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Analysis of paralytic shellfish toxins in seafood by liquid chromatography: A critical review of the methodologies developed

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ABSTRACT

Paralytic shellfish toxins (PSTs) are natural toxins produced by some microorganisms, especially during harmful algal blooms (HABs). Molluscs and other marine animals can accumulate significant amounts of these toxins, causing food poisoning. Due to the seriousness of this poisoning, the European Union has established a concentration limit and an official method based on liquid chromatography for their analysis. PSTs are very challenging analytes due to their very high polarity and occurrence of different isomers with varying toxicity towards humans. Current available extraction methods are adapted from a previous existing bioassay and not fully validated yet. Recovery efficiencies of the extraction procedure are occasionally low, and further clean-up can lead to highly variable results depending on the toxin and the matrix analysed. Detection of PSTs by mass spectrometry offers the ability to identify all toxins in a single analysis, which is an improvement over the current official method based on fluorescence detection prior derivatisation. As a drawback, normal phase chromatography is required, which tend to be less robust that more conventionally used reversed-phase chromatography used in the official method. New extraction techniques and recent advances in the mass spectrometry field (e.g., high-resolution mass spectrometry and ion mobility) have been barely applied yet to the analysis of PSTs. An increase in the frequency and extension of HABs due to global warming will lead to more severe impacts on health, environment and economy in coastal areas. An improvement of current existing analytical methods is therefore needed to allow for a faster and more accurate monitoring of PSTs.

1. Introduction

Paralytic shellfish toxins (PSTs) are potent neurotoxins that cause the interruption of nerve transmissions by blocking sodium channels and interrupting ion transport [1]. PSTs are produced mainly by marine microorganisms (e.g., dinoflagellates) and by some freshwater cyanobacteria [2], and can be accumulated and transferred through the trophic chain [3,4]. Among all the organisms exposed to PSTs, bivalve molluscs can accumulate the highest levels of these toxins through filter feeding. They can bio-accumulate high concentrations of PSTs without experience poisoning, becoming potential vectors of contamination towards animals that feed on them, including humans [5,6]. Poisoning by paralyzing toxins is a serious health threat as acute cases can provoke death in consumers [7]. The poisoning can be caused by the ingestion of both raw and cooked contaminated food, as PSTs are not thermolabile. The clinical effects manifest quickly and there is no antidote currently available [8]. Within the European Union, Regulation (EC) No 853/2004 sets a limit of 800 μg of saxitoxin (STX) equivalents per kg of shell-fish meat [9]. Due to their high toxicity towards humans, the concentrations of PSTs are not usually expressed in ng g^{-1} or μg g^{-1} as with other contaminants, but as the sum of the toxicity equivalents to STX, the most common toxin, for each PST. Table 1 shows the most widely analysed PSTs, all of them having the same core structure consisting on a trialkyl tetrahydropurine, with the purine ring containing two NH_2 groups. However, despite their chemical similarities, toxicity of PSTs can be very different depending on variations in functional groups connected the ring and ranges 0.01–2 Toxicity Equivalency Factors (TEFs).

On an environmental context, recent studies have demonstrated an steady increase of PSTs in molluscs associated to spatio-temporal expansions of dinoflagellates [8] and occurrence of harmful algal blooms (HABs). Being natural pollution episodes, the frequency of HABs varies depending on the year and location [11] and are therefore hard to predict [12]. As global warming continues, an increase in the occurrence of PSTs due to HABs [13] is forecasted due to the steady increase in ocean temperature. Continuous monitoring of the concentrations of

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PSTs is therefore required to protect environmental and public health and to minimize negative effects in the economy of some coastal areas, since the toxicity by PSTs may continue for weeks or months after algal blooms and significantly impact local fisheries and aquaculture. Consequently, the analytical procedures aimed to PST determination must be accurate, fast and reproducible.

From an analytical point of view, analysis of PSTs is very challenging due to: (i) the presence of multifunctional and complex structures that results in the existence of many analogs and isomers, and therefore, the need for very good chromatographic separation to distinguish them, (ii) the difficulty to achieve such chromatography due to the very high polarity of PSTs, (iii) their instability, since they can be transformed into each other, and (iv) different toxicities of individual analogs. Analysis of the occurrence of PSTs in bivalve molluscs started in the second half of the 20th century using the mouse bio-assay (MBA) [14]. This methodology is based on liquid-solid extraction of PSTs with water and later injection of the extract into a mouse. Death of the animal indicates that concentration of toxins is above safety levels for human consumption. For an easy-to-use and fast screening method, MBA has however noticeable drawbacks, including relatively poor sensitivity and precision due to variability in mice, existence of false positives, as well as ethical implications inherent to the use of animals for laboratory experimentation. According to the German Federal Institute for Risk Assessment (BfR), MBA should not be longer used as a reference method since results are strongly affected by the gender, weight and strain of the animal used, and they cannot be reproduced between laboratories, PST concentrations cannot quantified, and/or the sensitivity to detect toxic effects is occasionally not high enough [15]. For these reasons, the European Commission has recently set a new regulation (COMMISSION REGULATION (EU) 2017/1980) to switch from MBA to analysis of PSTs based on derivatisation followed by analysis using liquid chromatography with fluorescence detection (LC-FLD) [16]. LC-FLD allows the separation and determination of the different PSTs toxins, which was not possible to achieve through the MBA method. Several authors have already applied the new proposed methodology (or slight modifications of it) to the analysis of PSTs in various organisms, detecting a wide range of concentrations (Table 2), from 973.4 to 13600 μg STX eq/kg. After dinoflagellates blooms, concentrations of 4013 μg STX eq/kg in mussels, 4721 μg STX eq/kg in cockles, and 1176 μg STX eq/kg in razor shells were measured in Portugal [17]. It took at least 1 month after the blooms for the toxin values to fall below the safety levels imposed by European regulations in the case of cockle and razor shell, and 2 months for mussels. In Korea, concentrations of up to 7310 μg STX eq/kg were reported in mussels, remaining above the limits of the regulation for at least 1 month [18]. A quick and precise evaluation of PST levels is thus, not only a matter of environmental and health safety, but also has profound economic implications in coastal areas where aquaculture is heavily extended.

