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Analysis of paralytic shellfish toxins, potential chemical threat agents, in food using hydrophilic interaction liquid chromatography–mass spectrometry

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ABSTRACT

A novel method for determining paralytic shellfish toxin (PST) profiles in food was developed using a combination of silica and strong cation exchange (SCX) solid phase extraction (SPE) coupled to hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS). Besides the risk for natural contamination of seafood and drinking water, PSTs also pose potent threats through intentional contamination of food, due to their high toxicity and the wide distributions of toxin-producing algae. The new preparation method aim to maintain the samples' original toxin profiles by avoiding conditions known to induce interconversion or degradation of the PSTs. The method was evaluated for PST extraction from water, milk, orange juice, apple purée, baby food, and blue mussels (*Mytilus edulis*). The extracts were found to produce reproducible retention times in HILIC–MS/MS analysis. When an authentic toxic mussel sample was analyzed using the novel method, saxitoxin and gonyautoxin-3 were identified, in agreement with data acquired using the Lawrence pre-column oxidation high-performance liquid chromatography–fluorescence detection (HPLC–FLD) method. Overall recoveries of the PSTs from tested foods by the novel method ranged from 36% to 111%.

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1. Introduction

Paralytic shellfish toxins (PSTs) are neurotoxins that are naturally produced by certain genera of marine dinoflagellates (*Alexandrium*, *Gymnodinium* and *Pyrodinium*) and freshwater cyanobacteria (*Anabaena*, *Cylindrospermopsis*, *Aphanizomenon*, *Planktothrix* and *Lyngbya*) [1]. In the natural environment these organisms are known to cause harmful algal blooms (HAB) or red tides [2–5]. During a HAB the water can be highly toxic, threatening freshwater supplies and recreational waters [1]. In the marine environment the toxins are accumulated in organisms such as shellfish, crustaceans and mollusks feeding on the PST-producing dinoflagellates [1]. The toxins do not affect most of these organisms, but they are transferred through the marine food web to larger animals that they can severely harm, such as fish, birds and mammals, including humans [1]. Most human intoxication occurs through ingestion of contaminated shellfish. This can cause paralytic shellfish poisoning [6], as PSTs are potent neurotoxins that block sodium channels in nerve and muscle cells, and intoxication can lead to death from respiratory failure [7]. Due to the high toxicity of the PSTs, and the relative ease of their production through cultivation of toxin-producing dinoflagellates, they have attracted attention of military laboratories and saxitoxin (STX) was included in chemical weapon programmes during the cold war [8]. Based on its chemical properties, STX is not suitable for large-scale bulk dissemination techniques such as those used for classical nerve agents. Instead, it was weaponized in small-arms ammunition (e.g. flechettes) for clandestine applications, and is one of two biological toxins listed as chemical weapons in Schedule 1 under the Chemical Weapons Convention [9].

Besides the risk for natural contamination of seafood and drinking water, PSTs could also pose potent threats through intentional contamination of food, due to their high toxicity [6] and the wide distributions of toxin-producing algae [1,4]. The complexity of food production chains raises further concerns, as it heightens their vulnerability to attacks. Thus, the food sector has been identified as a potential target for chemical or biological terrorist attacks by the US Department of Homeland Security [10]. In such a scenario, the contamination levels are expected to be at or above the lethal level, significantly higher than the regulatory limits of the PSTs. An attack could contaminate over 100,000 consumers within 3–6 days, as illustrated by a simulated attack with botulinum toxin

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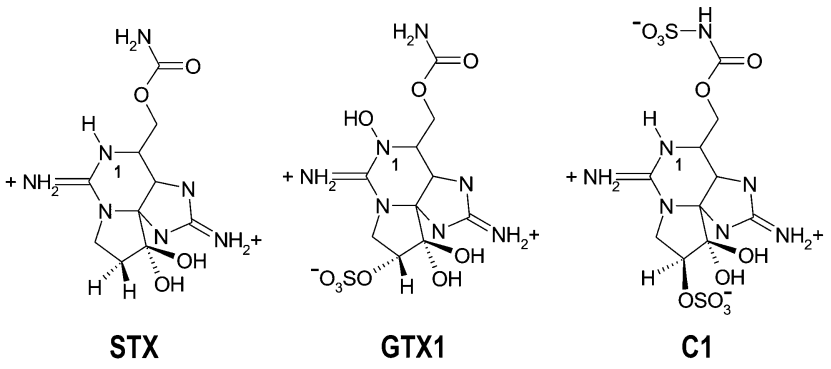

Fig. 1. Structures of STX, GTX1 and C1, with zero, one and two sulfonyl functional groups, respectively.

on the Californian milk supply chain [11,12]. After an attack it would be important to identify the chemical threat agent (CTA) used, and other relevant substances, such as byproducts from its production/synthesis or biologically related compounds and other contaminants related to the CTA. Such analytical information may provide valuable forensic information, for instance, it may be possible to use the resulting profiles to attribute toxins to materials of specific geographic origins, identify the production methods, distinguish between different samples, and/or link confiscated material to a perpetrator.

