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Ciclo XXXIV

"Development of methods alternative to the biological method for the rapid determination of marine biotoxin in bivalve molluscs"

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Summary

A hydrophilic interaction liquid chromatography method with high resolution mass spectrometry (HILIC-HRMS) have been used for the analysis of certified positive samples that results positive with Mouse Bioassay (MBA) and high-performance liquid chromatography with fluorescence detector (HPLC-FLD). Saxitoxins analyzed were STX, NEO, GTX1, GTX2, GTX3, GTX4, GTX5, C1, and C2. Official samples were diluted with blank matrices to assess concentration that were half the limit provided from EU Regulation (800 eq µg STX HCl/Kg). Extracts were tested with N2A assay. The cytotoxicity assay with ouabain (O) and veratridine (V) mainly detected GTX1-4 concentrations five times higher and six times lower than the established threshold (800 µg/Kg). Agilent SampliQ C18 column have been used for the purification of samples. All positive samples were revealed as "uncompliant" with the screening procedure.

Riassunto

Sono state condotte delle analisi in cromatografia liquida a interazione idrofila con spettrometria di massa ad alta risoluzione (HILIC-HRMS) su campioni risultati positivi nelle metodiche del mouse bioassay (MBA) e cromatografia liquida ad alte prestazioni con rivelatore di fluorescenza (HPLC-FLD). Le saxitossine analizzate sono state STX, NEO, GTX1, GTX2, GTX3, GTX4, GTX5, C1 e C2. I campioni ufficiali sono stati diluiti con matrici non contenenti analiti per valutare la risposta strumentale a concentrazioni di molto inferiori al limite normativo previsto dal Regolamento UE (800 eq µg STX HCl / Kg). Gli estratti di tali campioni (diluiti e non) sono stati analizzati mediante il test di citotossicità N2A. Tale test viene condotto con ouabain (O) e veratridina (V) e ha rilevato concentrazioni di GTX1-4 cinque volte superiori e sei volte inferiori alla soglia stabilita (800 µg/Kg). Per tale saggio si sono usate le colonnine Agilent SampliQ C18 al fine di purificare i campioni e diminuire l'effetto matrice. Non vi sono stati falsi conformi, e pertanto le metodiche si sono dimostrate sensibili ed efficaci ai test di screening.

Chapter 1

1. General introduction

Seas and oceans represent a source of fishery products. There is a lot of seafood that are used as human resources for proteins, minerals, and fatty acids. Fisheries and aquaculture contribute significantly to food security as suppliers of nutritious food. Aquatic food represents the main source of vital nutrients in developing countries [1], especially in small-scale fishing communities that are marginalized and at the bottom of the socio-economic system. In this context, fisheries and seafood are a generator of income and economic growth [2]. There was an increase in consumption since 2012. Per capita food fish consumption grew from 9.0 kg (live weight equivalent) in 1961 to 20.5 kg in 2018, by about 1.5 percent per year [3]. This expanding market, worth $ 401 billion, represents an excellent growth opportunity for developing countries such as Africa [4]. However, this delicate balance is going to change over time.

Climate change and global warming will have heavy consequences for aquatic systems and, therefore, the seafood market. In the future, oceans will absorb less CO_2, temperatures will be rising, and these will have implications for the fisheries and aquaculture sector [5]. Phytoplankton represents the first ring of Earth's carbon cycle and represent the base of the marine food web. Due to climate change, its biomass will decline by up to 20 percent by 2100 [6], [7]. The 6% of phytoplankton know extant species (300 of 5000) produce marine microalgae that are considered harmful to humans [8]–[10]. "Harmful Algal Bloom" (HAB) is a phenomenon that occurs when there is a high concentration of this species in water. It is a natural phenomenon that is characterized by the presence of scum and bad smells from water. HAB can be harmful to human health. In fact some microalgae can produce "biotoxins." These are secondary metabolites that can be lethal to humans [11], [12]. Costal shellfish species can accumulate these toxins and be unsafe for human consumption. Climate change increases HABs, especially in costal subtropical ecosystems where the concentration of harmful algae is rising [13]. During HAB, fishery activities must be delayed to the high presence of toxins (such as domoic acid) in fish and invertebrates [12] with negative socio-economic impact as result.

Bivalve shellfish use microalgae as food and accumulate toxins in their edible tissues [11] and therefore to consumers. Algal toxicosis is characterized by a plethora of symptoms that depend on the dose, the router of exposure, and the algal toxins [14]. Ingestion of contaminated seafood represents the most common route of exposure to algal or cyanobacterial toxins, skin contact, and inhalation can occur during recreational activities (swimming in contaminated water) (CDC, 2019). There are several syndromes associated with biotoxins intoxications, such as amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP), and diarrheic shellfish poisoning (DSP) [16]. Domoic acid (DA) is responsible for ASP, short-term memory loss or coma, and even death characterize ASP [17]. DA can be accumulated in clams and scallops and anchovies [18], [19]. The toxic effect has a high affinity for the glutamate receptor, which causes overstimulation and cause neurotoxic effects [20]. Saxitoxin (STX) and derivates of STX, around 40 different analogs, cause PSP that is characterized by paralysis after the consumption of contaminated shellfish such as clams [21], [22]. The toxicity is due to the blockage of the voltage-gated sodium channels and the resulting neuronal disorder [23]. There are a lot of human poisoning cases related to the exposure of marine biotoxins [24] correlated with the increasing cases of HABs in terms of frequency and geographical distribution [9], [11], [25]. This seems correlated with global warming. In fact, there are toxic microalgal that live in warmer conditions that appear in new places that now fit the ideal temperature [26]. Authorities have created monitoring programs with the purpose of limiting HABs and intoxication. The World Health Organization (WHO) and the Food Agricultural Organization (FAO) have set limits to guarantee safe products [27], [28]. In Europe, there are regulatory requirements created to manage the risk of the bivalve mollusks food chain in the pre-harvest phase of the food chain [29].

There are different methods to determine the presence of marine toxins in different matrices. Methods include *in vivo* to *in vitro* assays, immunochemical methods (sensors and enzyme-linked immunoassays), and analytical chemical methods. The mouse bioassay (MBA) was the first method for analyzing marine biotoxins in seafood [30]. It consists of an intra-peritoneal administration of seafood extracts to laboratory animals, followed by monitoring the symptoms and time to death. However, it has a high rate of false positives and negatives sample and a lack of sensitivity [24], [31], [32].

Analytical methods include liquid chromatography (HPLC or LC) or ultra-high-performance chromatography (UHPLC) associated with different detectors such as UV, tandem mass analyzers (MS/MS), high-resolution mass analyzer (HRMS), or fluorescence detector (FLD). Analytical techniques were chosen by FAO/IOC/WHO and European Regulation as reference control methods for marine toxins in shellfish [27], [27], [33]–[35]. Therefore, the Lawrence methods have been accepted as the reference method as an alternative to MBA. It is an HPLC-FLD method that uses acetic acid extraction and liquid chromatographic separation of the PSP toxins after their derivatization using hydrogen peroxide and periodate [36]. It has been validated through a collaborative trial and adopted as an Official Method [37].

2. Saxitoxins

2.1 Paralytic Shellfish Poisoning (PSP)

Paralytic Shellfish Poisoning (PSP) is a syndrome caused by consuming contaminated by a group of marine toxins referred to as saxitoxins (STXs) or Paralytic shellfish toxins (PST) [14]. This name is derived from saxitoxin (STX), and saxitoxins include more than 50 analogs [38], [39]. These toxins are produced by several dinoflagellates (*Alexandrium, Gymnodinium, Pyrodinium*) and by some cyanobacteria [40]–[42]. STXs can be found in contaminated seafood and predators of bivalve shellfish [43]. PSP is produced during HLB, and they are concentrated in the shellfish due to the filtration of toxic algae [44], [45]. However, this process is not homogeneous in the bivalve. It depends on the shellfish species and is influenced by environmental factors [46].

The first report of PSP syndromes was in California, United States, when six people died in 1920 due to the ingestion of contaminated seafood after a bloom of *A.fundyense* [47]. However, PSP diagnoses are reported worldwide in all continents due to different HBA phenomena [9]. Alexandrium spp. is primarily responsible for U.S. and Canadian PSP events [48], [49]. In addition, PSP cases have been recognized in Patagonia, the Philippines, Nicaragua, Southeast Asia, Latin America, and Europe, with a mortality rate that depends on the national medical system [50]–[52]. The syndrome is characterized by neurological disfunction that has a rapid onset (30 minutes to 3 hours) and a long duration (a few hours to a few days). Symptoms include:

- parathesias in perioral zone often spreading to neck and extremities [44], [53];
- gastrointestinal dysfunction (nausea, vomiting) [54];
- respiratory distress (that lead to death at high dose) [55];
- muscular weakness, drowsiness, incoherent speech [56].

The severity and the type of symptoms are dose-dependent [57]. Paresthesia in the mouth and lips are common in mild cases. Paralysis can affect the neck and face [50]. Death occurs for respiratory distress [58]. To date, the only treatment for

severe cases is artificial ventilation. Patients who survive PSP for 24 h without it have a big chance of surviving [27]. The paralytic effect of PSP is due to the high affinity of STXs in binding on site 1 of the voltage-dependent sodium ion channel in muscles and nerves [59]. This causes the interruption of nerve signal transmission and paralysis as a consequence [60]. According to EFSA, STX has a lowest-observed-adverse-effect-level (LOAEL) of 1.5 μg/kg [17].A more recent model set 0.37 μg/kg as the minimal critical dose (10% of the individuals exposed would have symptoms) [57]. Severe cases of PSP occur with the ingestion of 0.9 mg of the toxins [61], 0.4 mg STX equivalents per person can be fatal [62], [63].

2.1.1 European Legislation

European Union (EU) has established several regulations for monitoring and controlling the presence of STXs in bivalve shellfish. The Regulation (EC) No 853/2004 or "Hygiene Package" [64] set a limit value of 800 μg PSP/kg in shellfish meat. Fishery products that are not derived from bivalve mollusks, echinoderms, tunicates, and marine gastropods must not contain STXs. The presence of toxin-producing plankton in waters that produce shellfish must be monitored according to Regulation (EC) No 625/2017 (Commission, 2017). The monitoring is applied depending on harvesting periods, considered area, possible variations, and the risk assessment on toxins presence. With the prevention, shellfish contaminated products cannot be harmful [66].

Methods to determine the presence of toxins are regulated and change over time. The Regulation (EC) 627/2019 defines official controls' performance for marine biotoxins (Commission, 2021). Regarding STXs, the reference confirmatory method is described in Standard EN 14526 and consists of an HPLC-FLD method with pre-column derivatization with peroxide or periodate oxidation. The Regulation allows to use other internationally recognized validated methods; however, there are no LC-MS/MS official methods.

Europe has banned the MBA method since 2015 except for PSP analysis [68]. The Regulation (EC) 627/2019 removed this exception; therefore, live animals can no longer be used to analyze marine biotoxins regardless of the group of analytes. The methods must determine at least the following marine biotoxins:

a) STX, NeoSTX, gonyautoxin 1 and 4 (GTX1 and GTX4 isomers determined together), gonyautoxin 2 and 3 (GTX2 and GTX3 isomers determined together),
b) toxins N-sulfo-carbamoyl (B1), gonyautoxin-6 (B2), N-sulfocarbamoyl-gonyautoxin 1 and 2 (C1 and C2 isomers determined together), N-sulfocarbamoyl-gonyautoxin 3 and 4 (C3 and C4 isomers determined together);
c) toxins decarbamoyl dcSTX, dcNeoSTX, decarbamoylgonyautoxin-2 and -3 (isomers determined together).

EU legislation requires that the total toxicity be calculated using the toxicity equivalence factors (TEF), as recommended by EFSA or FAO OMS report, and it must be expressed in μg STX 2HCL equivalents/Kg for STXs. If new STX analogs with an established TEF are discovered, they shall be included in the analysis. TEFs can be found on the website of the European Reference Laboratory for marine biotoxins (Comunitary Reference Laboratory on Marine Biotoxines").

2.1.2 Chemical structure of SXTs and toxicity relationship

Saxitoxins was the first chemically characterized SXTs, and it is the most toxic among its analogs [70]. To date, more than 50 toxins are present in this group, and they share the core structure as shown in Table 1. STX is a tetrahydro-purine alkaloid with two guanidine groups. It is characterized by high polarity and solubility in polar solvents such as water, acid acetic, ethanol [71]. It is stable in acid media and forms a degradation product in alkaline conditions due to oxygen [72], [73]. It is a hygroscopic and not volatile compound [70]. STXs are characterized by substituents (Table 1) that heavily influence their stability and toxicology. N-Sulfocarbamoyl derivates can be hydrolyzed at high temperatures and with acid conditions and converted into carbamate derivates [73]. The predominant net charge into the physiological condition (≈ pH 7.4) is different among STXs due to the different substituents. STX is only characterized by two positive charges (+2). One positive net charge (+1) is defined NEO, B1, GTX2, GTX3, while C3 and C4 have one negative charge (-1) in physiological conditions [74]. The -OH group in R1 can influence the total net charge. In fact, among N-Sulfocarbamoyl derivates, only C3 and C4 have a negative predominant negative charge. The temperature can influence the stability of the molecules. STX solution can resist for 24 months at -20°C and -80°C, while at room temperature, the stability is reduced [75]–[77]. GTX1/4, NEO, GTX 2/3 are more thermolabile than STX [77].