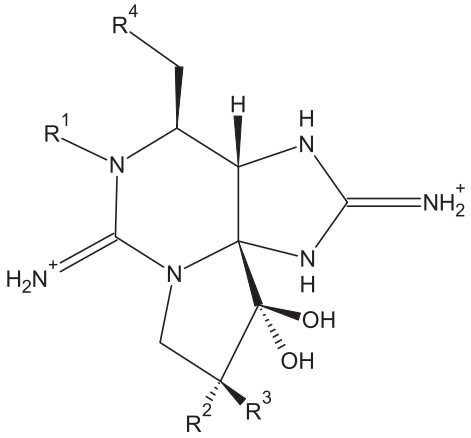
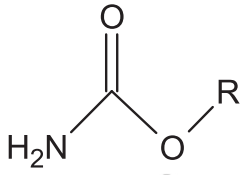
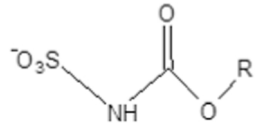
Table 2
PST concentrations reported in different locations and aquatic organisms using chromatographic methods.

Localization	Matrix	Maximum concentration measured (μg STX eq/kg)	Reference
Portugal	Fish	973	[21]
Italy	Molluscs	1922	[8]
Portugal	Bivalves	4721	[22]
Portugal	Mussels	4013	[17]
	Cockles	4721	
	Razor shell	1176	
Australia	Southern Rock Lobster	13600	[23]
Korea	Mussels	7310	[18]
United Kingdom	Sessile colonial fauna	2091	[24]
	Sunstar	1275	
Argentina	Mussels	402945	[25]
Chile		17772	
Uruguay		38804	
Mexico	Geoducks	3680	
China	Mussel	23300	[26]
	Clam	19000	
	Oyster	20300	

Nowadays, research on this topic is focused on improving the separation and determination of PSTs in bivalves while reducing analysis time. Due to very high polarity of PSTs, their retention and separation by reverse phase C18 columns is very challenging and requires pre-column derivatisation or ion pair reagent, as well as and post-column oxidation of the analytes to obtain fluorescent compounds [19]. To this end, new methodologies based on hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry (HILIC-ESI-MS/MS) have been recently proposed [20]. These issues can be overcome by the combination of newly developed HILIC phases and MS/MS with electrospray ionisation (ESI). A recent intercalibration exercise involving 21 laboratories and different species (mussels, oysters, clams, cockles, and scallops) has shown HILIC-ESI-MS/MS to be a viable alternative to the official LC-FLD method [16]. Concentrations from 1275 to 402945 μg STX eq/kg have been reported by several authors using HILIC-ESI-MS/MS approaches (Table 2).

In this critical review, we have analysed and compared the different

Table 1
Most commonly studied PSTs, their structures and TEFs.

Main structure	Name	R1	R2	R3	R4	TEF
	Saxitoxin (STX)	H	H	H		1
	Gonyautoxin-2 (GTX2)	H	H	OSO_3^-		0.4
	Gonyautoxin-3 (GTX3)	H	OSO_3^-	H		0.6
	Neosaxitoxin (NEO)	OH	H	H		2
	Gonyautoxin-1 (GTX1)	OH	H	OSO_3^-		1
	Gonyautoxin-4 (GTX4)	OH	OSO_3^-	H		0.7
	Gonyautoxin-5 (GTX-5)	H	H	H		0.1
	N-sulfocarbamoyl gonyautoxin-2 (C1)	H	H	OSO_3^-		0.01
	N-sulfocarbamoyl gonyautoxin-3 (C2)	H	OSO_3^-	H		0.1
	Gonyautoxin-6 (GTX-6)	OH	H	H		0.05
	N-sulfocarbamoyl-gonyautoxin-1 (C3)	OH	H	OSO_3^-		0.01
	N-sulfocarbamoyl-gonyautoxin-4 (C4)	OH	OSO_3^-	H		0.1
	Decarbamoylsaxitoxin (dcSTX)	H	H	H	OH^-	0.5
	Decarbamoylgonyautoxin-2 (dcGTX2)	H	H	OSO_3^-	OH^-	0.2
	Decarbamoylgonyautoxin-3 (dcGTX3)	H	OSO_3^-	H	OH^-	0.4
	Decarbamoylneosaxitoxin (dcNEO)	OH	H	H	OH^-	0.2

Source:Reproduced from World Health Organization [10].

methodologies applied to date for PST determination, with a special focus on those based on chromatographic techniques. There are other methods of determination such as capillary electrophoresis [27], capillary zone electrophoresis [28], receptor binding assay [29], enzyme-linked immunosorbent assays (ELISA) [30] or lateral flow immunochromatography [31], but their use is marginal compared to LC approaches. To the best of our knowledge, this is the first review available in which existing extraction and determination methodologies for PSTs in aquatic organisms are discussed. Previous reviews either compare different detection techniques for PSTs and/or focus on other environmental matrices such as surface water [2,30,32]. In this work, the results obtained by different authors working on the analysis of PSTs in mollusc tissues are compared and the difficulties to be overcome are pointed out. The review is organized in five sections: the first two sections are related to the analysis of the efficiency of the extraction process and the clean-up step, the following two sections discuss advantages and disadvantages in the use of LC-FLD and HILIC-MS/MS approaches for PST determination, and the last section is about the interpretation of the analytical results obtained.

