

Contamination Status of Lipophilic Marine Toxins in Commercial Shellfish from Spain, Chile and South East Pacific [†]

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Abstract: Lipophilic marine toxins in mollusc constitute an important threat to human health and high number of intoxications occur every year. These toxins restrict the progress of aquaculture, which is one of the fastest growing food sectors in the world. The region of Galicia (Spain), Chile and South East Pacific are commercially important producer of edible bivalve mollusc, however they have been subjected to recurring cases of shellfish farm closures in the last decade. This work aimed to study the lipophilic toxic profile of commercial shellfish (including emerging toxins) from these locations in order to establish a potential risk when ingested. For this, a total of 41 samples of Galician mussels (*Mytilus galloprovincialis*), 6 samples of mussels from Chile (*Mytilus chilensis*) and other 6 samples from South East Pacific (*Tawerea gayi* and *Meretrix lyrata*) were purchased in local markets and analysed by ultra-high-performance liquid chromatography system coupled to mass spectrometry (UPLC–MS/MS). Chromatograms from *Mytilus galloprovincialis* showed the presence of okadaic acid (OA), dinophysistoxin-2 (DTX-2), pectenotoxin-2 (PTX-2), azaspiracid-2 (AZA-2) and the emerging toxins 13-desmethyl spirolide C (SPX-13) and pinnatoxin-G (PnTX-G). Data showed that OA group toxins are the main risk in Galician mollusks, which was detected in 38 samples (93%) at levels close to the legislated limit, followed by SPX-13 that was detected in 19 samples (46%) in quantities of up to 28.9 µg/kg. Analysis from PTX-2, AZA-2, and PnTX-G showed smaller amounts, all below 3 µg/kg. Results also showed the presence of the emerging PnTX-G in mussels *Mytilus chilensis* at levels up to 5.2 µg/kg and AZA-2 and PTX-2 in clams *Tawerea gayi* up to 4.33µg/kg and 10.88µg/kg, respectively. Despite no potential risk through mussel ingestion was found for the emerging toxins (SPX-13 and PnTX-G), there is a need for robust methodologies that can detect a wide range of known or emerging toxins in different matrix due to the geographical expansion of marine toxins.

Keywords: lipophilic marine toxins; emerging toxins; *Mytilus galloprovincialis*; *Mytilus chilensis*; *Tawerea gayi* and *Meretrix lyrata*; UPLC-MS/MS.

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1. Introduction

Lipophilic marine toxins, which are produced by harmful microalgae and accumulate in the marine food chain, are of growing concern in our society [1]. *The legislated group of lipophilic marine toxins* includes: yessotoxins (YTXs) azaspiracids (AZAs), pectenotoxins (PTXs) and okadaic acid (OA) and its derivatives, the dinophysistoxins (DTXs). Only 13 compounds are regulated which are yessotoxin (YTX), homo-yessotoxin (Homo-YTX), 45-hydroxy-yessotoxin (45-OH-YTX), 45-hydroxy-homo-yessotoxin (45-OH-homo-YTX), azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2), azaspiracid-3 (AZA-3), pectenotoxin-1 (PTX-1), pectenotoxin-2 (PTX-2), OA, dinophysistoxin-1 (DTX-1), dinophysistoxin-2

(DTX-2) and dinophysistoxin-3 (DTX-3). In Europe, levels in shellfish for human consumption must be below 3.75 mg eq YTX/kg, 0.16 mg eq AZA and 0.16 mg eq OA/Kg (for the OA and PTX toxin group). The official detection method of these compounds is LC-MS/MS, according to the Regulation (EU) 2019/627, amending Regulation (EC) 2074/2005 [2,3].

Moreover, the presence of emerging lipophilic marine toxins from harmful phytoplankton called cyclic imines (CIs) is increasing in European waters [4–7]. This group comprises spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs) and pteriatoxins (PtTXs) and can be accumulated in the marine food chain. There are yet no official methods or regulatory limits for this toxin group. In this work, mussels and clams purchased in local markets and originally from 3 worldwide areas (Galicia, Chile and SouthEast) were analysed by the EU-Harmonised SOP for the determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS with the objective of investigating the potential consumer risks linked to their consumption.

2. Materials and Methods

2.1. Molluscs Acquisition and Sample Preparation

A total of 53 shellfish samples were purchased from December 2018 to December 2019. They were from 3 locations (Galicia, Chile and SouthEast Pacific). 41 samples from Galicia (*M. galloprovincialis*) were purchased fresh, 6 samples from Chile were frozen mussels (*M. chilensis*) and 6 samples from South East Pacific (*T. gayi* and *M. lyrata*) which were frozen clams. All molluscs were purchased in local markets in Lugo (Spain). Once in the laboratory meat molluscs were homogenized (Ultra Turrax™), stored in bags at $-20\text{ }^{\circ}\text{C}$ and preserved from oxygen and light. Each sample was a homogenate of the tissue of around 15 individual mollusc (minimum 100g).

