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Comparative analysis of pre- and post-column oxidation methods for detection of paralytic shellfish toxins

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ABSTRACT

Paralytic shellfish poisoning (PSP) toxins are highly toxic natural compounds produced by dinoflagellates commonly present in marine phytoplankton. Shellfish contaminated with these toxins create significant public health threat and economic losses to the shellfish industry. For this reason, several methods of high performance liquid chromatography (HPLC) with fluorescence detection have been developed in order to gain better knowledge of toxins profiles in shellfish and dinoflagellates samples. These methods have been subjected to continuous modifications to improve and shorten the run time of analysis in the routine monitoring control. In this paper, different samples are analyzed by pre- and post-column HPLC methods to compare toxin profiles. All PSP toxins were individually identified and quantified within the post-column oxidation method. However, although the pre-column oxidation method is significantly more sensitive and detects lower toxin levels, it provides a total amount of toxins that co-elute together, as GTX2 and 3, GTX1 and 4 and dcGTX2 and dcGTX3. The results obtained by both HPLC methods showed similar toxin concentration (expressed in μg/mL) in mussel samples, however when dinoflagellates samples were analyzed the toxin profile and concentration were different. In summary, the post-column oxidation method is accurate to determine the amount of each individual PSP toxin and to know the real toxic profile of non-transformed samples. In addition, this method is easy and faster to screen a large number of samples. The pre-column HPLC method is useful when mussel samples are analyzed even though the time required to prepare the samples is longer.

1. Introduction

Paralytic shellfish poisoning (PSP) are potent neurotoxins produced by several species of dinoflagellates such as *Alexandrium*, *Gymnodinium* and *Pyrodinium* (Kodama, 2000; Dell'Aversano et al., 2008). These toxins specifically block the excitation current in nerve and muscle cells, resulting in paralysis and other symptoms (Luckas et al., 2004). Hence, the accumulation of PSP toxins in shellfish creates a serious public health problem and affect to fisheries industry. For this reason many countries have monitoring and regulatory systems that include the routine sampling of shellfish flesh for the presence of biotoxins and the examination of water samples for the presence of toxin producing phytoplankton (Hallegraeff, 1995). The mouse bioassay (MBA), developed in 1937 to check toxicity in acidic extracts of mussels (Sommer and Meyer, 1937), is the worldwide PSP official method used in monitoring programmes to prevent human intoxication. However, several limitations, including the pH-dependant high variability and low sensitivity (Van Dolah and Ramsdell, 2001), in addition to the strong opposition to animal sacrifice, has led the search of alternative methods for PSP detection. These alternatives include immunoassays, receptor binding

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assay, cell assays (Van Dolah and Ramsdell, 2001), and several chemical methods. The most common chemical method uses a combination of high performance liquid chromatography (HPLC) with either pre- or post- column oxidation followed by fluorescence detection (Lawrence and Niedzwiadek, 2001; Thomas et al., 2006). The ability of PSP toxins to be easily converted into fluorescent derivatives has been the basis for their detection (Gago-Martinez et al., 2001). These methods have the advantage of detecting and quantifying individual PSP toxins by using standards, even though not certified material of each toxin is available (Ben-Gigirey and Villar-González, 2008). The two main methodologies used to analysis of PSP toxins involve an isocratic separation of the toxins followed by a post-column oxidation (Oshima, 1995) or a pre-column oxidation of toxins followed by a gradient separation of the oxidation products (Lawrence et al., 1995). In 2005, the Lawrence method has been adopted as an official method to detect PSP toxins and it has been recently approved by the EU for monitoring these toxins (AOAC, 2005; E.U.C, 2006). However, this method seems to be useful mainly for official PSP control in certain samples since its performance depends on the toxic profile of the sample (Ben-Gigirey et al., 2007). The major impediments to widespread use of this method are the co-elution of oxidation products and the amount of time required to process samples containing significant amounts of PSP toxins (Ben-Gigirey et al., 2007). In this sense, the post- column oxidation methods based on the Oshima method (Oshima, 1995) are suitable when a full quantification of PSP toxins is required (Franco and Fernández-Vila, 1993; Chen and Chou, 2002). Nowadays the post-column oxidations methods have been subject to continuous modifications to better and reduce of run times of analysis (Rourke et al., 2008). The pre- and post- column HPLC methods, despite the many benefits of each, which includes an increased sensitivity to low concentrations of toxins and less variability in the results, present also some drawbacks that should be resolved.