2. Extraction

Extraction efficiencies or extraction recoveries are often calculated by spiking tissues with analytical standards and comparing the concentrations obtained after extraction with those added. Unlike other more widely studied environmental contaminants such as polychlorinated biphenyls (PCBs) or polycyclic aromatic hydrocarbons (PAHs), there are not environmental contaminated reference samples (e.g., tissues and/or sediments) for PSTs. The only certified reference materials existing are in aqueous solution and are available from a few suppliers (e.g., NRC Canada, CIFGA Spain).

Extraction of PSTs from aquatic organisms usually consists in liquid-solid extraction using heated or boiling water with added acetic acid [17,33–35] or hydrochloric acid [36] and later centrifugation in order to precipitate and separate proteins from the extract [17,33–35]. Table 3 shows the recoveries obtained for every toxin by different research groups. After extraction, the supernatant is usually purified by solid phase extraction (SPE) and centrifuged again before injection into the LC system. Most of the available studies on PST determination use the aforementioned extraction procedure regardless the matrix. Such extraction method is directly inherited from the previously existing MBA method and has been used for decades, with some minor modifications, like the use of acetic acid instead of hydrochloric acid. Fully validation of the extraction method, however, is still pending for some toxins and/or organisms. The official method proposed by the EU [37], which is based on the protocol described by Lawrence and co-workers, consists in the extraction of PSTs using the MBA method with some minor modifications and addition of an extra clean-up step using C18 cartridges [37]. However, only the recovery of the cleaning step is reported due to the lack of certified material (contaminated tissues) according to Turner et al. [38]. To the best of our knowledge, only a few recent studies present some information on the extraction efficiency of the method for PSTs in selected organisms (Table 3), although in some occasions it cannot be discerned whether the recovery values are referred to the extraction step, the clean-up step, or both combined. These values often span over a very wide range depending on the toxin, analyte concentration and/or matrix, making the validation of the method completely necessary to account for changes in the extraction protocol and/or aquatic species analysed.

Table 3 shows extraction recovery percentages for specific PSTs. Their values are between 50% and 120% [17,33,34,39] and are in agreement with those previously reported using the MBA method, albeit recoveries lower than 50% for STX eq are occasionally reported [40,41]. Low recovery values for some toxins are occasionally attributed to losses by enzymatic activities (e.g., GTX5 and C1,2 can be converted into dcSTX [17]). In any case, extraction with acidified water seems to be the optimal choice, as very good extraction efficiencies are reported by Masias et al. [35] (> 90% for bivalves and gastropods) as well as Hignutt et al. [40] (between 95% and 104% in oysters). Van De Riet et al. [36] obtained recovery values usually over 100% in clams, mussels, scallops and oysters, ranging from 75% to 151%. On the negative side, variability is usually high due to the wide range of PSTs and organism species considered, and no information on the recoveries for some toxins such as GTX6 and C3/4 is currently available.

3. Clean-up

The clean-up process is aimed to remove interferences (e.g., salts, lipids and suspended particles) after the extraction that can hamper the chromatographic separation and/or detection by different techniques. In the case of PST analysis, clean-up is usually performed by using solid phase extraction (SPE) cartridges. SPE clean-up helps to minimize the presence of oxidation by-products in the chromatograms that may interfere with PST identification and/or quantification when LC-FLD methods are used (Fig. 1). It also reduces ionization suppression in the electrospray interface (matrix effect) when mass spectrometry is used for detection.

Clean-up efficiency is usually calculated by spiking PSTs to non-contaminated tissue extracts. In more recent works, the efficiency of the cleaning step only is also reported. Different SPE cartridges can be

Table 3
Recovery efficiencies of extraction (%) obtained for individual PSTs by different authors (n.a. = not available).

TOXIN	[33]	[17]	[34]	[39]	[35]	[36]
STX	65	93	90–93	79–100	96.6	89–113
NEO	128	97	97	61–106	95.3	75–105
dcSTX	76	114	77–114	76–93	95.3	n.a.
dcNEO	72	56	55–56	n.a.	n.a.	n.a.
GTX1	121	59	59	84–106	93.2	99–126
GTX4						103–132
GTX2	55	104	87–104	74–109	94.3	109–116
GTX3						103–115
GTX5	94	98	97–98	85–97	95.6	n.a.
GTX6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
dcGTX2	67	107	85–107	63–82	94.1	n.a.
dcGTX3						105–151
C1/2	52	74	71–74	73–90	93.5	n.a.
C3/4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
EXTRACTION	Acetic acid + heat	Acetic acid + heat	Acetic acid + heat	Acetic acid + heat	Acetic acid + heat	Hydrochloric acid
MATRIX	Mussel and flat oyster	Mussel, cockle and razor shell	Cockle	Clam and processed king Scallop	Mussel, clam and gastropod	Mussel, clam, scallop and oyster
TECHNIQUE	LC-FLD	LC-FLD	LC-FLD	LC-FLD	LC-FLD	LC-FLD

