBER OF OXIDATION PRODUCT**		
	Compound	IO_4^-	H_2O_2
No-N-OH	*STX*	1	1
	GTX2,3	1	1
	C1,2	1	1
	GTX5 (B1)	1	1
	dcSTX	2	2
	dcGTX2,3	2	2
N-OH	*GTX1,4*	3	0
	NEO	3	0
	dcNEO	2	0
	GTX6 (B2)	3	0
	C3,4	3	0

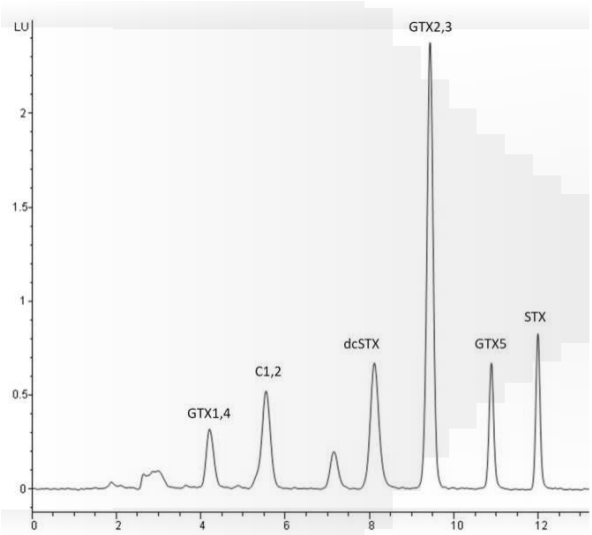


Figure 6: PSP mix standard for screening (periodate oxidation)

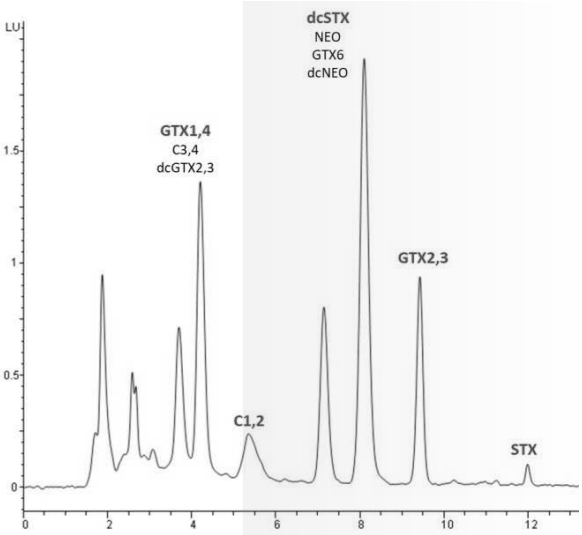


Figure 7: Contaminated mussel sample with SPE C18 cleanup (periodate oxidation)

Due to the lack of fractioning step (Figure 5, Section C), the quantitation procedures for chromatographic coelutions are carried out against the individual toxins having the higher toxicity (See table 4).

Table 4: The main chromatographic peaks of individual toxins with higher TEFs are used for semi quantitation when coelution is observed.

Coelution	**Identify as:**
GTX6	NEO
NEO	
C3,4	GTX1,4
GTX1,4	
dcGTX2,3	
dcSTX	dcNEO (*)
dcNEO	

(*) dcSTX higher TEF than dcNEO but only can be detecte using peryodate oxidation.

a) ***Semiquantitation***:

Calculate the response factor (R_F) for each toxin in the standard (screening standard).

$$R_F = \frac{\text{Peak Area}_{(\text{Standard})}}{\text{C } (\mu\text{M})_{(\text{Standard})}}$$

Toxins are individually quantified using chromatographic data. Each peak identified as toxin in sample (C_X) is assessed against the response factor.

$$C\ (\mu M)_X = \frac{\text{Peak Area}_{(\text{sample})}}{R_F}$$

Calculate the toxicity equivalent to saxitoxin, using:

$$C\ (\mu M\ STX.\ eq)_{Total} = \sum(TEF_{(toxin)} \times C(\mu M)_{toxin})$$

Each toxin is semi-quantified in terms of total toxicity (µg STX eq/Kg):

$$C_{(\mu g\ STX\ eqv\ /\ Kg)} = C(\mu M\ STX\ eqv)_{total} \times MW \times \frac{V(mL)_{extrac}}{m\ (g)_{shellfish\ meat}} \times Dil.$$

Where,

$C_{(\mu M\ STX\ eq)}$: concentration of toxin in extract equivalent to saxitoxin

MW: STX molecular weight (372.2 g/mol)

Dil.: dilution factor

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

Where, $\sum_{n=1}^{} C_i(\ \mu g\ STX\ /Kg)$

C_i is toxicity equivalent of saxitoxin for each toxin identified in sample extract.

EXPRESSION OF THE RESULTS

PSP toxins not detected in sample, or signal/noise is less than three, must be reported as ND, limit of detection (LOD) value must be indicate.

Samples with toxicity values above the LOD but under quantitation limit (LOQ, S/N≤10) must be reported as <LOQ , the LOQ value must be indicate.

Samples with toxicity values semi quantified higher then LOQ and lower the 500 - 600 µg STX eq./Kg, must be report as < RL (regulatory level)

Samples showing semi quantified PSP at level higher than 500 - 600 µg STX/Kg should be processed to complete quantitation.

METHOD FEASIBILITY:

Use adequate sequence analysis, avoid column overload, or cross contamination from the matrix.

In order to help toxin identification, the most significant secondary peak must be detectable.

A non-oxidized sample extract must be run in HPLC-FLD. Potential interferences from natural fluorescent compounds must be identified if the results are to be used for official control purposes.

Matrix modifier extract oxidized with periodate must be run in HPLC-FLD. Check any peaks which can interfere with the identification and quantitation of toxins in shellfish extract.

Use semi quantitative analysis to estimate total toxicity (µg STX.2HCl eqv/Kg) in samples extracts, proceed with total quantitation when value is higher than range 500 - 600 µg/Kg.

Use semi quantitation to estimate the total toxicity and complete quantitation to determine the real total toxicity in sample.

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