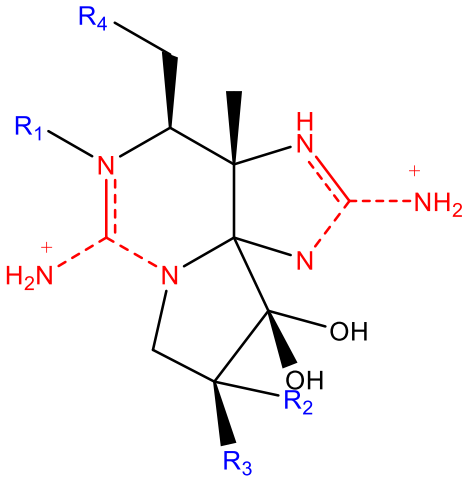


Figure 1: Molecular structure of STXs, with positive charge fully delocalized over the pyrimidine and imidazole groups (red).

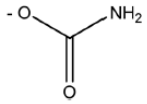
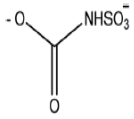
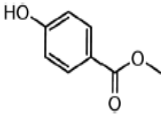
R1	R2	R3	R4	Toxin	Molecular weight	STX-subgroup
–H	–H	–H		STX	299	
–OH	–H	–H		NEO	315	
–OH	$–OSO^{3-}$	–H		GTX1	411	
–H	$–OSO^{3-}$	–H		GTX2	395	
–H	–H	$–OSO^{3-}$		GTX3	395	
–OH	–H	$–OSO^{3-}$		GTX4	411	
–H	–OH	–H		M2α	315	Carbamate
–H	–H	–OH		M2β	315	
–H	–OH	–OH		M4	331	
–H	–OH	–OH		M6	333d	
–OH	–OH	–H		M8α	331	
–OH	–H	–OH		M8β	331	
–OH	–OH	–OH		M10	347	
–OH	–OH	–OH		M12	349d	
–H	–H	–H		GTX5 or B1	379	
–OH	–H	–H		GTX6 or B2	395	
–H	$–OSO^{3-}$	–H		C1 α	475	
–H	–H	$–OSO^{3-}$		C2 β	475	
–OH	$–OSO^{3-}$	–H		C3 α	491	
–OH	–H	$–OSO^{3-}$		C4 β	491	
–H	–OH	–H		M1 α	395	N-Sulfocarbamoyl
–H	–H	–OH		M1 β	395	
–H	–OH	–OH		M3	411	
–H	–OH	–OH		M5	413	
–OH	–OH	–H		M7 α	411	
–OH	–H	–OH		M7 β	411	
–OH	–OH	–OH		M9	427	
–OH	–OH	–OH		M11	429	
–H	–H	–H		dcSTX	256	
–OH	–H	–H		dcNEO	272	
–OH	$–OSO^{3-}$	–H	–OH	dcGTX1 α	368	Decarbamoyl
–H	$–OSO^{3-}$	–H		dcGTX2 α	352	
–H	–H	$–OSO^{3-}$		dcGTX3 β	352	
–OH	–H	$–OSO^{3-}$		dcGTX4 β	368	
–H	–H	–H		GC3	376	
–OH	–H	–H		GC6	392	
–OH	$–OSO^{3-}$	–H		GC4 α	488	Benzoate(para-hydroxy-)
–H	$–OSO^{3-}$	–H		GC1 α	472	
–H	–H	$–OSO^{3-}$		GC2 β	472	
-OH	-H	$–OSO^{3-}$		GC5 β	488	
-H	-H	-H	–H	doSTX	240	Deoxydecarbamoyl
-OH	$–OSO^{3-}$	-H		doGTX1	352	Deoxydecarbamoyl
-H	$–OSO^{3-}$	-H		doGTX2	336	Deoxydecarbamoyl

Table 1: STXs toxins and their main substituents

These instability or degradation conditions are essential and can influence the result expression. As described above, the results are expressed as TEF, and therefore molecules degradation that occurs during analysis can influence the quantitative estimation of STXs found in the sample. TEF, in fact, is the "toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in

the same group." It is correlated with "potency", a pharmacology term that indicate the dose to obtain a certain effect [78]. In the case of toxins, we indicate the median lethal dose (LD_{50}) that is the dose that kills half the population of animals tested. Regarding STXs, the route of exposure is mainly oral. Initially, TEFs were obtained from the MBA test, which is not a toxicological parameter correlated with acute toxicity [78].

Toxin	By MBA[a]	LD 50 by i.p. injection[b]	LD 50 by i.p. oral administration[c]	EFSA proposal[d]	FAO/WHO proposal[e]
STX	1.00	1.00	1.00	1.00	1.00
NEO	0.92	3.12	2.54	1.00	2.00
GTX1	0.99	1.90	0.93	1.00	1.00
GTX4	0.73			0.70	0.70
GTX2	0.36	0.76	0.57	0.40	0.40
GTX3	0.64			0.60	0.60
GTX5(B1)	0.06	0.22	0.06	0.10	0.10
GTX6(B2)	-	0.12	<0.017	0.10	0.05
C1	0.01		0.04	-	0.01
C2	0.10			0.10	0.10
C3	0.01			-	0.01
C4	0.06			0.10	0.10
dcSTX	0.51	0.79	0.37	1.00	0.50
dcNEO	-	0.06	0.22	0.40	0.20
dcGTX1	-			-	-
dcGTX2	0.15		0.11	0.20	0.20
dcGTX3	0.38			0.40	0.40
dcGTX4	-			-	-
M2	-			0.30	-

Table 2. Note: (mouse unit) is defined as the amount of toxin to kill a male mouse weighing 20 g after 15 min one MU. a) Estimated by MBA[79]. b) From LD_{50} by i.p. injection.[78], [80]. c) Oral LD_{50} [72], [79]. d) Proposed by CONTAM Panel[81]. e) Proposed by the expert of FAO and WHO [78].

To date, the most accurate way to set TEFs is data from human cases, and the least is *in vitro data* [78]. MBA is considered less accurate than oral or intra peritoneal LD_{50}/toxicity data in the animal. TEFs are shown in Table 2. Based on Table 2 and Table 1 is clear that the group that differed for R4 substituent (carbamate, N-Sulfocarbamoyl, Decarbamoyl, etc.) have different toxicity. In decrescent order of toxicity, there are carbamate, Decarbamoyl, N-Sulfocarbamoyl groups. The polarity of STXs is correlated with their toxicity, in fact, decarbamoyl toxins are more polar than carbamate toxins, and NEO is the most toxic among all STXs. The hydroxyl group present in NEO in R1 could

be associated with higher toxicity. This association is also in GTX1/4, which has hydroxyl substituent in N1 and is more toxic than GTX2/3. Another substituent such as -$OCONH_2$, –$OCONHSO_3^-$,-OSO_3^- can seem to influence STXs toxicity [70]. Due to the high toxicity, several analytical, molecular, and *in vivo* techniques were developed to increase consumers' safety and deepen the knowledge on STXs.

3. Determination of STXs

3.1 *In vivo* and *in vitro* techniques

3.1.1 Mouse bioassay (MBA)

MBA was applied for the first time in 1937 to analyze STXs in shellfish [82]. The standardized method became an official AOAC method with the AOAC 959.08 [83]. The method was accepted as the gold standard for PSP analysis. It consists of homogenizing the edible parts in aqueous HCl. The extract is filtered and injected into replicate mice. The injection is intraperitoneal (i.p.). The mouse should die between 5 and 7 minutes, or the quantitative evaluation cannot be done. The mouse units (MUs) can be calculated through the correlation between time and death. An i.p. injection that kills a 20g mouse in 15 minutes has one MU. MBA was used massively in the past; however, it has several serious drawbacks. Salts and metals can influence the analysis. Only the total toxicity values can be calculated, and no information on which STXs were present in the matrices.

Furthermore, the MBA protocol can quantify STXs at the current EU regulatory limit value, but not below approximately 370 µg STX equivalents/kg shellfish meat, which is far above the concentration compatible with the acute reference doses (ARfD) set by EFSA for STX-group toxins (0.5 µg STX eq./kg body/weight). As discussed previously, STXs toxins are thermolabile. Therefore, the boiling process with HCl during extraction may result in the conversion of less toxic analogs into more toxic ones and, thus, overestimating the toxicity depending on the toxin profile. It was officially banned in Europe with the Regulation (EC) 627/2019 in favor of analytical techniques. However, an MBA essay can be useful to assess the toxicity of unknown biotoxins.

3.1.2 Receptor binding assay (RBA)

The high affinity for STXs to site 1 of Na^+ channels was used to detect these marine biotoxins through binding activity [84]. The assay uses tritiated radiolabeled STX(^{3}H-STX) subjected to the competitive displacement from STXs. The radiolabeled standard is added to the sample extracts. The amount of it that is not bound to the Na+ channel is measured using scintillation counting. United States approved this method and has been accepted as AOAC official method (2011.27) and performs well compared to MBA and HPLC methods [85]. The technique can be used in several bivalve mollusks, and the extraction procedure is similar to the MBA [86]. The main drawbacks of this method are the use of STX(^{3}H-STX) in the analysis and the high variability in the assay due to the membrane that can be heterogeneous [87], [88].

3.1.3 Cell-Based (Cytotoxicity) Assays (CBAs)

CBAs are functional assays used to analyze toxin or toxin class that can affect the survival of cell type [89]. The accuracy is higher when the survival rate depends on the interaction between toxin-receptor [90]. There are commercial products based on CBA that can detect STXs toxin (MIST™)[91], and its performance was comparable with the MBA method for quantifying toxicity associated with STXs in shellfish [92]. However, some problems can occur with this assay. For example, some compounds in the matrices can interfere with the cellular response [93]. For these reasons, more specific cell lines and methods have been developed for STXs analyses. One of them is the Neuro-2A Assay that uses Neuro-2A (N2A) mouse neuroblastoma cell line (ATCC, CCL-131). N2A cells are seeded into a plate with 96 slots and allowed to grow for a maximum of 24h. After removing the medium, cells are exposed for a period to the test solution. The survival rate can be assessed using a dye and a microplate spectrophotometer. STX's targeted approaches employ the VGSC-blocking toxins ouabain and veratridine to increase Na+ inflow, followed by STX, which restored the cells by blocking this influx and maintaining viability. The effect is dose-dependent and assessed by visual scoring of each sample [56]. Hayashi *et al.* (2006) compared N2A assay with the HPLC method and a correlation index over R^2 0.9 [94].

3.1 Chemical Assays

HPLC associated with different revelators has been used for STXs analysis. Fluorescence detection (FLD) or direct ultraviolet (UV) cannot be used without STXs derivatization because these marine biotoxins do not contain chromophore or fluorophore groups. The first method developed utilizes FLD derivatize molecules to obtain the iminopurine derivates [95]. However, this method was not able to distinguish toxins and profile information. Due to the high hydrophilic characteristics, a non-polar stationary phase (C18 chain) is used as a column.

In contrast, the mobile phase is polar and composed of aqueous buffers and organic solvents. There is also an HPLC method that uses ion-pairing and ion-exchange, or hydrophilic interaction liquid chromatography (HILIC) [46], [96]. To date, the methods currently validated for routine control of PSP toxins are PCOX and Pre-Cox or Lawrence method [87], [97]. There are no collaborative studies currently available for LC-MS methods.

3.1.4 Liquid Chromatography with Fluorimetric Detection

PCOX methods utilize a derivatization procedure that occurs before or after the column. Different methods were validated with different results. STXs include different molecules with different substituents, and therefore the separation can be difficult. In 1984, *Oshima et al.* used alkaline-oxidation to create strongly fluorescent derivates. However, it was not efficient for all STXs [79]. Eleven years later, they resolve all analytes with three different isocratic runs and a long column with an optimized pH, mobile phase, oxidant composition, and a C-19 solid-phase extraction (SPE) [98]. The periodate was the oxidant, and the derivatization was post column. Three stages were needed to separate STXs, 1)C-toxins, 2) gonyautoxins, and 3)NEO, STX, and dcSTX. The procedure was expensive and time-consuming; furthermore, dcNEO and NEO were not separable. The method was improved and refined in the following years [99]–[101]. Two chromatographic runs were needed (instead of 3), new HPLC column and new ion-pairing reagent were used. Among STXs analyzed, only GTX5 continued to be hard to resolve. This method was used by *Riet et al.* that conducted a single-laboratory validation study followed by a collaborative study for analyzing STX, NEO, dcSTX, dcGTX2, dcGTX3, GTX1-5, C1, and C2 in mussels, oysters, and scallops [102], [103]. It was

approved by AOAC in 2011 as an official method and used in the United States [104]. The method is the Official Method of Analysis (OMA) AOAC 2011.02. A scheme of the method can be found in Table 3.