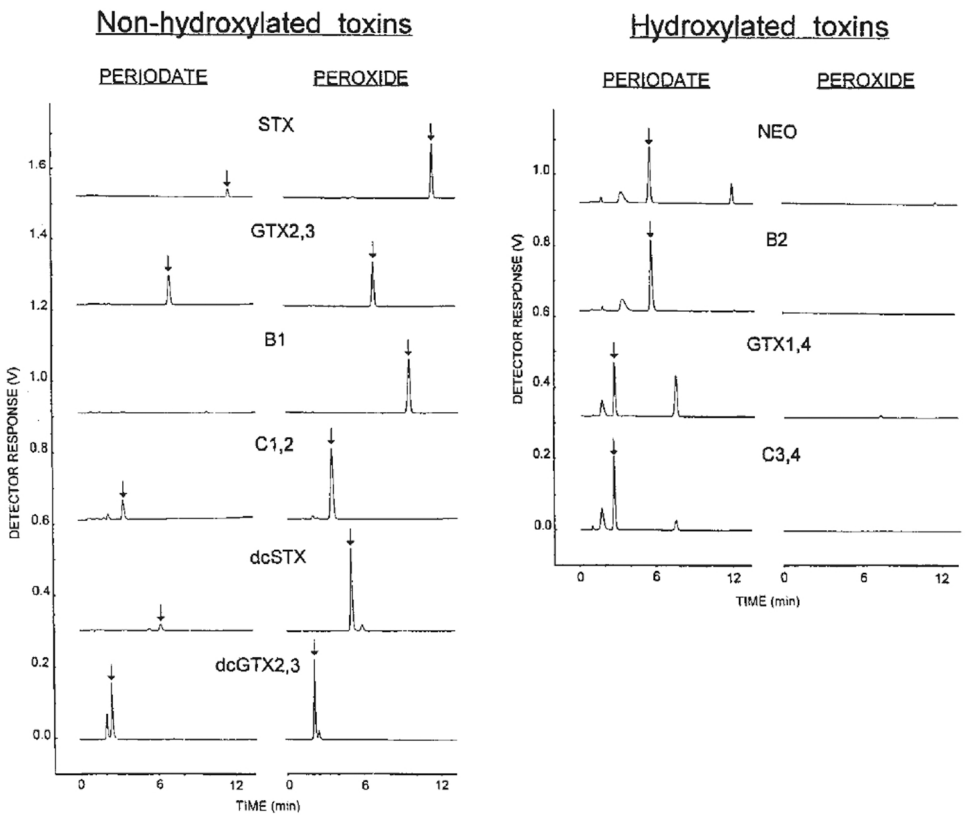


Fig. 1. Chromatograms obtained by Lawrence et al. [16] using pre-column oxidation and LC-FLD.

used for clean-up, but their efficiency (% of PSTs extracted after SPE) is not reported in all cases. So far, C18 cartridges [37,42,43] haven been proven to achieve better extraction efficiencies than carbon cartridges [38,44,45], as well as lower variance between analytes (Table 4). In some cases, carbon cartridges causes heavy losses for specific analytes, such as C1, for which the recovery efficiency can drop below 30% [45]. On the other hand, other toxins such as NEO show improved results [38]. HLB [46] and non-porous graphitized carbon [47] cartridges can be also viable alternatives for clean-up of PST extracts although their use is more limited.

Other than SPE, there are alternative approaches for sample purification. For instance, Mattarozzi et al. used QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) and obtained satisfactory recoveries for most PSTs in mussels (79–112%) [48] and clams (from 62% to 99%) [49]. Another approach used by Wang et al. [50] consisted on amino-modified multiwalled carbon nanotubes, yielding recovery percentages from 71% to 98%. Both alternatives (QuEChERS and carbon nanotubes) are therefore comparable to most commonly used SPE cartridges in terms of extraction efficiency. Finally, a comparative study was performed by Zhang et al. [51] by using both SPE and matrix solid-phase dispersion (MDSP) as clean-up strategies. The first technique had a recovery range from 40% to 170%, whereas values between 45%

Table 4
Efficiency of clean-up (%) obtained for individual PSTs by different authors (n.a. = not available).

Reference	[42]	[43]	[44]	[45]	[38]	[46]	[47]
Cartridge	C18	C18	Porous graphitic carbon	Graphitized carbon	Amorphous graphitized polymer carbon	Oasis HLB	Non-porous graphitized carbon
Matrix	Mussel, Oyster, Ark shell, Sea squirt, Styela clava	Scallop, Mussel, Clam	Mussel, Clam, Scallop, Oyster	Mussel	Mussel, Oyster, Clam, Scallop	Mussel	Scallop, Clam
STX	83 – 108	86–99	79–83	107	116	96	n.a.
NEO	n.a.	84–103	72–74	80	153	99	n.a.
dcSTX	n.a.	n.a.	78–93	105	96	97	n.a.
dcNEO	n.a.	n.a.	64–72	74	126	93	n.a.
GTX1	89–109	80–100	74–79	82	80	97	90–95
GTX4	91–111	82–97	73–76	74	62	95	78–92
GTX2	87–109	82–117	67–83	83	82	97	87–95
GTX3	91–111	83–100	70–84	71	65	94	83–92
GTX5	n.a.	n.a.	72–86	82	76	110	86–91
GTX6	n.a.	n.a.	n.a.	n.a.	n.a.	110	82–90
dcGTX2	n.a.	n.a.	80–119	78	75	96	83–95
dcGTX3	n.a.	n.a.	64–69	76	65	96	80–92
C1	n.a.	86.42–107.86	63–78	28	45	110	81–94
C2	n.a.	82.27–104.5	66–76	74	73	100	88–96
TECHNIQUE	LC-MS/MS	LC-MS/MS	LC-FLD	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS

and 100% were observed from the latter. In this sense, MDSP seems to be a slight improvement over SPE due to lower variability while achieving similar extraction efficiencies.

4. Liquid chromatography with fluorescence detector

Analysis of PSTs by liquid chromatography using reverse phase columns and fluorescence detectors (LC-FLD) is the main alternative and replacement to the MBA method. To achieve enough retention of toxins in the column (typically a C18 type) and detection by the FLD detector, derivatisation is required. Pre-column oxidation of PSTs is the most widely used approach [8,11,17,21,22,33–35,39,52–58] and consists on a periodate or peroxide oxidation [16]. The periodate reagent is added to the sample extract after clean-up and permitted to react, followed by addition of concentrated acetic acid. Peroxide oxidation, on the other hand, relies on using H_2O_2, NaOH and glacial acetic acid to the extract. After complete oxidation, derivatised PST are stable for 24 h, except for NEO and GTX1.4, which after 8 h showed a degradation of 30%. Excitation and emission wavelengths selected for the determination of the fluorescent derivatives formed in both cases are 340 nm and 390 nm, respectively (Fig. 1). The aforementioned protocol was developed by Lawrence et al. [59] in 1991, tested in oysters, clams, and mussels ten years later [37], and finally proposed as the official method for PST analysis after an intercalibration exercise involving 18 laboratories [16, 60]. Although the method has significant advantages over the previously official method (MBA), such as the capability of determining the concentrations of different toxins and avoiding the sacrifice of animals, there are some other flaws. The most noticeable drawbacks are: long times required for pre-oxidation of PSTs, inability to distinguish between different isomers of each toxin [36,61] and some toxins yielding several and/or equal oxidation products (Fig. 1). The latter may hamper PST quantification and thus result in less accurate data, and it is usually overcome by using 2 different reactions and separation chromatographic runs. Fig. 1 shows several toxins such as NEO, GTX1,4 and C3,4 that cannot be determined using peroxide as reactive. On the other hand, STX, dcSTX and C1,2 are poorly detected when periodate is used, therefore confirming the necessity of using two reactive and two chromatographic runs. In addition, the same oxidation products can be formed from different toxins, as it is the case of GTX1,4 and C3,4.

Post-column derivation oxidation (PCOX) was introduced later as an improvement of the aforementioned method, using ion-pair reagents to modify the retention time of PSTs. However, to the best of our knowledge, this alternative has been less used [18,36,40,44,62,63]. The original protocol for PCOX of PSTs was published in 1995 [64], requiring three different analyses per sample while changing column and/or reagents between analysis [65]: one for STX and NEO, another for GTX toxins, and a third one for C toxins (later reduced to two by combination of GTX toxins, STX and NEO in a single run). Additionally, PCOX requires an extra module after chromatographic separation where the oxidation of toxins occurs with certain reagents. In the method presented by Thomas et al. [65], optimal determination was achieved maintaining the reaction coil at 80 °C, using ammonium phosphate with periodate as oxidation reagent, and acidifying the column effluent with nitric acid prior to fluorescence detection. The chromatographic run is greater than 80 min. After validation by several laboratories in a joint exercise [66], the proposed PCOX modification was also accepted as an official method for PST determination. Although analysis times are reduced using PCOX instead of pre-oxidation, some drawbacks such as the need of changing the column between analysis and shorten LC column life due to the mobile phases used (A: 20 mM heptane sulphonate and 10 mM ammonium phosphate, B: 30 mM ammonium phosphate and 11% acetonitrile, and C: 10 mM ammonium phosphate) still exist. The improvement proposed by Rey et al. [44] to avoid multiple injections per sample requires using a porous graphitic carbon stationary phase instead of silica-based columns.

Evaluation of the sensitivity of LC-FLD methods for PST analysis is often presented through the calculation of the limits of detection (LODs) and/or limits of quantification (LOQs). Determination of the variation of the signal due to the matrix effect is usually not required due its limited influence over FLD detectors, as it was demonstrated by Soliño et al. [21]. Nevertheless, matrix match calibration has been also occasionally used [52,55]. To compare the sensitivities of the different methodologies published based on LC-FLD is not straightforward, since different authors tend to use different concentration units and/or to present the LOD and LOQ values either as the sum of STX equivalents of all toxins or as individual toxins. Available data on LODs or LOQs for PSTs span over a wide range, from very low LODs such as 0.07 – 4 ng/g [54] to higher LOQs (e.g., 29 – 107.5 ng/g) [21]. If we compare μg STX eq/kg, we also found a wide range depending on the matrix and the toxin considered: 0.5 – 13 μg STX eq/kg [33], 4 – 131 μg STX eq/g [39], or 45 – 227 μg STX eq/kg [35]. These last values are close to those obtained by the MBA method (280 μg STX eq/kg [58]). Using PCOX simplifies the analysis of PSTs and, according to the results of DeGrasse et al. [67], lower LOD values could be obtained. However, whether this is true or not also depends on the paper considered. Thus, when comparing the results from both the PCOX and MBA methods, sometimes results are quite close. Low LODs ranging from 0.7 to 41 μg STX eq/kg have been obtained [61], nevertheless most of papers had LOQs ranging from 10 to 390 μg STX eq/kg.