Structurally, PSTs are perhydropurine skeletons fused with a five-membered ring and two guanidinium groups. They also possess a hydrated ketone, a unique structural feature stabilized by the electron-withdrawing guanidine moieties linked to the carbonyl α-carbon. There are three major groups of PSTs, which can be distinguished by the absence/presence of sulfonyl functionalities, as illustrated in Fig. 1 The saxitoxins (saxitoxin, neosaxitoxin; NEO, decarbamoyl-saxitoxin; dcSTX and decarbamoyl-neosaxitoxin; dcNEO) lack these functional groups, gonyautoxins (GTX1–5 and dcGTX2–3) contain a sulfate moiety, while the N-sulfocarbamoyl-gonyautoxin group (C1–4) contain a sulfate and a N-sulfocarbamoyl moiety [13,14]. The toxins within these groups differ in the substitution of hydroxyl groups and decarbamoylation. The guanidinium and sulfonyl functionalities confer high polarity and water-solubility. The guanidine moieties of saxitoxin have reported p*K*a's of 8.1 and 11.5, respectively [15,16]. Thus, at neutral and weak alkaline pH, STX is singly or doubly protonated (with +1 and +2 charges, respectively). At a pH higher than 11.5, the deprotonated, uncharged molecule is the most abundant species. The two sulfonyl functionalities are both negative charged at neutral and basic pH and their presence will reduce the net charge of the PST analogues carrying them. The charge distribution among PSTs can be used for their separation by cation exchange chromatography.

Under regulatory sea food monitoring, the mouse bioassay (Official reference method, AOAC Method 959.08) [17] and the pre-column derivatization and florescence detection (FLD) HPLC method (AOAC Method 2005.06) [18] are the two currently approved methods for PST detection/quantification in the European Union [19]. These methods are very sensitive, but do not provide full information on the identities of individual PSTs. The post-column derivatization HPLC–FLD method offers a way to individually identify epimers [20]. In the mouse bioassay, the total toxicity of a sample is assessed and expressed as "mouse units" (which can be converted to saxitoxin di-hydrochloride equivalents). In the Lawrence method samples are cleaned up on a C18 SPE column followed by a weak cation exchange (WCX) column [18]. The PSTs are detected as fluorescent periodate and peroxide oxidation products by HPLC–FLD. None of these fractions are amenable to LC–MS detection since the C18 flow-through fraction contains polar interfering substances and the WCX fraction is eluted by solutions with high NaCl concentrations, resulting in poor electrospray ionization efficacy [21]. The pre- and post-column derivatization HPLC–FLD methods are time consuming and requires dedicated analytical systems and skilled staff for data interpretation [22]. From a forensic perspective a more generic analytical approach is desirable to allow identification of CTAs in their unmodified chemical states. For this purpose a liquid chromatography tandem mass spectrometry (LC–MS/MS) method would be more suitable. Several methods have been published for analyzing saxitoxin in seafood, water or algae, involving solid phase extraction or filtration followed by hydrophilic interaction liquid chromatography (HILIC) LC–MS/MS analysis [23–28]. However, in our experience these sample preparation methods are insufficiently robust due to interfering substances that compromise chromatographic reproducibility, i.e. cause excessive variation in retention times among samples of different food types. The variation can be reportedly reduced, for seafood samples, by using a method involving use of graphitized-carbon SPE [29]. However this method includes an initial boiling procedure and the risk for PST conversion is still present. Hence, the variation in LC retention time of PSTs associated with matrix effects was recently discussed as an important area of improvement at the evaluation meeting of the Saxitoxin Proficiency Test of the EU-project EQuATox (www.equatox.net). Therefore, a sample preparation method which gives consistent HILIC chromatography across matrices and minimizes toxin interconversions is desirable. Here we present an alternative solid phase extraction approach based on use of silica and strong cation exchange (SCX) SPE columns, coupled to HILIC UPLC with an amide chromatographic column and MS/MS detection.

2. Experimental

2.1. Chemicals and material

Ammonium acetate (BioXtra, 98%), sodium hydroxide (reagent grade), hydrochloric acid (ACS reagent, 37%) and formic acid (reagent grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA), HPLC grade acetonitrile from Fisher Scientific (Loughborough, UK), and hypergrade acetonitrile for LC–MS/MS analysis from Merck (Darmstadt, Germany). Water was purified with a Milli-Q Plus ultra-pure water purification system (Millipore, Bedford, MA, USA). STX (65.0 μM), NEO (65.6 μM), dcSTX (65.0 μM), dcNEO (39.4 μM), GTX1[a] (60.4 μM), GTX2[b] (114.2 μM), GTX3[b] (43.3 μM), GTX4[a] (19.7 μM), GTX5 (65.0 μM), dcGTX2[c] (116.0 μM), dcGTX3[c] (26.1 μM), C1[d] (113.4 μM) and C2[d] (33.9 μM) reference standards (a–d: mixed epimeric pairs) were acquired from the Canadian National Research Council (Halifax, Canada). The following four silica SPE columns were evaluated: SampliQ Silica, 1 mL, 100 mg, from Agilent Technologies (Santa Clara, USA); Isolute Si, 100 mg, 1 mL, from Biotage (Uppsala, Sweden); Chromabond SiOH, 1 mL, 100 mg

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