The Pre-Cox method (Lawrence method) involves the derivatization of a sample extracted prior to the chromatographic analysis. The first attempt was performed by *Lawrence et al.* [105]. Hydrogen peroxide was used together with periodate for the derivatization. N1 hydroxylated toxins react with periodate to form fluorescent products, but not with permanganate, while non-N1-hydroxylated STXs react with both reagents. The method was modified and improved. In 2006, this method was accepted as AOAC official method 2005.06 [87] and implemented in the European Commission legislation in 2005 [106].

EXTRACTION	
- 5 g shellfish homogenate - Single extraction in 0.1 M HCl (boiled 5 min) - Adjust to pH 2-4 before and after boiling	
EXTRACT CLEAN-UP	
TCA precipitation Filter extract (0.2 μm)	
Gonyautoxins/NEO/dcSTX/STX	**C-toxins**
HPLC	**HPLC**
Agilent Zorbax bonus RP column, 150 x 4.6 mm, 3.5 μm	Thermo beta basic 8, 250 mm x 4.6 mm, 5 μm
OXIDATION	**OXIDATION**
Oxidant and acid flow rate 0.4 ml/min	Oxidant and acid flow rate 0.4 ml/min
FLD	**FLD**
25 min/sample	25 min/sample

Table 3: Schematization of steps involved in the PCOX method AOAC 2011.02.

Since 2017, this method has been considered the official testing method for STXs in the EU (Commission, 2017). The advanced method includes two steps of solid-phase extractions (SPE): one to purify the sample and the second to elute different fractions of STXs and different chromatography runs. A scheme of the method can be found in Table 4. It seems clear the main drawbacks of this method. It requires much time and cannot

distinguish isomers with different toxicities [108]. It includes several extraction steps, clean-up, different analytical runs, and oxidations procedures. It is not applicable in all laboratories due to the costs and requires a lot of labor, mainly because official control laboratories must give results in a short time [109]. Four injections are needed to analyze and quantify all STXs listed, (1) SPE C18 peroxide derivatization, (2) SPE COOH periodate fraction F2 and (3) F3, (4) underivatized C18 fraction to assess the absence of fluorescence interfering molecules. It is 1 hour for each sample, extraction excluded. Furthermore, there are co-elution issues for several STXs. In "screening" mode, that is, without the derivatization procedure, it is a valuable tool for the reduction of quantification procedure [37], [46], [110].

EXTRACTION

- 5 g shellfish homogenate
- Duplicate extraction in 1 % acetic acid (1st step boiled 5 min)
- Centrifugation
- Combine extracts
- Dilute to 10 ml

EXTRACT CLEAN-UP

C18 SPE

Adjust to pH 6.5 ± 0.5 with 1 M NaOH

PRESENCE OF NON-HYDROXYLATED STXs	**PRESENCE OF N-1-HYDROXYLATED STXs**
(dcGTX2,3, C1 ,2, dcSTX, GTX2,3, GTX5 or STX)	(GTX1 ,4, GTX6,C3,4 dcNEO or NEO)
PEROXIDE DERIVATISATION	**SPE-COOH**
Quantification of non-hydroxylated toxins	Preparation of F1, F2, F3
HPLC-FLD	**PERIODATE DERIVATISATION**
RP C18150 x 4.6 mm, 5 pm 15 min/sample	**HPLC-FLD** RP C18150 x 4.6 mm, 5 pm 15 min/sample

Table 4: Schematization of steps involved in the Pre-Cox method (Lawrence) AOAC 2005.06.

3.1.5 Liquid Chromatography with Mass Spectrometric Detection

The time needed for quantification is a disadvantage of both AOAC 2005.06 and AOAC 2011.02 methods. Therefore, LC-MS methods are being developed to analyze SXTs toxin. Mass spectrometric detection is used to quantify lipophilic biotoxins [111]. In the past, several LC-MS methods were reported to detect and quantify SXTs in

shellfish tissues [112]–[114]. Electrospray ionization mass spectrometry (ESI-MS) can perform ionization of STXs with high efficiency. The choice of the right ion source is essential. It can influence the method's performance. Fast atom bombardment (FAB) or atmospheric pressure chemical ionization (APCI) was not able to produce mass spectra of STX standards and therefore were not developed methods with these ion sources [115]. ESI is considered a "soft" form of ionization because ions are formed without significant fragmentation. This is important since a hard source fragmentation can result in the loss of SO_3 for STXs with the sulfonic acid with a predominant cursor that is the same between different STXs such as gonyautoxin-2 and neo-saxitoxin [114]. In ESI, the flow from LC passes through a capillary with a charge and produces a charge separation at the surface of the liquid with the formation of a Taylor cone. With the evaporation of solvents, the size of droplets is reduced, and the excess charge creates ion that goes in the vacuum chamber [116]. Selected reaction monitoring (SRM or MRM) is the most common approach for STXs analysis.

The ion source is not the only parameter that can heavily influence an LC-MS/MS method. STXs are hydrophilic molecules with molecular analogies, and the extraction procedure and instrument settings need to be refined and improved. The main parameters will be discussed in the following paragraphs.

3.1.5.1 Hydrophilic Interaction Liquid Chromatography (HILIC)

The term HILIC was proposed by Alpert in 1990 [117] and consisted of a hydrophilic stationary phase and an organic mobile phase. These characteristics are different from normal phase (NP, polar stationary phase, and lipophilic mobile phase), reversed-phase (RP, non-polar stationary phase, and polar mobile phase), ion exchange (IEC, stationary phase with ionizable functionalities that retain analytes of interest). In HILIC, the mobile phase is typically composed of acetonitrile and a minimum percentage of water of 3% [118], [119]. HILIC performs better than other techniques with polar analytes, especially with STXs [119]–[123]. Several HILIC methods were validated for STXs toxins analysis [34], [123]–[126]. HILIC offers several advantages. The high percentage of organic solvents in the mobile phase is compatible with the most common phase extraction (SPE) and dispersive SPE (d-SPE) as QuEChERS [118]. This is important because extracts often contain ions and salts that can cause ion suppression in

the MS source [112], [127]. Organic solvents have low viscosity and are therefore allowed to work with higher flow rates without increasing the column pressure. The chromatography run can resolve analytes thanks to two different mechanisms: adsorption and partitioning. The low percentage of water is adsorbent onto the polar stationary phase and creates a layer of water in the stationary phase. Other solvents influence the entity of this mechanism into the mobile phase. It is reduced in methanol-water solution compared with acetonitrile due to the affinity of methanol with polar stationary phase [128]. The partitioning mechanism depends on the polarity of molecules and is between molecule-water layer and molecule-mobile phase. This mechanism give retention and is influenced by hydrogen and polar interactions between analytes and water layer [118], [119]. Common stationary phase utilized in HILIC are amide (TSK-gel Amide-80, Waters Xbridge Amide), zwitterionic (SeQuant HILIC column), and unbonded silica (Phenomenex Kinetex) [129]. Solvents for the partitioning of polar analytes are in crescent order of polarity: acetone, acetonitrile, isopropanol, ethanol, methanol, water [119].

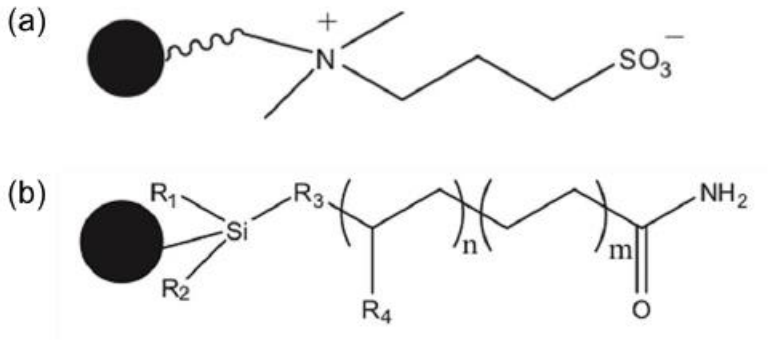


Figure 2: Functional group of ZIC HILIC (a) and TSKgel Amide80 (b) [130].

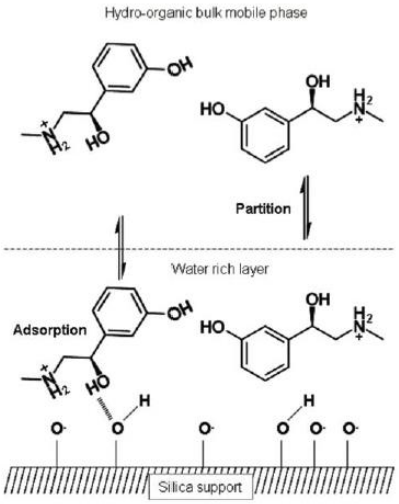


Figure 3: Partition and adsorption mechanism in HILIC [131]

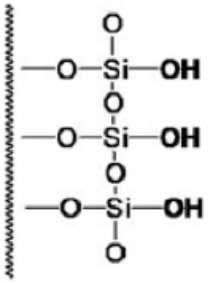


Figure 4: Underivatized silica [126]

3.1.5.2 Electrospray ionization

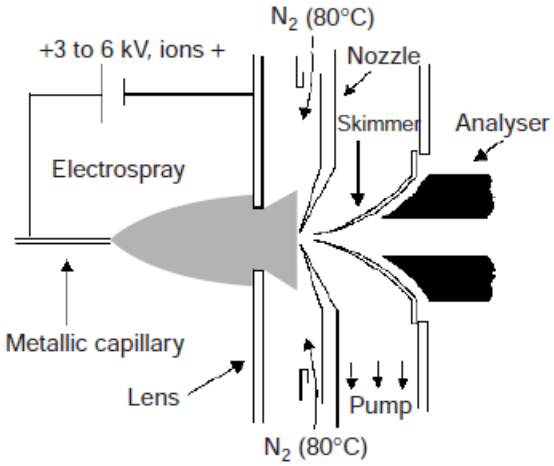


Figure 5: Scheme of an ESI.

ESI started as an ion source for protein analysis and biological applications [132]. It consists of a strong electric field at atmospheric pressure where a liquid passes through

this field [133]. The potential to create the field is typically from 1 to 4 kV, and the capillary can be heated. The liquid comes from a capillary with a low flux and is subjected to the field, leading to an accumulation of charge at the surface of the liquid. These conditions create droplets that will continue to lose solvent and increase the surface charge [134]. As droplets lose all solvents, gas-phase ions are formed and transferred into the mass spectrometer due to an applied potential and the high vacuum [135], [136]. Droplets from gas-phase ions due to the electric field. Droplets at low voltages appear as spherical, higher voltage deform droplets into a "Taylor cone" and release tiny droplets [137]. This can occur before the limit given by the Rayleigh equation because of the deformation of droplets [137]. The charge present in droplets released deforms the droplets again, forming a new Taylor cone. This mechanism is repeated until gas-phase ions are formed [133].

3.1.5.3 Mass-analyzer for MS/MS

The mass spectrometer produces a mass spectrum from the measures of the ion current versus the mass to charge ratio (m/z). There are different mass analyzers, single quadrupoles (Q) or triple quadrupoles (QqQ), ion traps, time of flights (TOF), electrostatic trap or orbitrap, etc. Each of these different mass analyzers has been used for SXTs analysis. Quadrupole time-of-flight (qTOF) MS has been used for quantification of STXs in cyanobacteria [138] ion-trap MS for the analysis of STXs in mollusks [139]. There are a plethora of papers that use quadrupole or electrostatic trap for STXs determination or screening [115], [140], [141]. Quadrupole analyzers are made up of four rods of circular sections disposed of in parallel. An ion that is entering the space between the rods will be drawn towards the rod of the opposite charge. The first that described the principle of the quadrupole was Paul and Steinwegen in 1953 [142]. In traveling along the z-axis and are subjected to the influence of quadrupolar alternative field and constant field resulting from the potentials upon the rods. [143] Due to the electric fields that accelerate ions, the ions maintain their velocity along the z-axis and are subjected to accelerations along the x and y-axis. If x and y do not reach $r_{0,}$ the ion will continue its trajectory without colliding with rods and is detected [144].

