The analysis of lipophilic marine toxins

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Abstract

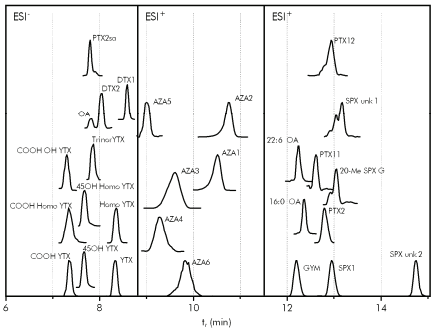
Consumption of lipophilic marine toxin contaminated shellfish can lead to severe intoxications. Methods described in European Union (EU) legislation to test for the presence of these toxins are based on a mouse or rat bioassay. These assays are unethical and have a poor sensitivity and selectivity. For this reason there is an urgent need for alternative methods. Most promising alternatives are the methods based on liquid chromatography - tandem mass spectrometry (LC-MS/MS). A LC-MS/MS method with alkaline chromatographic conditions in which we were able to separate and analyze the most important toxins in a single analysis was developed. Furthermore, a clean up procedure based on solid phase extraction (SPE) was developed. A combination of SPE clean up and alkaline chromatographic conditions resulted in reduced matrix effects for all matrices tested (mussel, scallop and oyster). The developed SPE & LC-MS/MS method was in-house validated using EU Commission Decision 2002/657/EC. With respect to accuracy, repeatability, reproducibility and decision limit the method performed well. The method also performed excellently in view of possible new limits that are 4- to 5-fold lower than current limits for some toxins. A collaborative study was also performed for the most important toxins of the lipophilic marine toxin group.

Introduction

Marine toxins (phycotoxins) are natural toxins produced by at least 40 species of algae belonging mainly to the dinoflagellates and diatoms (Gerssen et al., 2010a). Phycotoxins can accumulate in various marine species such as fish, crabs or filter feeding bivalves (shellfish) such as mussels, oysters, scallops and clams. In shellfish, toxins mainly accumulate in the digestive glands without causing adverse effects on the shellfish itself. However, when substantial amounts of contaminated shellfish are consumed by humans, this may cause severe intoxication of the consumer (Aune and Yndestad, 1993; Botana et al., 1996; Jeffery et al., 2004). Based on their chemical properties marine toxins can be divided in two different classes: hydrophilic and lipophilic toxins. Toxins associated with the syndromes Amnesic Shellfish Poisoning (ASP) and Paralytic Shellfish Poisoning (PSP) are hydrophilic by nature and have a molecular weight (MW) below 500 Da. Toxins responsible for Neurologic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Azaspiracid Shellfish Poisoning (AZP) and other toxins such as pectenotoxins (PTXs), yessotoxins (YTXs) and cyclic imines [spirolides (SPX) and gymnodimine] all have as common denominator a MW above 600 Da (up to 2 000 Da). These toxins have strong lipophilic properties and are generally called lipophilic marine toxins. European Union (EU) legislation prescribes animal tests (mouse or rat) as the official method for control of lipophilic marine toxins in shellfish (Anon, 2005). More than 300 000 test animals (mostly mice) are used annually for routine monitoring of lipophilic marine toxins in shellfish within the EU. Besides the ethical aspects of this cruel animal test, it also contradicts with other EU legislation which states the reduction, refinement and replacement of animal tests (Anon, 1986). Furthermore, these animal tests can produce false positive results and have a poor sensitivity and selectivity. In this paper the development of an alternative method for the determination of lipophilic marine toxins is described, based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

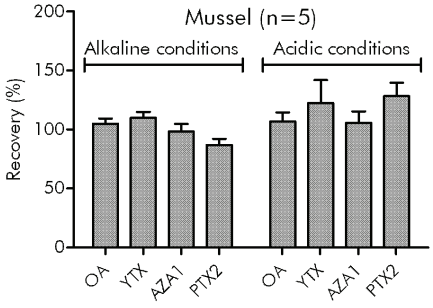
Liquid chromatography mass spectrometry

Traditionally, LC-MS/MS methods used acidic chromatographic conditions for the determination of lipophilic marine toxins (Fux et al., 2007; Quilliam et al., 2001). However, under acidic conditions peak shapes as well as separation of some toxins was poor. With alkaline chromatographic conditions, an aceto-nitrile/water gradient containing ammonium hydroxide (pH 11), the limit of detection (LOD) for OA, yessotoxin (YTX), gymnodimine (GYM) and 13-desmethyl spirolide C (SPX1) was improved two- to three-fold (Gerssen et al., 2009b). This improvement is mainly due to improved peak shapes. A major advantage of the developed alkaline method is that toxins can be clustered in retention time windows separated for positively and negatively ionized molecules. Therefore, there is no need for rapid polarity switching or for two separate runs to analyze a sample. With this method at least 28 different lipophilic marine toxins can be analyzed in a single run. Separation of the most prominent lipophilic marine toxin groups comprising okadaic acid (OA), dinophysistoxins (DTXs), YTXs, azaspiracids (AZAs) and SPXs was achieved (Figure 1).