5. Hydrophilic interaction liquid chromatography with tandem mass spectrometry

The most recent analytical methods aimed to separation, identification and quantification of PSTs in bivalves and other environmental samples are based on the combined use of HILIC columns and tandem mass spectrometry (HILIC-MS/MS). The main advantage over LC-FLD based methods is that pre and/or post-column oxidation of the toxins is not mandatory. Additionally, and due to the very high capability of HILIC phases to retain high polar compounds and to separate them, even PST isomers can be distinguished during analysis. This feature is very important to assess the level of PSTs in terms of μg STX eq/kg with high confidence since the toxicity may differ up to 10 times depending on the isomer considered. The main disadvantage of using HILIC columns instead of more conventional C18 reverse phase columns is that the stability of the first is not as good as for the latter, requiring longer equilibration times before and after each run and more careful preparation of the mobile phase.

Among the most widely used HILIC columns to separate PSTs [24,26, 38,42,43,45–48,50,51,55,68–74], TSK-gel Amide-80 [26,42,43,46,48, 51,68,70,73,74] and Acquity UPLC BEH Amide [24,38,45,47,50,69,71] are the first in the list, consisting in spherical silica particles that are covalently bonded with carbamoyl groups and a trifunctionally bonded amide phase, respectively. Other column types such as Agilent Poroshell 120 HILICZ [24], Waters Acquity Glycan UPLC [55], and ZIC-HILIC HPLC [72] have been also employed but to a lesser extent. The most commonly mobile phases tested are H_2O and acetonitrile (ACN) with modifiers like formic acid (F.A.), ammonium acetate (A.A.), ammonium formate (A.F.), and ammonia (NH_3). Despite the relatively limited choice in terms of HILIC columns and mobile phases, we have found considerable variations in the gradients used for different authors to separate the same groups of PSTs. Chromatographic separations involving high temperatures (60 ºC) and even flow changes are not rare [38,45,50] (Fig. 2). In order to avoid longer chromatographic runs and complex re-equilibrations gradients between each injection [20,45], a isocratic regime could be effective in providing enough separation of PSTs [47] under very defined conditions (e.g., 38% of a ACN:H2O mixture at acidic pH).

Determination of PSTs by mass spectrometry after HILIC separation has been mostly achieved through triple quadrupole instruments operating in multiple monitoring reaction (MRM) due to their high sensitivity and specificity. In most cases, quasimolecular ions that correspond