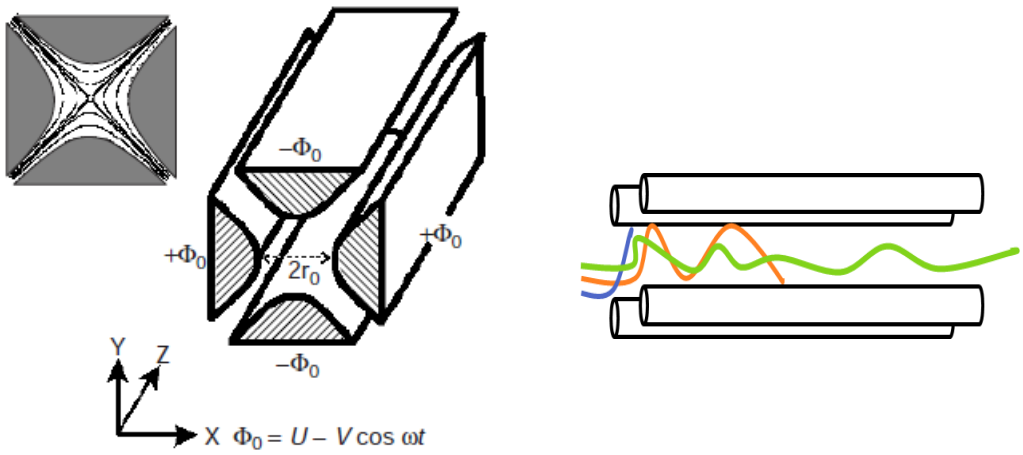


Figure 6: (on the left) Quadrupole with hyperbolic rods and applied potentials, the equipotential lines are represented on the left [134]. (on the right) example of ions that cross quadrupole. Detected ions cover the green trajectory. The revelator will not detect blue and orange ions because they collide on the rods [135].

Quadrupole has a mass limit of 4000 Th, a resolution FWHM (m/z) of 2000, an accuracy of 100 ppm [134]. Quadrupoles can focus ions from a mass range towards the axis without affecting the kinetic energy of the ions. Quadrupoles can transport the ion from one compartment of the mass spectrometer to another and can connect regions with different pressure. For this reason, they can be helpful as collision cells. The triple quadrupole instrument is made up of three single quadrupoles in series. Quadrupole mass spectrometers are symbolized by upper case Q during radio-frequency quadrupole with a lower-case q. The basic scheme of a triple quadrupole is QqQ, where the central quadrupole is used as a collision cell. In fact, a high-pressure collision gas (as helium, nitrogen, or argon) is introduced into q and an ion entering the quadrupole undergoes an n number of collisions [135]. Collisions between the precursor ions and the neutral gas atoms fragment the precursor ions into productions in a process called “collision-induced dissociation” (CID). A schematic representation of a triple quadrupole can be seen in Figure 6.

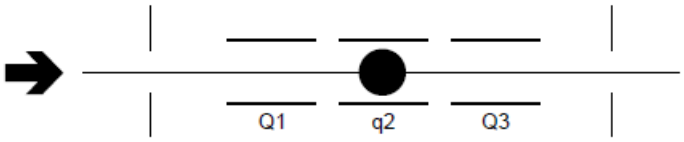


Figure 7: Scheme of a triple quadrupole. The first and the last (Q1, Q3) are mass spectrometers, while q2 is a quadrupole using radiofrequency only [134].

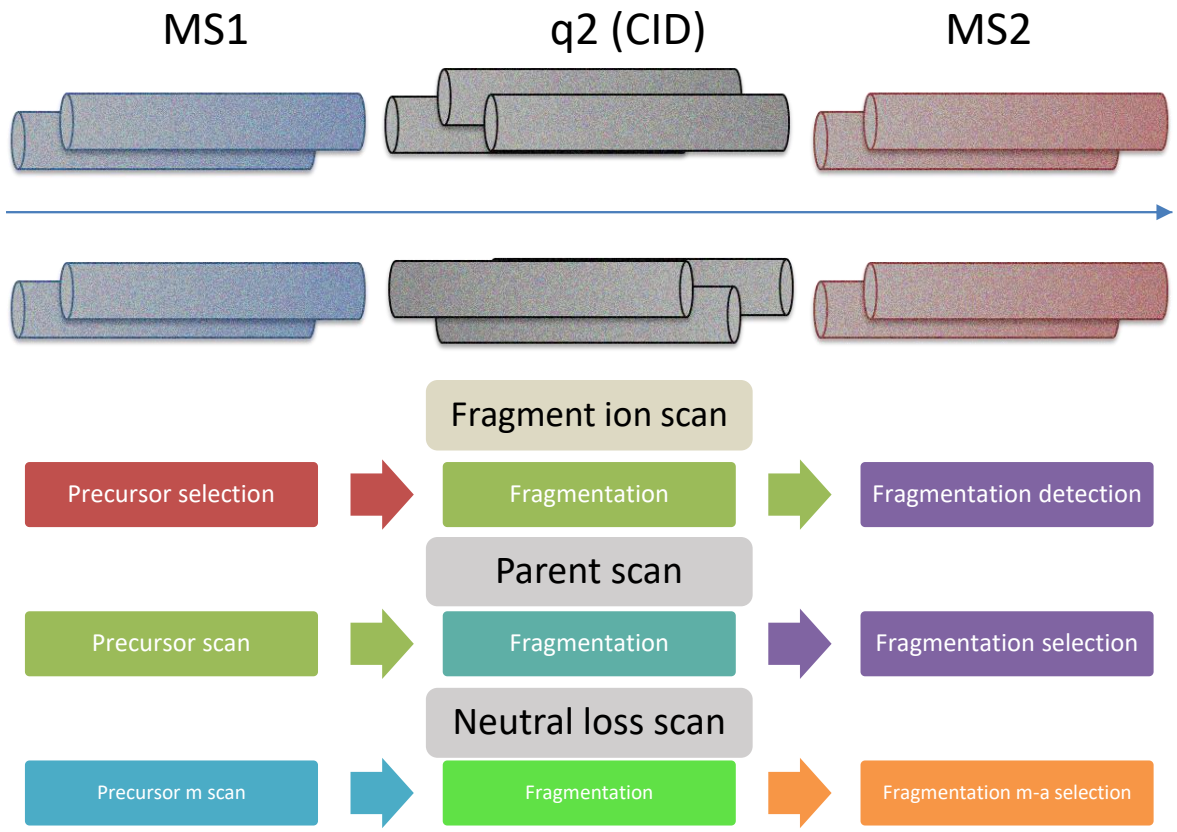


Figure 8: A schematic diagram of a triple quadrupole and three of the possible configurations that can be used. In the presented configurations, MS1 and MS2 are used as mass analyzer while q2 as a CID.

This configuration can be used in several ways. The "Fragment ion scan" consists of selecting an ion with a chosen m/z ratio with MS1. The ion collides inside q2 and creates a fragment that MS2 analyzes. In "parent scan", MS2 focuses on a selected ion while MS1 scans the masses. Only the ion that produces the selected mass through fragmentation is detected in this case. Finally, in "neutral loss scan," both MS1 and MS2 are scanned with a constant mass (m) offset between the two (a).

Another mass analyzer is the ion trap, instead of filtering ions traveling through the mass analyzer (as quadrupole), ion trap stores ions in two or three dimensions. There are, therefore, 2D (linear) and 3D ion traps. They are composed of four rod-shaped electrodes subjected to a direct current (DC) and an alternating current (AC) and confine and store ions due to the electromagnetic fields produced by these currents. The main radiofrequency (leading RF) is an AC applied to the electrodes and confine ions radially [145].

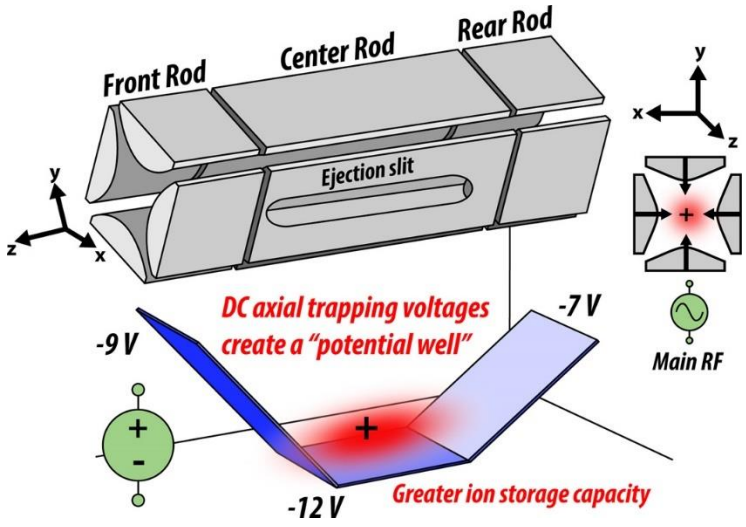


Figure 9: Scheme of a two-dimensional ion trap. Main RF is the AC applied to all four rods [145].

Another ion trap type is the Fourier-transform mass analyzers (FTMS) or Orbitrap. It is formed by an electrode with a barrel that is cut into two equal parts and separated by a small gap. The diameters of the central electrode are under 8 mm, while the external one is 20 mm [134]. The ions enter in Orbitrap tangentially into the gap and start to oscillate simultaneously into the axial dimension while are orbiting around a central electrode. The Orbitrap calculates the frequency during this oscillatory movement. Ions that move from one section to another produce a current that Orbitrap measures. Through Fourier transformation, time oscillation data are converted into *m/z* data.

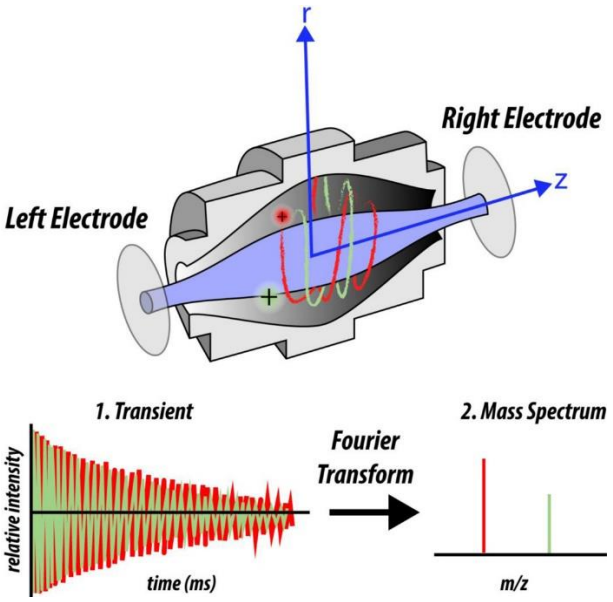


Figure 10: Scheme of an Orbitrap analyzer [145].

The frequency of this spiral movement is directly correlated with the m/z ratio. There are other differences between orbitrap and quadrupole. Orbitrap is subjected only to DC voltage, and the external electrode is at ground potential. It allows to measure all the *m/z* together and simultaneously and has higher resolution and accuracy [134]. Both revelators were used to detect STXs in bivalve, and several spectra were recorded [112], [146], [147].

Table 5 are reported the mass measurement data obtained in HRMS analysis by *Pearl Blay et al.* [146].

Toxin ID	Ion detected	Calculated m/z	Measured m/z	Measured std. Dev.	Error (ppm)
C1	$[M+NH_4]^+$	493.07657	493.07688	0.000165	0.64
C2	$[M+NH_4]^+$	493.07657	493.07693	0.000144	0.73
GTX2	$[M+H]^+$	396.09321	396.09326	0.000095	0.21
GTX1	$[M+H]^+$	412.08812	412.08823	0.000117	0.29
dcGTX2	$[M+H]^+$	353.08739	353.08735	0.000113	0.21
GTX3	$[M+H]^+$	396.09321	396.09321	0.000124	0.24
GTX4	$[M+H]^+$	412.08812	412.08821	0.000070	0.23
dcGTX3	$[M+H]^+$	353.08739	353.08733	0.000147	0.30
GTX5	$[M+H]^+$	380.09829	380.09824	0.000093	0.18
STX	$[M+H]^+$	300.14148	300.13123	0.000040	0.84
NEO	$[M+H]^+$	316.13639	316.13619	0.000049	0.64

Table 5: Mass measurement data reported by Pearl Blay et al. during HRMS analysis [146]

4. Validation procedure for an analytical method

4.1 General introduction

Method validation is a requirement necessary in analytical chemistry activities. The requirements in standards such as ISO / IEC 17025 [148], ISO 15189 [149], and ISO 15195 [150] clarify what is method validation. Validation is specified in ISO/IEC 17025, and it is "t*he confirmation by examination and the provision of objective evidence that the particular requirements for specific intended use are fulfilled."* The validation process has as its purpose the validity of use of a method, by evaluating parameters useful for this purpose (technical characteristics, applicability, analytical performance, etc.). The parameters derived from the validation process can be considered characteristics of the "good-service" that the laboratory is able to provide, and there give an idea of the reliability of the data coming from an analysis. Validation therefore has a function both internal to a laboratory. In fact, it can be a tool for checking the performance of an instrument and the skills of an operator who is external to the laboratory because he provides parameters that highlight the quality of the analytical data with other laboratories.