Matrix effects

It is well known that LC-MS/MS analysis is sensitive to matrix effects (signal suppression or enhancement). This is also the case for lipophilic marine toxins. Therefore, the potential of solid phase extraction (SPE) clean up has been assessed to reduce matrix effects in the analysis of lipophilic marine toxins. A large array of ion-exchange, silica-based and mixed function SPE sorbents was tested. The toxins were best retained on polymeric sorbents. Optimization experiments were carried out to maximize recoveries and the effectiveness of the clean up. This was done by optimization of the wash and elution conditions. Matrix effects were assessed using either an acidic or an alkaline chromatographic system as described in earlier publications (Gerssen, et al., 2009b). In combination with the alkaline LC method this resulted in a substantial reduction of matrix effects to less than 15%, while in combination with the acidic LC method approximately 30% of the matrix effects remained (Figure 2). A combination of the SPE method with the chromatography under alkaline conditions was the most effective (Gerssen et al., 2009a).



In-house validation

Before a method can be officially used in the EU for routine analysis, the method needs to be validated. The in-house validation was performed for the quantitative analysis of OA, YTX, AZA1, PTX2 and SPX1 in shellfish extracts [mussel (*Mytilus edulis*), oyster (*Cassostrea Gigas*), cockle (*Cerastoderma edule*) and ensis (*Ensis directus*)]. Dinophysistoxin-1 (DTX1), -2 (DTX2) and azaspiracid-2 (AZA2) and -3 (AZA3) were not included in the study because the certified standards were not available at that time. The validation was performed using the EU Commission Decision 2002/657/EC as guideline (Anon, 2002). Validation was performed at 0.5, 1 and 1.5 times the current EU permitted levels, which are 160 µg/kg for OA, AZA1 and PTX2 and 1 000 µg/kg for YTX. For SPX1 400 µg/kg was chosen as target level as no legislation has been established yet for this compound. The method was

validated for determination in crude methanolic shellfish extracts and for extracts purified with solid phase extraction (SPE). The toxins were quantified against a set of matrix matched standards instead of standard solutions in methanol. In order to save valuable standard the toxin standards were spiked to the methanolic extract instead of the shellfish homogenate. This was justified by the fact that the extraction efficiency is high for all relevant toxins (>90%). The method performed very well with respect to accuracy, intra-day precision (repeatability), inter-day precision (within-lab reproducibility), linearity, decision limit (CCα), specificity and ruggedness. For crude extracts the method performed less satisfactory with respect to the linearity (<0.990) and the change in LC-MS/MS sensitivity during the series (>25%). This decrease in sensitivity could be attributed to contamination of the LC-MS/MS system. SPE purification resulted in a greatly improved linearity and signal stability during long series (more than 20 samples). Recently the European Food Safety Authority (EFSA) has published a number of opinions on the various toxin groups. The EFSA has suggested that in order not to exceed the acute reference dose the levels should be below 45 µg/kg OA-equivalents and 30 µg/kg AZA1-equivalents. If these levels are adapted in legislation this means a 4-5 fold lower permitted limit than the current one. For these toxins a single day validation was successfully conducted at these levels (Gerssen et al., 2010b).

Outlook

Now the developed method has been in-house validated, the next step is a full collaborative study. This collaborative study was performed in 2010. In total 13 laboratories participated in this study. Statistical evaluation was performed according to AOAC guidelines for collaborative study procedures (appendix D). HorRat values were good, ranging from 0.71 for AZA total group toxicity till 1.60 for YTX. The final report of this study is under preparation and will be published in the beginning of 2011. Furthermore, recently it is decided to change EU legislation. The new legislation will prescribe the use of LC-MS/MS as the reference method for the analysis of lipophilic marine toxins instead of the animal assay. The method described in this paper can than be adopted as an official method for routine analysis and the mouse and rat bioassay can be finally abolished.
Furthermore, to our opinion more research is needed for the production and isolation of lipophilic marine toxins and method development on functional assays and other new emerging toxins such as palytoxins, cyclic imines and ciguatera toxins.

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