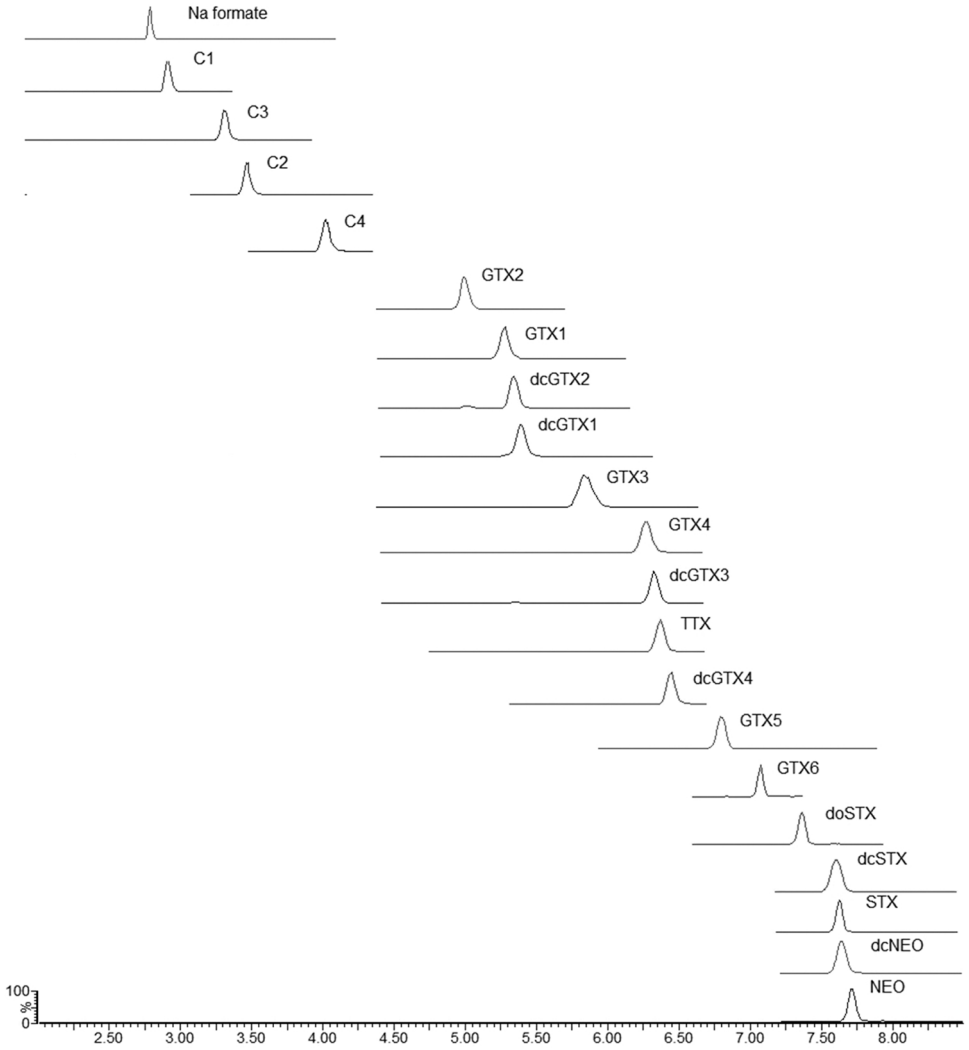


Fig. 2. Chromatograms obtained by Turner et al. [20] using HILIC columns and MS/MS.

to the protonated (ESI+) or deprotonated (ESI-) form of the molecule are used for fragmentation, although the use of precursor ions where a radical has been lost is also reported. A secondary MRM transition for identity confirmation is often not present or, if it is, it may be shared by several toxins. For instance, transitions 412 > 332 and 412 > 314 (+) are shared among GTX1 and GTX4 [42,43,46–48], so, a good chromatographic separation is required even in MS/MS. A strategy to overcome such limitation could be using mixed ionization mode (ESI+/-) as ionization of many PSTs can occur in both positive and negative mode. To the best of our knowledge, this approach has not been used in PST analysis yet, but for other biological compounds such as lipids [75]. In any case, a very efficient separation of all isomers is essential to achieve reliable identification and quantification by mass spectrometry, which makes chromatographic separation the most critical step to be optimized in the analysis of PSTs.

The effect of ion suppression in the electrospray ion source must be also properly evaluated, since it can heavily affect the abundance of PST ions and, therefore, hamper their quantification. Only a few authors have presented some results related with this topic. Rey et al. [76] reported matrix effects ranging from − 86.5 (signal loss) to 38.6% (signal enhancement), which are comparable to the results presented by Yang et al. [43] (from −80% to 150% depending on the analyte). Other researchers such as Mattarozzi et al. [48] and as Shin et al. [42] observed suppressions of the MS signals due to matrix effect that were from 13% to 89% of the total signal intensity. Zhang et al. [51] did a comparative study to evaluate changes in the matrix effect using several dilutions and clean-up steps, achieving optimal results (signal intensity losses between −3% and 58%) when MSDP was used before HILIC-MS/MS determination. Analytical procedures used by other authors such as Ochi et al. resulted in negligible matrix effects, allowing the quantification of PSTs directly by using external calibration curves [47]. Wang et al. [50] and Turner et al. [38] also obtained relatively low signal suppression (< 30%) for most toxins. All the aforementioned results indicate that changes in the sample matrix, clean-up steps and/or MS instrument used could significantly affect the results because of varying matrix effects. To compensate for these variations, the use of matrix-match calibrations or matrix-matched standards [42,43,46,48,50,55,72] is considered to be the best approach to obtain more accurate results considering the lack of commercially available isotopically labelled (e.g., deuterated or ^{13}C) toxins that could be used as internal standards.

Sensitivity of the currently available HILIC-MS/MS methods is, in general terms, higher than when using other analytical techniques. LODs and LOQs are often lower than those for previously existing LC-FLD methods, and are found in a relatively narrow range: 0.5 – 62 μg STX·2HCl eq/kg [45], 3.43 – 35.46 μg/kg [26], 0.26 – 39.4 μg STX eq/kg [47], 9.11 – 39.3 μg/kg [50], and 1.32–11.29 μg/kg [43], as examples. From a regulatory point of view, these results are good enough to discontinue the use of the MBA approach and, hence, avoid sacrificing animals [72]. Faster sampling processing in comparison to LC-FLD methods may be also critical during HAB episodes as it allows for

shorter reaction times of authorities to implement measurements.

6. Toxicity assessment

The interpretation of results after chromatographic analysis of paralytic toxins in terms of toxic equivalent factors (TEF) is somehow controversial. TEF is defined as 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" [77]. According to Botana et al. [77], establishing TEFs is not a straightforward process as many factors are implied, including available data on intoxications in humans and results from acute toxicity in animals and in vivo assays. Back in 1937, Sommer & Meyer [78] published a work in which dose-death time relationship in mice was used to establish STX toxicity. Such relationship was assumed to be virtually equal for all analogues of STX for the upcoming decades. It was only until different PST analogues could be isolated that their toxicities could be tested in different organisms to establish the new and more TEF values used nowadays. Such TEFs are, however, are presented in ranges rather than using unique values and are still open for further modifications [77]. For instance, Perez et al. [79] evaluated the toxicity of PSTs according to their ability to inhibit voltage-dependent sodium currents and reported that NEO and dcSTX were more toxic than STX. On the other hand, the criteria established by the World Health Organization [10] results in a TEF for dcSTX that is half of that for dcSTX. Alonso et al. [80] found a large variation of TEFs depending on the channel subtype selected in toxicity tests, reporting equal TEF values for the analogues GTX1, GTX4, STX and NEO, which is in agreement with the values used by the European Food Safety Authority (EFSA) [81].