2.2. Analysis of Lipophilic Marine Toxins

Molluscs were extracted and analysed following the EU-Harmonised Standard Operating Procedure (SOP) for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS [8], including the emerging toxins SPXs and PnTXs. Analysis were performed by a 1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent G6460C Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The toxins were separated using a column AQUITY UPLC BEH C18 ($2.1 \times 100\text{ mm}$, $1.7\text{ }\mu\text{m}$, Waters) at $40\text{ }^{\circ}\text{C}$. Mobile phase A was 100% water and B acetonitrile-water (95:5), both containing 50 mM formic acid and 2 mM ammonium formate. The gradient program with a flow rate of 0.4 mL/min was started with 30% B and then a linear gradient to 70% B in 3 min. After an isocratic hold time linear of 1.5 min at 70% B and return to the starting conditions of 30% B in 0.1 min. Finally, 30% B was kept for 1.99 min before the next injection. Source conditions were: $350\text{ }^{\circ}\text{C}$ of drying gas temperature with 8 L/min flow, nebulizer gas pressure of 45 psi (Nitrocrafter NCLC/MS from Air Liquid, Madrid, Spain), sheath gas temperature of $400\text{ }^{\circ}\text{C}$ and a flow of 11 L/min. The capillary voltage was set to 4000 V in negative mode with a nozzle voltage of 0 V and 3500 V in positive mode with a nozzle voltage of 500 V. Analysis were performed in multiple reaction monitoring (MRM) acquisition mode, selecting two transitions per molecule. Transitions were: 45-OH-homo-YTX (m/z 1171.5 > 1091.5, m/z 1171.5 > 869.5), 45-OH-YTX (m/z 1157.5 > 1077.5, m/z 1171.5 > 871.5), Homo-YTX (m/z 1155.5 > 1075.5, m/z 1155.5 > 869.4), YTX (m/z 1141.5 > 1061.5, m/z 1141.5 > 855.4), PTX-1 (m/z 892.5 > 821.5, m/z 892.5 > 213.2), PTX-2 (m/z 876.5 > 823.5, m/z 892.5 > 213.2), AZA-1 (m/z 842.5 > 824.5, m/z 842.5 > 806.5), AZA-2 (m/z 856.5 > 838.5, m/z 856.5 > 820.5), AZA-3 (m/z 828.5 > 810.5, m/z 828.5 > 792.5), OA/DTX-2 (m/z 803.5 > 255.1, m/z 803.5 > 113.2) and DTX-1 (m/z 817.5 > 255.1, m/z 817.5 > 113). Certified reference materials were provided by Cifga (Lugo, Spain) and from The Institute for Marine Biosciences, National Research Council (Halifax, NS, Canada).

3. Results

3.1. *Mytilus Galloprovincialis* Toxin Levels

OA and DTX-2 were the main toxins in Galician molluscs. Figure 1 represent lipophilic marine toxin levels. OA and DTX-2 were detected in the 93% of the samples (7% exceeding the legal limit 160 µg/kg) and followed by SPX-13 detected in the 46% of the samples up to 28.9 µg/kg. PTX-2, AZA-2 and PnTX-G were present in lower levels, 37% of the samples showed PTX-2 (0.7–2.9 µg/kg), 29% AZA-2 (0.1–1.8 µg/kg) and 12% PnTX-G (0.4 µg/kg–0.9 µg/kg). Chromatogram of the major toxins, OA and DTXs are represented in Figure 2.

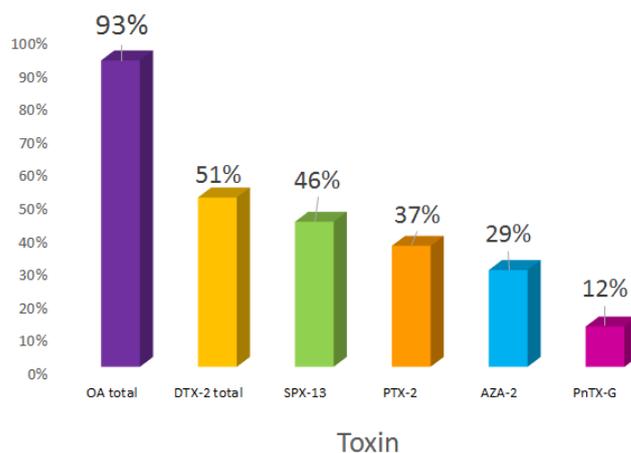


Figure 1. Lipophilic levels of *Mytilus galloprovincialis* from market.