The validation of a method is must be done when the laboratory wants to use nonstandard methods, laboratory-designed/methods, standard methods used outside their intended scope, and change and modify some standards methods [148]. In addition, the standard method needs to be *verified* by the end-user and not validated [149]. For the validation procedure, some performance characteristics must be evaluated during the method validation. If an internal method is validated, it is considered equal to an official method. However, regarding STXs, the European Legislation is clear. The only international validated method valid is the Lawrence Method [67, p. 627]. Furthermore, the Commission Regulation (EU) 2019/627 specifies that STX, neoSTX, GTX1, GTX4, GTX2, GTX3, B1, B2, C1, C2 C3, C4, dcSTX, and dcNeoSTX must be analyzed.

Chapter 2: Significance and Aim of Research investigation

There are analytical difficulties for STXs analyses. It is hard to obtain CRM, and this has a high cost. The confirmatory method provided from the UNI EN 14526/2017 is time-expensive, and there are no LC-MS/MS methods that are internationally validated as required from (EC) Regulation 2019/627. Therefore, we decided to investigate the uniformity of several screening analytical techniques. Five positive samples confirmed with MBA and HPLC-FLD method were analyzed with HRMS and CBAs. The results were collected, and a comparison between methods was made.

Based on the reasons highlighted above, the aim of this research was achieved with a two-phase approach:

1. Bibliography research for an HRMS-HILIC method allows analyzing the most representative toxins in the samples. A screening method was found. Results were similar to those obtained with the HPLC-FLD and MBA analysis.

2. Bibliography research and application for CBA assay on the same actual samples;

3. Comparison between HRMS results and those in CBA.

Figure 11: Available techniques for the STXs analyses. "Celeste" arrows identify molecular techniques, "red" the mouse bioassay, "blue" the analytical techniques for the screening procedure and in "green" the quantitative analytical method.

Chapter 3: Chemical investigation of *Mytilus* spp. from Sicily. Screening method by LC-HRMS/MS

1. Introduction

Phycotoxins can accumulate in seafood, including fish and shellfish; therefore, their levels are regulated [161]–[163]. Monitoring the presence of phycotoxins in seafood is vital due to the risk for consumers and tourism. In fact, as a health concern, the member state of the European Union must carry out official monitoring of this toxin class in shellfish [29].

The presence of biotoxins in *Mytilus galloprovincialis* and clams (*Venerupis decussata*) collected in Sicily have been reported [164]. *Dell'Aversano et al.* collected samples in Syracuse Bay (Sicily, Italy) during an HBA. They found a very high STXs contamination with a value of 10851 μg saxitoxin equivalents per kg of shellfish tissue. The authors suggested activating monitoring programs for STXs. The presence of other phytotoxins has been reported in other parts of Italy. A five-year study in Tortolì Lagoon (Sardinia, Italy) reported the presence of okadaic acid group toxins and pectenotoxins2 (PTX2) in mussels [165]. The incidence of positive samples was 3.57% (39/1090), and only mussels had a detectable amount of water.

As potentially toxic phycotoxins have repeatedly been reported in Italy, especially Sicily, we developed a screening method to detect STXs. To date, does not exist official HRMS screening method, so we want to reduce this gap. A screening test can be helpful to increase the number of detectable samples and understand the epidemiological situation in Sicily deeply. We focused on regulated hydrophilic toxins known to cause problems in terms of public health. Lipophilic toxins are analyzed in LC-MS/MS and therefore were not investigated.

In this study, we report chemical analyses of *Mytilus galloprovincialis* collected in Syracuse harbors on the Ionian coast of Sicily. These samples were already analyzed in MBA and FLD [166].

Analyses were carried through LC-HRMS. The method used were the same as reported by *Boundy et al.* (2015) [112].

2. Material and methods

2.1 Chemicals, reagents, and materials

Ultrapure water was obtained from the Milli Q purification system (Merck, Darmstadt, Germany). Acetonitrile, ammonium format, and formic acid were purchased from Sigma-Aldrich (Amsterdam, The Netherlands). Toxin standards solution of decarbamoyl (dcSTX), neosaxitoxins (NEO), saxitoxin (STX), and gonyautoxins (GTX1, GTX2, GTX3, GTX4, GTX5) and N-sulfocarbamoyl-gonyautoxins (C1&C2) were purchased in 0.5 mL glass ampoules from the National Research Council Canada (Halifax, Nova Scotia). Each ampoule contains a certified concentration of toxin dissolved in aqueous 3mM aqueous hydrochloric acid. According to the supplier's instructions, all reference standards were stored at -20°C or + 4°C according to the supplier's instructions. Polytetrafluoroethylene (PTFE) 0.45 µm filter, 0.45 µm NYLON filter, and 0.22 µm PTFE filters were purchased from Merck KGaA (Merck, Darmstadt, Germany). Amorphous graphitized polymer carbon Supelco ENVI-Carb 250 mg/3 mL cartridges were bought from Sigma-Aldrich (P/N: 57088, St. Louis, MO, USA).

Stock Solution	**CRM-00-STX**	**CRM-00-NEO**	**CRM-00-GTX1&4**		**CRM-00-GTX2&3**		**CRM-00-GTX5**	**CRM-00-C1&2**	
Primary STD	STX	NEO	GTX1&4		GTX2&3		GTX5	C1 & C2	
Primary Conc. (µmol/L)	66.3	52.2	GTX1 66.4 GTX4 27.3		GTX2 56.3 GTX3 20.6		47.6	C1 84.4 C2 24.2	
Volume (µL)	100	100	100		100		100	100	
Final Volume (mL)	1	1	1		1		1	1	
Final conc. (nmol/L)	6630	5520	GTX1 6640 GTX4 2730		GTX2 5630 GTX3 2060		4760	C1 8440 C2 2420	
Working solutions	**CRM-00-STX (nmol/L)**	**CRM-00-NEO (nmol/L)**	**CRM-00-GTX1&4 (nmol/L)**		**CRM-00-GTX2&3 (nmol/L)**		**CRM-00-GTX5 (nmol/L)**	**CRM-00-C1&2 (nmol/L)**	
S1-1/50 dil	133	131	121	39	228	87	130	227	68
S2-1/100 dil	66.3	65.6	60.4	19.7	114,2	43,4	65	113	33,9
S3-1/200 dil	33.15	32.8	30.2	9.85	57,1	21,7	32,5	56,7	16,95
S4-1/500 dil	13.26	13.12	12.08	22.33	22,84	16.19	13	22,68	18.43
S5-1/1000 dil	6.63	6.56	6.04	1.97	11,42	4,34	6,5	11,34	3,39
S6-1/2000 dil	3.31	3.28	3.02	0.985	5,71	2,17	3,25	5,67	1,695

Table 6: Mixed stock preparation and concentration and calibration standard concentrations.

2.2 Sampling plan and sample collection

Mussels' samples (*Mytilus edulis*) that resulted as negative with MBA assay were used as blank material for HRMS analysis. Positive samples were obtained from IZS Sicilia. These samples were analyzed by Centro Ricerche Marine (National Reference Laboratory for Marine Biotoxins, Cesenatico). They were known to contain GTX1&GTX4, GTX2&GTX3, C1& C2, C3&C4, and STX. Positive samples were stored at -20°C until analysis. All mussels were defrosted when needed for the analysis under running water. The tissue was homogenized using a B-400 mixer (Büchi, Flawil, Switzerland) for 5 minutes. The temperature was kept at +5°C

during homogenization with an ice bath. The homogenate was divided into 5 g aliquots in polypropylene containers. This process was observed for both negative and positive samples.

Sample	%STX	%GTX5	%GTX 2,3	%C1,C2	%GTX 1,4	%neoSTX	%C3,4	STX eq ug STX HCl/Kg
1	2.27	3.72	14.59	11.22	51.41	3.1	13.70	5310
2	1.85	3.35	15.45	9.60	54.25	3.00	12.50	5227
3	2.09	3.69	14.24	7.47	59.41	3.44	9.66	5647
4	-	-	25.09	-	74.91	-	-	948
5	-	-	19.91	-	80.09	-	-	723

Table 7: STXs toxins in positive samples. Results are expressed as a percentage of STXs. For example, there are the concentrations expressed as eq STX HCl/Kg in the last column.

2.3 Equipment

SPE cartridge Supelco™ EnviCarb (250 mg, 3 mL), Supel QuE™ PSA/ENVI-Carb (EN) Tube 1, Supel QuE™ PSA/ENVI-Carb (EN) Tube 2, and Strata-X™ (500 mg, 3 mL) were obtained from Sigma Aldrich™ (Castle Hill, NSW, Australia). A Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ was used as a mass spectrometer (Thermo Fisher Scientific, CA, USA) with a heated electrospray ionization source (HESI-II). LC analysis was carried out with a Thermo Fischer Transcend II VIM UHPLC System (Thermo Fisher Scientific, CA, USA); the column heater was HotSleeve™+ and MultiSLEEVE controller. HILIC columns used in this study were Acquity UPLC™ BEH Amide 150 x 2.1, 1.7 µm, obtained from Merck™ KgaA (Darmstadt, Germany).

2.4 Analysis procedure

Positive samples were diluted with blank material to obtain the desired concentration.

Sample	STX eq ug STX HCl/Kg	%STX	%GTX5	%GTX 2,3	%C1,C2	%GTX 1,4	%neoSTX	%C3,4*
A	5310	2.27	3.72	14.59	11.22	51.41	3.1	13.70
B	5227	1.85	3.35	15.45	9.60	54.25	3.00	12.50
C	5647	2.09	3.69	14.24	7.47	59.41	3.44	9.66
D	948	-	-	25.09	-	74.91	-	-
E	723	-	-	19.91	-	80.09	-	-

Sample	Theoretical amount STX (STX eq ug HCl/Kg)	Theoretical amount STX (µgSTX.2HCleq/kg)	Theoretical amount GTX5 (µgSTX.2HCleq/kg)	Theorical amount GTX 2,3 (µgSTX.2HCleq/kg)	Theoretical amount C1,C2 (µgSTX.2HCleq/kg)	Theoretical amount GTX 1,4 (µgSTX.2HCleq/kg)	Theoretical amount neoSTX (µgSTX.2HCleq/kg)	Theoretical amount C3,4* (µgSTX.2HCleq/kg)
d1 - 1/2	474	-	-	119	-	355	-	-
e1 - 1/2	362	-	-	72	-	290	-	-
d1 - 1/4	237	-	-	59	-	177	-	-
e1 - 1/4	181	-	-	36	-	145	-	-
d1 - 1/6	158	-	-	40	-	118	-	-
e1-1/6	121	-	-	24	-	96	-	-

*Table 8: Positive samples with MBA and FLD method [166] tested with the HRMS method reported by Boundy et al. [112]. Samples were diluted with mussels that had STXs.*Were not analyzed with HRMS method.*

The approach for the screening procedure was semi-quantitative. The following parameters were calculated:

1) Response factor (R_F) for each STXs;

$$R_F = \frac{Peak\ area_{STD}}{C\ (uM)_{STD}}$$

2) The concentration for each STXs;

$$C\ (uM)_X = \frac{Peak\ area_{Sample}}{R_F}$$

3) The toxicity equivalent (STX);

$$C\ (uM\ STX.eq)_{Total} = \sum\ (TEF_{(toxin)} x C(uM)_{toxin})$$

4) The concentration of the toxins was calculated in the semi-quantitative approach

$$C_{\ ug\ STX\ eqv/Kg} = C\ (uM\ STX.eq)_{Total}\ x\ MW\ x\ \frac{V\ (mL) extrac}{m\ (g) shellfish\ meat}\ x\ Dil.$$

5) Total toxicity of the sample

$$\sum_{i=1}^{n} C_{\ ug\ STX\ eqv/Kg}$$

A reference level "RL" of 400 µg/kg STX was chosen. Were identified:

1. "<LOD" when area pick/noise <3, "<LOQ" when area pick/noise <10;
2. "<RL" when the quantification value was within the quantification range of the semi-quantitative method (200-400 µg/kg STX).
3. ">RL" Value over the proposed RL.

3. Results and discussion

3.1 Biotoxin Reaction Monitoring Transitions

STXs transition in MS has already been reported by several authors [112], [141], [167]. The geometry between mass spectrometers and ion sources can affect the performance of detectors, and for these reasons, it is essential to test standards when possible. The mass was obtained with the direct infusion without column. Both positive and negative mode was investigated.