In scientific literature, as it has been already mentioned in previous sections, there is not a unified criterion to express the results obtained after LC-FLD or HILIC-MS/MS analysis. Some authors express the results as concentrations whereas others do it as toxic equivalents. The latter can be seen as an attempt to compare their data with those obtained using the MBA approach. As LC methods are expected to be more widely used in the near future once MBA is progressively abandoned, a unified criterion on this respect is required. Due to the capability of LC methods to separate toxins and to determine their individual concentrations, we consider that presenting the data as concentrations rather than TEFs would make more sense for both scientific purposes. In this way, it would be easier to compare the sensitivity of different analytical methods as well as results from monitoring campaigns.

7. Conclusions and future trends

We have reviewed over 40 manuscripts in which PSTs are extracted from organisms such as mussels [18,22,38,46,48,54,55,58,68–70,72], fish [13,21,63], clams [38,47,49,52,55,62,71,72], cockles [22], razor shells [22], abalone [56], scallop [38,47,51,73], lobster [23], oysters [38], shellfish [8,43,57,82], bivalves [11,26,50,53], benthic organisms [24], and other invertebrates [63]. In about 80% of these papers authors did not fully conducted assessment of extraction and/or clean-up efficiency, which are critical for method validation. Analytical parameters and figure of merits of the reviewed papers are summarized in Table S1. In our opinion, and due to the relatively high number of species susceptible to bio-accumulate these toxins, the analytical methodologies should be re-evaluated in every type of tissue for a more reliable determination of PSTs This is of key importance considering that the data obtained have direct implications not only towards coastal environments affected by HABs but also towards human health and local economies.

We have observed that, over the last 30 years, most of the effort regarding PST determination has been put into the separation and detection of PSTs by using different chromatographic approaches. This is understandable due to the very polar nature of the toxins and quick advances in analytical techniques. However, the extraction of the toxins has not developed further or evolved in the last decades as it is still mainly based on the original MBA method. In those studies where extraction efficiencies using acidic water were determined, important losses of analytes (> 50%) during the extraction and/or clean-up steps were often reported, as well as very wide ranges of recovery percentages that vary significantly from one study to another in spite of all researchers using very similar analytical protocols. Determination of recoveries of the extraction and clean-up steps by separate is still pending to identify potential losses when employing the same methodology over different organisms. This aspect is even more critical as it has been shown that some toxins can be converted into others due to the heat used during extraction [51]. Alternative extraction techniques such as pressurized liquid extraction (PLE), ultrasonic assisted extraction (UAE) or microwave assisted extraction (MAE) could be also tested and evaluated in order to improve the extraction procedure firstly developed for MBA.

Regarding separation and determination of PSTs, this is the most challenging aspect during method development due to toxins being complex mixtures of very high polar isomers for which reverse-phase columns are not suitable (unless prior derivatisation of analytes is performed). Newly introduced HILIC phases are the workhorse nowadays and in the near future to achieve the chromatographic determination of all the toxins in a single chromatographic run. Different authors have reported satisfactory separation of the analytes using different mobile phases and gradients. However, current available HILIC methods still lack the robustness of more conventionally C18 reverse phase based protocols as slight changes in the mobile phase composition can have a dramatic effect on the retention time and elution order of PST isomers. Determination of toxins by MS/MS at trace levels is more straightforward than achieving a good chromatographic separation but the first is heavily dependent on the latter as MRM transitions are not unique for each PST. An exhaustive review of LODs or LOQs obtained by LC-FLD and HILIC-MS/MS reveals that, in spite of the wide range of values and different measurement units reported by different authors, both techniques can achieve sensitivities that are very similar or superior to those obtained by the MBA method. Such results confirm that animal bioassays can be progressively abandoned in favour of newly developed chemical methods. There are still, however, some challenges associated to PST detection by HILIC-MS/MS and are mainly derived from suppression/enhancement of the analyte signals due to matrix effects. Matrix match calibration is currently the easier approach to overcome this issue, although synthesis of isotopically labelled internal standards and reference materials would be desirable.

Finally, new saxitoxin derivatives are currently being identified [74], and subsequently the existing and new analytical methodologies to come should be validated for them. In this sense, Medina-Elizalde et al. [71] have raised concern about M-Toxins since, in spite of the lack of certified material or standards, they have recently estimated that these toxins can be responsible of 49% of the total toxicity in some organisms. These new toxins are probably PST metabolites or transformation products produced in shellfish, since they have not been detected in microalgae. Research on this topic is under way [68,71,83] but advances are hampered by the unavailability of reference standards yet. Newly discovered toxins have been not reported in bivalves and other marine organisms for human consumption (despite the fact that its existence had been known for several years already) because they are not detected by currently used LC-FLD and HILIC-MS/MS protocols [25]. To the date, at least 57 products analogous to STX have been already identified [4] and some of them could potentially be regulated to establish safety limits in food. Other than improving extraction and clean-up procedures, analysis of existing and new PSTs could hugely benefit from using high resolution mass spectrometry (HRMS), which have been barely applied to this field. New HRMS instruments, some of which incorporate ion mobility spectrometry (an analytical technique that separates gas-phase ions based on their size and shape), can provide valuable complementary structural information through accurate mass measurement and by adding an additional dimension and separation step for co-eluting PST isomers [84,85].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.teac.2022.e00190.

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