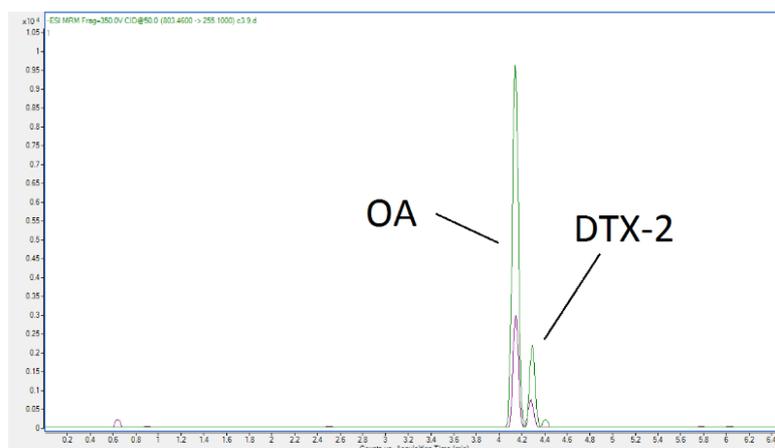


Figure 2. Chromatogram of OA and DTX-2 from a mussel sample *Mytilus galloprovincialis*.

3.2. *Mytilus Chilensis* Toxin Levels

Results from *M. chilensis* showed just the presence of the emerging PnTX-G in all samples at levels up to 5.2 µg/kg. This is the first time that PnTX-G is found *M. chilensis*. Figure 3 represent the chromatogram where PnTX-G is eluting in the minute 3. No other lipophilic toxins were found in the samples.

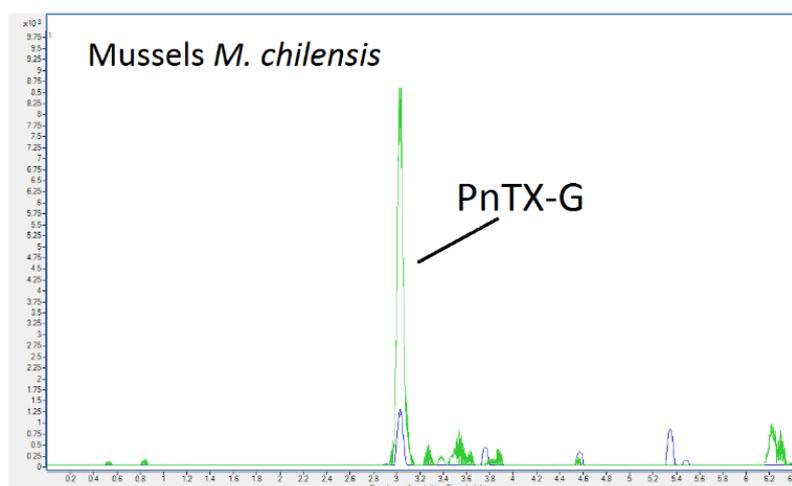


Figure 3. Chromatogram of PnTX-G from a mussel sample *Mytilus chilensis* from market.

3.3. *Tawera Gavi* and *Meretrix Lyrata* Toxin Levels

Chromatograms from clams *T. gayi* showed the presence of AZA-2 and PTX-2 at levels up to 4.33 µg/kg and 10.88 µg/kg, respectively (Figure 4). No lipophilic toxins were found in *M. lyrata* shellfish.

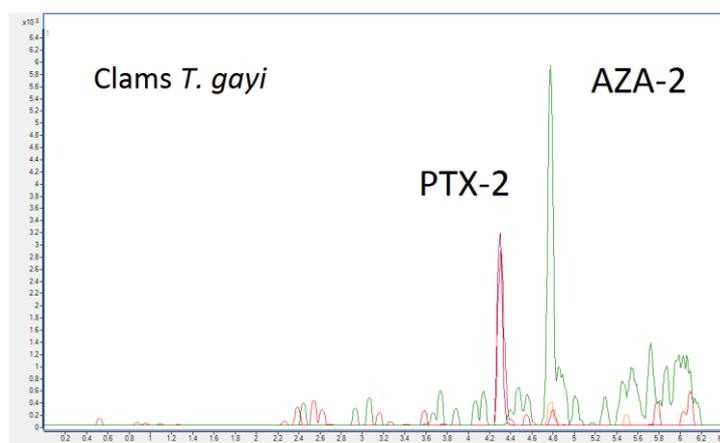


Figure 4. Chromatogram of PTX-2 and AZA-2 from a mussel sample *Tawera gayi* from market.

4. Conclusions

OA group toxins continue being the main lipophilic toxins in Galician molluscs. These toxins were detected in the 93% of the samples, followed by SPX-13, detected in the 46% of the samples. PnTX-G is confirmed in commercial mussels from Chile for the first time. However, the low levels found (<6 µg/kg), means that there is no potential risk through mussel ingestion for the emerging toxin PnTX-G. Although it does not seem to pose a potential risk through mussel consumption for the emerging toxins (SPX-13 and PnTX-G), the presence of new analogs must be considered in the shellfish safety monitoring programmes by LC-MS/MS methods.

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