Toxin	Formula	Detected ion (+)	Measured m/z	Detected ion (-)	Measured m/z
STX	$C_{10}H_{17}N_7O_4$	$[M+H]^+$	299.1342 u	$[M+HCOO]^-$	344.1324 u
NEO	$C_{10}H_{17}N_7O_5$	$[M+H]^+$	316.1363 u	$[M+HCOO]^-$	360.1273 u
GTX1	$C_{10}H_{17}N_7O_9S$	$[M+H\text{-}SO_3]^+$	332.1314 u	$[M-H]^-$	410.0735 u
GTX2	$C_{10}H_{17}N_7O_8S$	$[M+H\text{-}SO_3]^+$	316.1363 u	$[M-H]^-$	410.0735 u
GTX3	$C_{10}H_{17}N_7O_8S$	$[M+H]^+$	396.0933 u	$[M-H]^-$	410.0735 u
GTX4	$C_{10}H_{17}N_7O_9S$	$[M+H]^+$	412.0882 u	$[M-H]^-$	410.0735 u
GTX5	$C_{10}H_{17}N_7O_7S$	$[M+H]^+$	380.0983 u	$[M-H]^-$	378.0837 u
C1, C2	$C_{10}H_{17}N_7O_{11}S_2$	$[M+H\text{-}SO_3]^+$	396.0932 u	$[M-H]^-$	474.0355 u

Table 9: Measured mass obtained from HRMS in both positive and negative mode.

STX, NEO, and their dicarbamoyl derivates gave $[M+H]^+$ ions while gonyautoxins and and their derivates lost the hydroxysulfate group at C-11.

3.2 Stationary and mobile phase selection

The column selection represents an essential phase during the validation of an analytical method. Typically, it is one of the first steps when reference standards are available. STXs are polar molecules, and therefore HILIC chromatography has been tested several times. Acquity UPLC™ BEH Amide 150 x 2.1, 1.7 µm, obtained from Merck™ KgaA (Darmstadt, Germany), was tested for STXs. It is characterized by ethylene bridged hybrid (BEH) particle that improves the retention of polar analytes and can resist high pH variations [168].

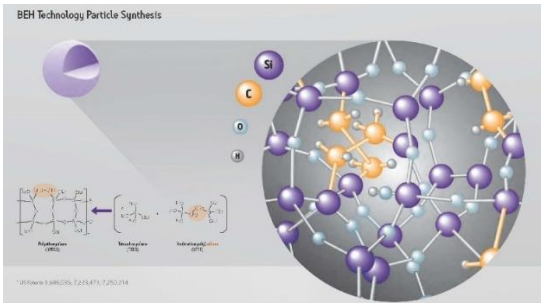


Figure 12. BEH technology from Waters https://www.waters.com/waters/it_IT/BEH-%28Ethylene-Bridged-Hybrid%29-Technology/nav.htm?locale=it_IT&cid=134618172

This column was tested because it was successfully applied to similar methods [169].

The nature of the mobile phase is critical for developing an efficient method for the determination of STXs. The high polarity of STXs requires a high amount of organic solvent in the mobile phase (methanol or acetonitrile). Reverse-phase chromatography is possible; however, it requires a high amount of ion-pairing reagents that need to be added to the mobile phase but can interfere with ionization and ion source [170]. HILIC mobile phase does not require ion-paring reagents, so the ionization efficiency is not reduced. The percentage of organic modifier heavily influence the retention time of STXs. It increases with a high percentage of organic modifier and polarity of the solute.

For this reason, the order of elution is typically gonyautoxins, followed by STX and NEO and dicarbamoyl derivates at the end [141], [171]. Acetonitrile and methanol are the most common organic modifiers. The mobile phase used was the same as described by Turner et al. [169]. Briefly, mobile phase A was water with 0.015% formic acid + 0.06% ammonium hydroxide, and B was 70% ACN-0.01% formic acid. Methanol is protic solvents, while ACN aprotic can create a hydrogen bond with the active polar sites on the BEH site of the Acquity UHPLC column. Thus, producing a more hydrophobic stationary phase for replacing water molecules and analytes with hydrogen-bond capability is retained less [172].

For this reason, the percentage of organic modifier influence the retention time of STXs [173]. Acetonitrile provided sharper peaks while methanol reduced or changed analytes' retention time. For this reason, we prefer to avoid methanol. Regarding ammonium formate buffer, it is required because without STXs were not retained. The gradient used was the same as described by dell'Aversano et al. [164].

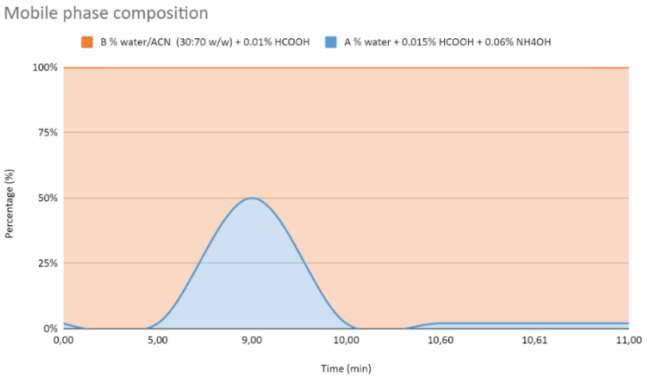


Figure 13: Mobile phase for STXs analysis.

3.3 Extraction procedure

Once the mobile and stationary phase is established, the extraction method is an important parameter to consider. The matrix can retain analytes in the sample. There are extraction procedures used worldwide and can serve as a good starting point. Most procedures use boiling techniques to extract samples [123], [141], [164], [169]. An example of this is the extraction provided by the AOAC MBA method [123]. We decided to follow the extraction procedure described by Boundy [112]. This method is based on a single-step dispersive extraction using 1% acetic acid. The SPE was conducted with graphitized carbon Supelco ENVI-Carb 250 mg/3 mL. The scheme for the extraction procedure tested is presented in the next Figure. The most common solvents during the boiling procedure are acetic acid or hydrochloric acid [174]. However, in acid condition, the STXs can be converted to more toxic forms and therefore the total toxicity can be overestimated [63]. This phenomenon happens especially with hydrochloric acid [36], where some STXs (C1, C2, and GTX5) can be hydrolyzed into toxic analogs (GT2, GT3, STX). The use of acetic acid has demonstrated the best overall recovery [175], [176], STXs extraction by AcOH solvent is higher than that by the HCl [175], [176]. More recent HILIC-MS/MS methods have abandoned HCl in favor of AcOH [112], [123]. The boiling procedure can cause the conversion of STXs in M toxins. However, heat treatment increases the overall recovery of STXs [177] and therefore is preferred. Clean-up methods significantly increase the sensitivity of the analysis. Two-step extraction with clean-up procedure was already adopted from several authors [127], [141]. In STXs analysis, there is strong signal interference due to the matrix effect. STX and NEO signals are enhanced while suppressing other signals [178]. This effect is significant in STXs analysis since results must be expressed as μg STX 2-HCL equivalent/kg as required from European Regulation (EC) 2019/627 [67]. Regarding the SPE, ENVI-CARB^{TM} cartridge (250 mg/3 mL. Supelco) has been used in other methods successfully with high recoveries that range from 75 to 125% [112], [179]–[181].

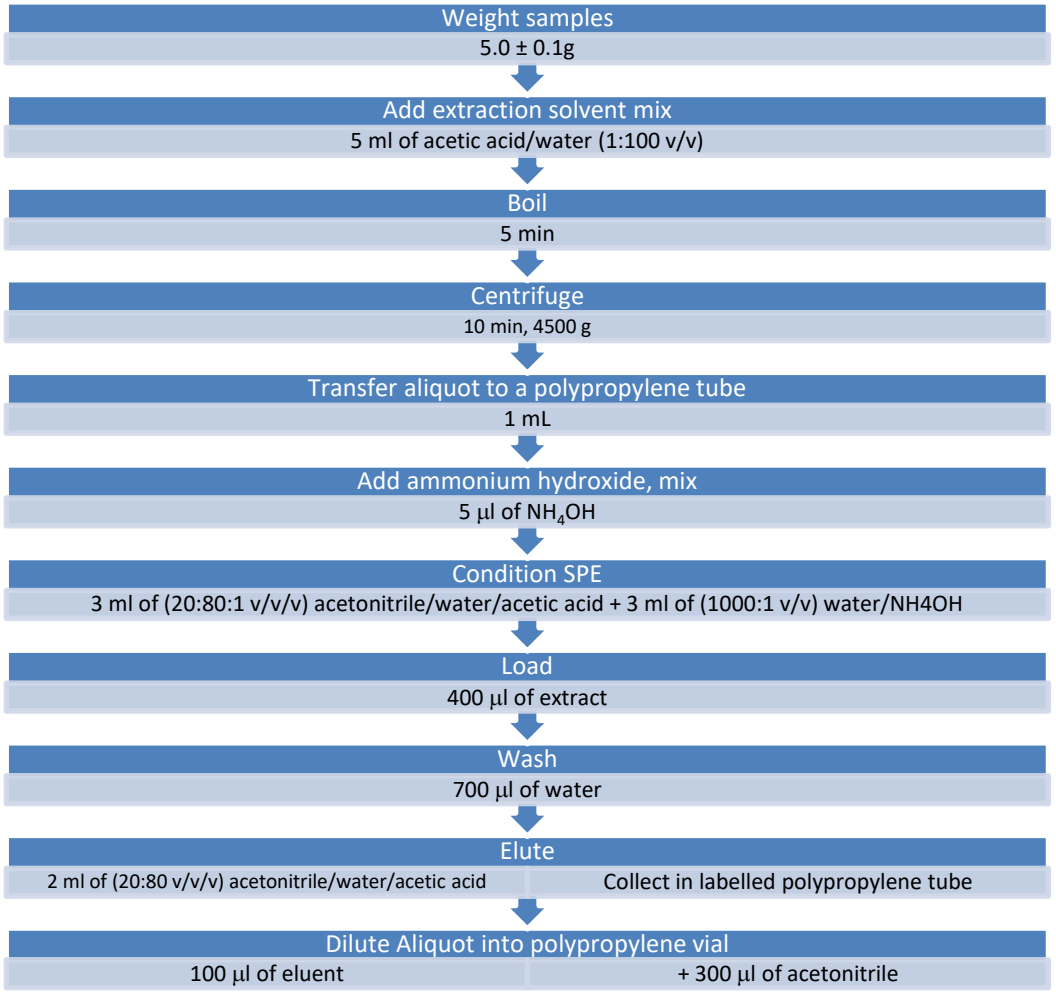


Figure 14: Extraction procedure according to Boundy et al. [112].

Superclean ENVI-Carb is a non-porous carbon that was already tested for the STXs extraction in mussels and was effective for the matrix interferences, in particular salts that can influence chromatography and suppress electrospray ionization [112], [127], [141]. The process was conducted as described by Boundy et al. [112]. The cartridge was activated with 3 mL of acetonitrile/water (20:80) containing 1% acetic acid, equilibrated with 3 mL of 0.1% (v/v) ammonium hydroxide (25%). An amount of 400 µl of the extracts was loaded into the cartridges. Then, 700 µl of water was used to wash, and 2 mL of 20% acetonitrile/water (v/v) containing 1% acetic acid was used for eluting. 100 µl of the eluate was combined with 300 µl of acetonitrile (final volume 400 µl).

3.4 Screening Procedure

The extraction method reported by *Boundy et al.* [112] was tested on positive samples and their respective dilutions. Over 400 µg/kg STX was identified as ">RL." The results of the screening procedure are reported in the Table above. Certified positive samples (FLD method)

result in each case over the "RL". The screening method did not yield false negatives, even with the lowest concentration in diluted samples. Concentration under 200 μg/kg STX eq was expressed as "<LOQ."

Sample	FLD results (STX eq ug STX HCl/Kg)	MBA results	Samples extracted (STX eq ug STX HCl/Kg)	HRMS results
A	5310	Positive	4220	Positive (>RL)
B	5227	Positive	4070	Positive (>RL)
C	5647	Positive	4530	Positive (>RL)
D	948	Positive	802	Positive (>RL)
E	723	Positive	570	Positive (>RL)
Diluted samples	**Theoretical amount (STX eq ug STX HCl/Kg)**	**Samples extracted (STX eq ug STX HCl/Kg)**	**Underestimate percentage (%)**	**HRMS results**
D1-1/2	474	402	15.19	Positive (>RL)
E1-1/2	362	280	22.65	<RL
D1 – 1/4	237	142	40,08	<LOQ
E1 – 1/4	181	90	50,21	<LOQ
D1 - 1/6	158	79	50	<LOQ
E1 – 1/6	121	60	50,07	<LOQ

Table 10: Results of HRMS analyses. The extracted sample with the Boundy method has a dilution factor of 2 [112].