PSP toxins can be *in vitro* transformed by pH and/or temperature effect (Vale et al., 2008), in addition bivalves that accumulate the toxins can also transform them (Franco and Fernández-Vila, 1993). For these reason, the sample source and the processing procedures as well as the standard composition are important items when the toxin profile of a sample will be HPLC studied. In the present work, several samples from different dinoflagellates (non-converted) and shellfish (converted) were analyzed by pre- and post- column HPLC methods in order to compare the PSP toxins profile obtained by both methods.

2. Material and methods

2.1. Chemicals and solutions

HPLC grade methanol, acetonitrile, acetic acid, sodium hydroxide, periodic acid, disodium hydrogen phosphate (Na_2HPO_4) and hydrogen peroxide were obtained from Panreac Quimica S.A. (Barcelona, Spain). Ammonium formate was purchased from Sigma Aldrich (Spain).

Peroxide oxidant was 10% hydrogen peroxide aqueous solution. Periodate oxidant was prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate and 0.3 M Na_2HPO_4, then adjusted pH to 8.2 with 1 M NaOH using pH meter.

Standards of PSP toxins; saxitoxin (STX), neosaxitoxin (NEO), decarbamoylsaxitoxin (dcSTX), gonyautoxins 1 and 4 (GTX1,4), gonyautoxins 2 and 3 (GTX2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX2,3), GTX 5 (B1), and C1 and C2 were provided by NRC Certified Reference Material Program (Institute for Marine Biosciences, Halifax, Canada) for the identification of each toxin.

2.2. Samples preparation

Several samples (1–13) from different PSP producer dinoflagellates were used. *Alexandrium tamarense* (CCMP1598 strain) was purchased from Bigelow Laboratory for Ocean Sciences (ME, USA), and several *Alexandrium* spp. samples were obtained from the west coast of Spain. The dinoflagellates were filtered through a 10 μm mesh and the cells were re-suspended in acetic acid/ethanol (3:1). Then, the cells were ultrasound lysed (three cycles of 20 s) and centrifuged at 3000 rpm for 5 min. The supernatant was separated from the pellet, evaporated to dryness and finally dissolved in 0.03 M acetic acid.

Several toxins extracts (14–17) isolated from contaminated mussels from Galicia, Spain were used. The extracts were obtained after whole flesh extraction (ethanol/acetic acid) followed by purification through weight exclusion chromatography (Alfonso et al., 1993).

The extracts, either from dinoflagellates or mussels, were cleaned from residues in centrifuge tubes (with 0.45 μm filters) before HPLC analysis by both methods. Aliquots of 100 μL of the extracts were directly injected in a 20 μL loop in the post-column oxidation method. For analysis by pre-column oxidation method, the extracts were previously oxidized with hydrogen peroxide or periodate. For the peroxide oxidation 25 μL of 10% (w/v) aqueous H_2O_2 were added to 250 μL of 1M NaOH in a 1.5 mL vial and mixed. Then 100 μL of standard solution or sample extract were added to the vial. The reaction was allowed for 2 min at room temperature. Then 20 μL of glacial acetic acid was added to stop the reaction, the solution was mixed, and 25 μL were injected into the HPLC system. For periodate oxidation 100 μL of standard solution or sample extract were added to 100 μL of deionized water in a 1.5 mL vial. Then 500 μL of periodate oxidant were added to the vial and mixed. The solution was allowed to react at room temperature for 1 min, and then 5 μL of glacial acetic acid were added and mixed. The mixture was allowed to stand for at least 10 min at room temperature before injecting 100 μL into the HPLC system.

2.3. HPLC

2.3.1. HPLC with post-column oxidation

A modification of Oshima's (Oshima, 1995) HPLC method (Vale et al., 2008) was used to identify PSP toxins. To separate the toxins an AquaSep column, reversed-phase C8 (5 μm, 4.6 × 250 mm) from ES Industries Chromega Columns was used with different mobile phases depending on the toxin group. The mobile phase to identify the GTX toxins group (acetonitrile-free) was constituted by 2 mM