The HRMS results are consistent with the STXs present in all samples extracted. Therefore, the screening test underestimates the total theoretical toxicity of STXs present in samples. The underestimation was from a minimum of 15.19% (sample D1-1/2), from a maximum of almost 50,21 % in samples with a low concentration of STXs (D1-1/6, D1-1/4, E1-1/4, E1-1/6). Therefore, the method performed better with a high concentration of STXs, a characteristic that is well suited to a screening test. Some STXs were not quantified due to the lack of analytical standards (C3, C4, and dcNEO). However, C3 and C4 are characterized by a low TEF [182] and must be at high concentration to contribute significantly to the total toxicity. Further studies are needed. First is a validation procedure that can assess specificity, sensitivity, matrix effect, linearity, repeatability, method recovery, relative standard deviations (RSDs), and intra-day and inter-day variations.

Chapter 4: Cell-Based (Cytotoxicity) Assay (CBAs)

1. Introduction

Several works in the bibliography show that the presence of PSP on cell cultures can be highlighted by exploiting their ability to bind VGSCs by competing with the binding of other molecules. Among these, the most used are ouabain (O) and veratridine (V) [183]. These toxins are used for the dose-response curve for neurotoxins acting on the voltage-gated sodium channel (VGSC) [184]. Ouabain is a cardioactive glycoside whose best-known action is the inhibition of Na / K ATPase, the ubiquitous plasma membrane enzyme responsible for transporting Na and K ions across membranes using ATP as a driving force [185]. Cardiac glycosides are a class of natural products traditionally used to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias [186]. These glycosides are found as secondary metabolites in various plants, including Strophanthus spp. (ouabain), *Digitalis lanata* and *Digitalis purpurea* (digoxin, digitoxin), *Scilla maritima* (proscillaridin A), *Nerium oleander* (oleandrin, oleandrigenin) but also in frogs (some frog-poisons contain bufadienolides like bufalin, marinobufagenin) [187], [188]. It has been shown that Na / K ATPase, in addition to its pump function, is able to act as a receptor, activating various signal cascades [189]. Veratridine, a steroid alkaloid, can selectively open voltage-dependent sodium channels and prevent their inactivation, allowing the presence of some functional characteristics typical of mature neurons, such as membrane ion channels, to be detected in differentiated serotonergic neurons in vitro voltage-gated for sodium and calcium [190].

Combining the two molecules at specific concentrations causes high cell mortality [184]. STXs can compete with the two drugs in binding with VGSCs, promoting cell survival instead. However, an important limitation to the use of cell cultures is represented by the system's high sensitivity towards components that may persist in the mollusk extract (matrix effect) and by the cell susceptibility to low pH values of the extract.

2. Materials and Methods

2.1 Cell culture

Mouse neuroblastoma cells (Neuro-2a, ATCC CCL-131) were grown in flasks of 75 cm 2 with MEM (Minimum Essential Medium) supplemented with 10% heat-inactivated fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 50 g/mL streptomycin, and 50 units/mL penicillin. Cultures were maintained at 37 °C in a humidified air–CO2 atmosphere (95 + 5). In both media formulations, the fetal bovine serum was reduced from 10 to 5%. Subcultures were carried out every 4-5 days when the growth of the monolayer was found to be confluent. The nitrogen

2.1.1 Extraction procedure

Sixty homogenates of MEL resulted negative to mouse test were used to optimize the PSP extraction method.

The homogenates were subjected to the PSP extraction process according to the AOAC 959.08 method. A control group was directly processed on Neuro 2° cell; A second group was subjected to a modified and innovative purification method based on columns by affinity *Agilent SampliQ C18* (Agilent Technologies). Subsequently, the extract was dried and resuspended in a culture medium before the cell bioassay. Positive control of 0.5% phenol was used to demonstrate the system's ability to produce a reproducible cytotoxic response.

Details of the PSP extraction are represented as follows:

A 10 g aliquot of the homogenized sample was weighed and transferred into a 15 ml sterile conical tube. About 10 ml of HCl 0.1M were added to the solution and vortexed for 3 minutes. The pH of the solution was maintained at 3±1 with NaOH. The solution was heated at 100°C for 5 minutes.

Subsequently, the solution was stored at room temperature. After a pH evaluation, the solution was centrifugated at 3000 rpm for 5 minutes. Subsequently, the surnatant was recovered, put into a 50 ml tube, and raised up to 20 ml with ultrapure water. Finally, the solution was stored overnight at +4°C then centrifugated at 3000 rpm for 5 min for another recovery of the surnatant.

2.1.2 Purification step

Before the purification, the SampliQ C18 cartridges were conditioned with 5 ml of methanol. Subsequently, a vacuum was applied, and the eluent was removed. The cartridges were stabilized with Ultrapure water. One ml of the samples was added into the cartridges then the eluent was removed after vacuum. Subsequently, the cartridges were washed with 5 ml of ultrapure water; the eluent was removed again after vacuum. The last elution steps were carried out by adding 5 ml of methanol into the cartridges; the eluent was collected after vacuum. Subsequently, 2.5 ml of isopropanol was added to the cartridge for another eluent collection after vacuum. The samples were dried under nitrogen flux to remove any traces of methanol and isopropanol. The pellet of the samples was resuspended with 1 ml of MEM culture with 1x of antibiotics. The samples obtained were ready for cell bioassay.

2.1.3 Cell bioassay

Culture plates for cell bioassay were prepared in the function of the samples to be tested. The samples were examined in triplicate. First, cell suspensions were plated into microtiter 96-well culture plates using 200 µl per well of Neuro-2a cells at 30.000 cells/100 µl. One column of the plates was used for the cells viability control (KC). The cultures were then incubated for 24 h at +37°C (5% CO2).

2.1.4 Inoculation of the samples

After 24 hours, the culture medium was removed from the plate. Then the plate was structured for the inoculation as follows:

1) Line 1: 100 µl of culture medium as KC.

2) Line 2: 200 µl of samples in triplicates and 200 □l of positive control

3) From line 3: 100 µl of culture medium performing scalar dilutions (in base two).

One hundred µl was removed from the last dilution (1:32). Finally, the plate was incubated for 24h at +37°C (5% of CO 2).

After the incubation, the plate was observed by optical microscopy for the cytotoxic effect evaluation (ETC).

Twenty μl of MTS reagent CellTiter 96 ® (Promega, USA) at +37°C were added to all the wells and incubated for four h at +37°C (5% of CO2). The plates were immediately read on a Thermo automated multiwell scanning spectrophotometer at 490 nm. The test was considered reliable if:

- KC does not show morphological alterations attributable to ECT (viability 100%);

- The PTC shows decreasing ECT intensity, proceeding in the reading, from TQ at the 1:32 dilution.

The reading on the spectrophotometer carried out at 490 nm allows us to correlate the intensity of the purple color produced, measured in O.D., with the percentage of viable cells. Therefore, the percentage of viable cells was calculated as follows:

$$\% \text{ Cell viability} = \text{M O.D. X} / \text{M O.D. KC} \times 100.$$

Where:
M O.D.x is the mean of the optical densities produced by the sample in triplicate;

M O.D.KC is the average of the optical densities produced by the KC;

Therefore, Samples are considered cytotoxic if the cell viability percentage is <70%. Samples are considered non-cytotoxic if the cell viability percentage is >70%.

2.1.5 Optimization of the competitive in vitro assay on Neuro-2a cells

For the optimization of the competitive assay, the standard toxin Gonyautoxin 1-4 (GTX1-4) and concentrations of Ouabauine (O) and Veratridine (V) can induce 80% of cell mortality were used (1 mM and 0.1 mM, respectively). After 24 hours, the culture medium was removed from the plate. Then the plate was structured for the inoculation as follows:

- 100 μl of culture medium (MEM with antibiotics 1X and SFB 10%) in all wells of the KC line;

- 100 μl of culture medium containing 12 μl of GTX1-4 toxin (80 μM/L), in three wells as toxin control (without drugs);

- 100 μl of medium containing 1 mM of Ouabauine in three wells as Ouabauine control;

- 100 μl of medium containing 0.1 mM of Veratridine in three wells as Veratridine control;

- 100 μl of medium containing 0.1 mM of Veratridine and one mM of Ouabauine in three wells as Veratridine and Ouabauine controls;

- 50 μl of culture medium containing the mixture of the two drugs O and V (1 mM and 0.1 mM, respectively) in all wells, excluding KC and controls;

- In line 2: 50 μl of culture medium containing GTX 1-4 toxin, previously diluted, obtaining a final concentration of 4000 μg/Kg;

- Lines 3-7: medium containing doubling dilutions of the toxin analyzed (from 4000 μg/Kg to 125 μg/Kg).

The plate was incubated for 24 h at +37°C (5% of CO 2). The reading of the plates was carried out by spectrophotometry analysis as described above. In addition, the same competitive test was conducted on an extract of MEL experimentally contaminated with the standard toxin GTX1-4 and subsequently purified with Agilent SampliQ C18 columns, in the presence of O and V (1 and 0.1 mM, respectively).

2.1.6 Analyses of HRMS purified samples

The samples extracted and analyzed with the HRMS method were tested with the N2A assay. Acetonitrile was evaporated with a gentle nitrogen stream at +30°C and resuspended in MEM. The samples tested were:

1. Sample A, B, C, D, E;
2. D1-1/2 was obtained by diluting with blank matrix D in a ratio of 1:4.
3. D1-1/4 was obtained by diluting with blank matrix D in a ratio of 1:4.
4. D1-1/6 was obtained by diluting with blank matrix D in a ratio of 1:6.
5. E1-1/2 was obtained by diluting with blank matrix E in a ratio of 1:4.
6. E1-1/4 was obtained by diluting with blank matrix E in a ratio of 1:4.
7. E1-1/6 was obtained by diluting with blank matrix E in a ratio of 1:6.

3. Results

3.1 Cytotoxicity test on Neuro-2a cell line

A plate with the samples examined is shown in the Figure below.

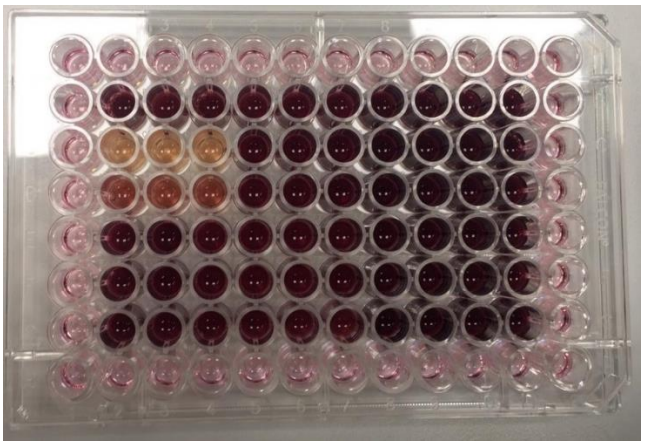


Figure 15: Plate for the CBAs assay.

The samples extracted and tested directly on Neuro-2a cells in 96-well culture plates showed a cytotoxic effect with vitality values <70% up to 1:4 dilution. Conversely, all the extracts purified with the innovative method proposed in this work, consisting of Agilent SampliQ C18 columns, dried and re-suspended in culture medium, did not show any cytotoxic effect, giving viability values >70%. The 0.5% phenol solution, used as a positive control, always produced marked cell mortality (from 95 to 70%) up to the 1:2 dilution. The HCl 0.1 M control produced a cytotoxic effect up to 1:4 dilution (Figure 16).

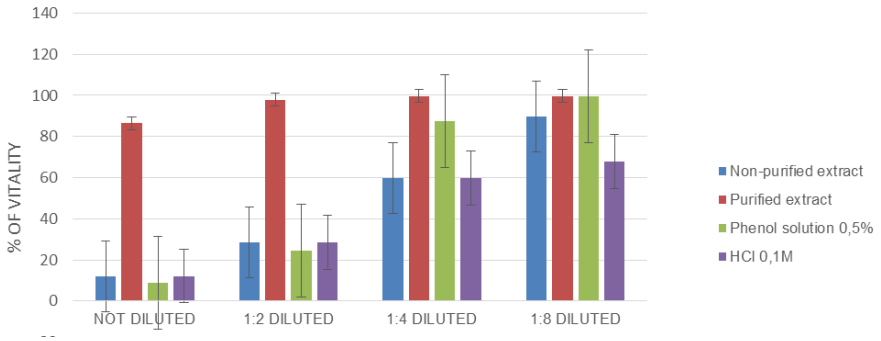


Figure 16: Effects of non-purified shellfish extracts, purified shellfish extracts, and controls on Neuro-2a cells after 24 h of exposure. Data are expressed as a percentage of cell viability.

Conversely, all the extracts purified with the innovative method proposed in this work, consisting of Agilent SampliQ C18 columns, dried and re-suspended in culture medium, did not show any cytotoxic effect, giving viability values >70%. The 0.5% phenol solution, used as a positive control, always produced marked cell mortality (from 95 to 70%) up to the 1:2 dilution. The HCl 0.1 M control produced a cytotoxic effect up to 1:4 dilution (Figure 16).

3.1.1 Competitive assay on Neuro-2a cells

Neuro-2a cells were susceptible to the combined action of the two drugs O and V at concentrations of 1 mM and 0.1 mM, respectively, showing vitality values <30%. On the other hand, GTX1-4 toxin used alone did not produce any cytotoxic effect on Neuro-2a cells, showing cell viability values >70%. Furthermore, cell mortality induced by the combination of drugs was inhibited in the presence of the GTX1-4 toxin, tested into a concentration range from 4000 µg/kg to 125 µg/kg, showing cell viability values greater than 50% (Figure below). The same results were obtained with the shellfish sample contaminated with the toxin.

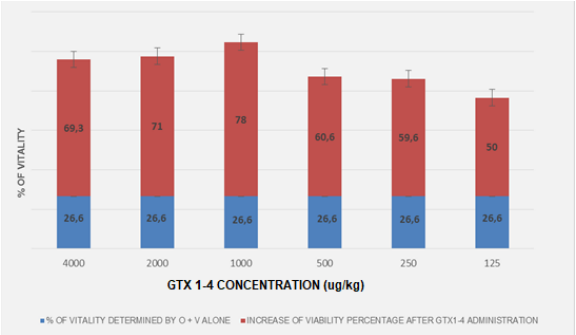


Figure 17: Effects of GTX1-4 on cell viability. Red bars represent the percentage of cell viability; blue bars represent the effect of Veratridine and Ouabain (1 mM and 0.1 mM, respectively) on cell viability (%).

3.1.1 Analyses of HRMS samples with Neuro-2a Cells assay

Samples extracted for the HRMS method were tested with the competitive assay on Neuro-2A Cells. Each sample was tested with veratridine and ouabain at 1 mM and 0.1 mM. The official samples tested had an STX eq ug STX HCl/Kg range from 723 (E) to 5647 STX eq ug STX HCl/Kg (C).

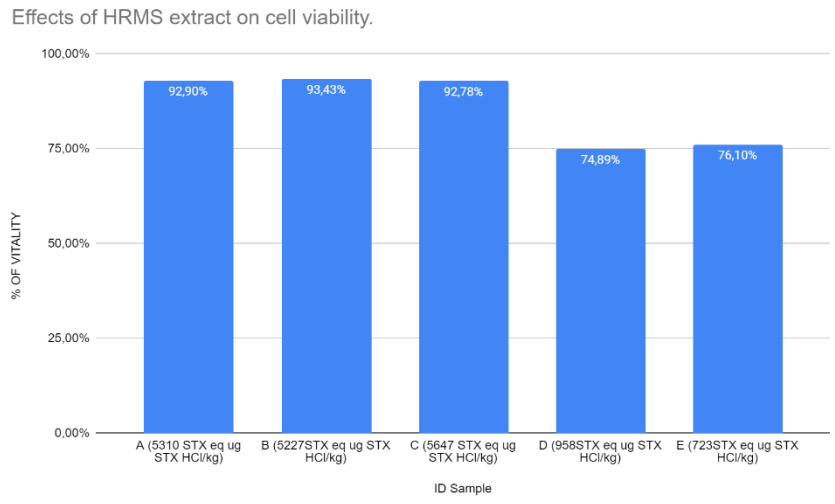


Figure 18: Effects of HRMS extract on cell viability. Bars represent the percentage of cell viability (%). Veratridine and Ouabain were constant at 1 mM and 0.1 mM respectively.

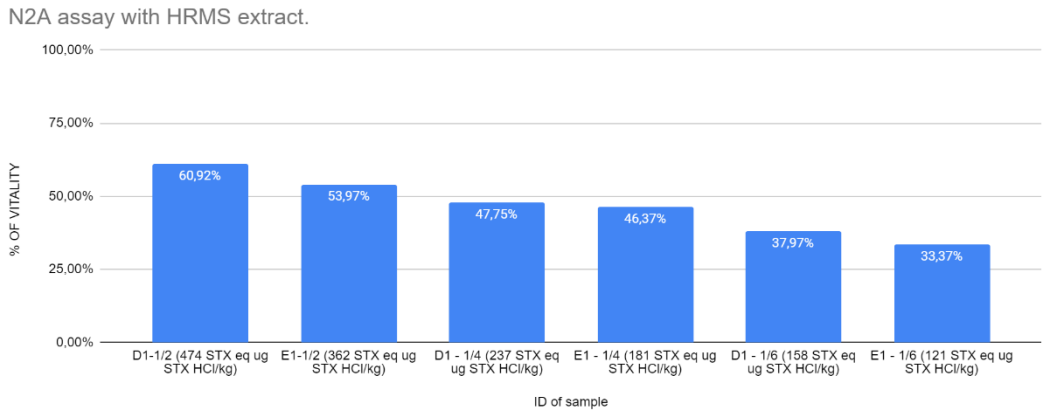


Figure 19. Effects of HRMS extract on cell viability. Bars represent the percentage of cell viability (%). Veratridine and ouabain were constant at one mM and 0.1 mM, respectively. The concentration reported was the theoretical amount.

ID Sample	Theoretical amount (STX eq ug STX HCl/Kg)	HRMS results (STX eq ug STX HCl/Kg)	Vitality rate
D1-1/2	474	402	60.92 %
E1-1/2	362	280	53.97 %
D1 - 1/4	237	118	47.75%
E1 - 1/4	181	90	46.37%
D1 - 1/6	158	79	37.97%
E1 - 1/6	121	60	33.37%

Table 11: Samples extracted for the HRMS method and analyzed with N-2A assay.

4. Discussion

The non-purified samples tested directly on the cells confirmed the presence of a cytotoxic effect due to the nature of the matrix and the use of acid (HCl) in the extraction process. This cytotoxicity was evident up to the 1:4 dilution, beyond which the viability values were above 70%. The purification process with Agilent SampliQ C18 columns and the subsequent drying and resuspension of the pellets in the culture medium contributed to eliminating the interference of the matrix and the lethal effect due to the presence of acids and alcohols, used both in the extraction and purification process, promoting cell viability values higher than 70%, starting from the maximum concentration of the sample.

The Neuro-2a CBA is a specific method for detecting toxins that target VGSCs. For VGSC- blocking toxins, the reduction of cytotoxicity, or cell rescue, in the presence of O/V shows a typical sigmoidal dose-response curve [191], [192]. In the case of STX, Cañete and Diogène (2008) [193] reported a maximum cell rescue (40–50%) at a concentration of 100 nM in comparison with the modified method proposed in this work. The interference of PST-negative matrices in the presence of O/V must be considered to ensure the reliability of the results. The competition for VGSCs between STX and monovalent and divalent cations such as Li^{+}, Cd^{+2}, Ca^{+2}, and Zn^{+2} [194]–[196] could explain, in part, the observed interference of the

PST-negative matrices in the Neuro-2a CBA. Taken together, the results obtained by the modified method proposed in this work suggest that the toxicity and interference in cell rescue of PST-negative extracts will depend on the extraction method and matrix. Sato et al. (1988) [197] reported that interferences, such as NaCl, KCl, and polypeptide at concentrations greater than 3.5%, or meat extract concentrations above 2%, cause errors in the quantification of fortified samples.

The matrix effect in the detection of PSTs has also been reported in other toxicological methods, including the MBA [17], [33], [63]. Turner et al. (2011) [123] showed that the MBA underestimates PST quantification two- to three-fold in biological matrices with high concentrations of zinc and manganese. The lethal effect induced by the 0.5% phenol solution on the Neuro-2a cell line demonstrated the ability of the cell system to produce a reproducible cytotoxic response. Moreover, Neuro-2a cells proved to be sensitive to the combined action of the two drugs (O and V), while the GTX1-4 toxin did not induce any lethal effect on the cells when used alone.

In the competitive assay, the presence of different concentrations of GTX1-4, used directly on the cells or added to the shellfish extracts (spiked samples), inhibited the lethal effect of the drugs. Furthermore, this essay mainly detected GTX1-4 concentrations five times higher and six times lower than the established threshold (800 µg/Kg). These results support the validity of the competitive Neuro-2a cell-based assay as a method suitable to highlight the presence of different types of PSP toxins in shellfish, exploiting the interaction with sodium channels.

HRMS extracts were tested with an N2A assay. As a result, the minimum value of cells vitality (33.37%) was obtained with the minimum calculated toxicity of 60.25 STX eq µg STX HCl/Kg. In comparison, concentration near the 800 eq µg STX HCl/Kg limit gives a cell vitality percentage of at least 74.89%. Therefore, we can consider as "negative" all HRMS extracted samples that give a cell vitality of a maximum of 50% with the N2A assay. For this reason, the next goal for this modified method could be its validation on a large scale, using wild shellfish samples and carrying out inter-laboratory tests to validate its effectiveness and reproducibility.

The release of urban, industrial, agricultural, and livestock effluents into the marine environment, together with the significant climate changes worldwide, contribute to increasing the risk of new algal species and new algal biotoxins in the marine environment. Another risk factor is represented by the international trade in shellfish products that could promote the diffusion of these compounds, maintaining a high level of risk worldwide [198]. Impacts of

climate variability and future climate change on harmful algal blooms and human health [199]. Therefore, a rapid in vitro biological assay could be a helpful screening tool together with the recommended analytical methods to ensure continuous surveillance and monitoring of shellfish and guarantee public health. The interaction of cells in culture with different molecules always induces a biological response that is detectable both at the functional and morphological levels. For this reason, cell-based assays are suitable for these purposes, and their use in toxicology is growing, resulting in many cases substitute entirely for in vivo assays. However, the thorough knowledge of mechanisms of interaction between already existing biotoxins and future biotoxins and cell structures and the deriving effects represents a crucial step that can lead to the development of valid in vitro methods or the implementation of more sensitive, accurate, and rapid analytical methods such as the LC-HRMS method proposed in this thesis.

Chapter 5: Conclusions and future work

5.1 Conclusions and Recommendations

Certified positive samples with MBA and HPLC-FLD methods were tested with screening procedures involving analytical (HRMS) and cellular (N2A) assays.

The analytical method reported by *Boundy et al.* (2015) [112] was fully adapted to HRMS analyses, and the results of the method were adequate for a screening test that allowed to analyze several STXs in accordance with the in-house reverence level (400 μg/kg STX). C3-C4 and dcNEO toxins were not analyzed due to the lack of standards. HRMS screening procedure provided good results, and false compliant samples were not reported, even at concentrations well below the reference limit of 800 STX HCl eq/kg.

However, the HRMS method underestimates the concentration in all the samples analyzed. For these reasons, it was more reasonable to use for our method an RL (400 STX HCl eq/kg) that is not the EU limit set by 853/2004 [64].

N2A assays gave results following analytical methods and suitable for a screening test. The purification process with Agilent SampliQ C18 columns eliminates the interference of the matrix and the lethal effect of acids and alcohols. The SPE eliminated one of the biggest problems typical of CBAs assay. This assay was able to detect GTX1-4 concentrations five-time higher and six-time lower than the established threshold (800 μg/Kg). The test on the extracted HRMS samples showed a correlation between concentration calculated with the HRMS method and cell vitality. Samples with total toxicity under 800 STX HCl eq/kg had a cell vitality under 77% in all cases.

The different techniques proved to be complementary and adequate for the screening test. One of the problems of analytical techniques is that the commercial availability of certified reference materials is critical and not always available for all STXs. In fact, in HRMS, were not possible to analyze C3-C4 and dcNEO toxins. This can lead to an underestimation of the total toxicity of the samples. These issues were mitigated with the N2A assay that does not require all analytical standards and can quantify even unknown toxins.

5.2 Future work

Further work should be performed to ensure the relevance of the techniques used. First, the method must be validated, and the uncertainty in the current analytical and cellular techniques must be investigated. Not all toxins were analyzed in HRMS, and therefore new STXs will be considered in the validation procedure. The percent recovery was not evaluated, and therefore the concentration of all extracted can be different from the calculated ones. The underestimation in HRMS analyses is probably due to the extraction procedures that were not appropriately conducted or matrix effects. This can affect also extract that were analyzed in N2A competitive assay.

N2A competitive assay on the extract showed a correlation between STXs total toxicity and cell vitality. However, even in this case, a validation procedure is needed to assess a maximum and a minimum value of cell vitality that correspond to the EU Regulation limit.

Chapter 6: References

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