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# Heat treatment and the use of additives to improve the stability of paralytic shellfish poisoning toxins in shellfish tissue reference materials for internal quality control and proficiency testing

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ABSTRACT

The need for homogenous reference materials stable for paralytic shellfish toxins is vital for the monitoring and quality assurance of these potent neurotoxins in shellfish. Two stabilisation techniques were investigated, heat treatment through autoclaving and the addition of preserving additives into the tissue matrix. Short and long-term stability experiments as well as homogeneity determination were conducted on materials prepared by both techniques in comparison with an untreated control using two LC-FLD methods. Both techniques improved the stability of the matrix and the PSP toxins present compared to the controls. A material was prepared using the combined techniques of heat treatment followed by spiking with additives and data is presented from this optimised reference material as used over a two year period in the Irish national monitoring program and in a development exercise as part of a proficiency testing scheme operated by QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe) since 2011. The results were indicative of the long-term stability of the material as evidenced through consistent assigned values in the case of the proficiency testing scheme and a low relative standard deviation of 10.5% for total toxicity data generated over 24 months.



## 1. Introduction

Marine biotoxins causing paralytic shellfish poisoning (PSP) are mainly produced by some marine dinoflagellates which can find their way through the food chain to humans via filter feeding bivalves such as mussels, clams, oysters and scallops, causing a range of symptoms including paralysis and even death (EFSA, 2009). The potentially grave consequences following consumption of these toxins can put significant pressure on monitoring laboratories to ensure contaminated product does not reach the marketplace.

The use of matrix reference materials (RMs) in the analytical quality control and quality assurance of marine biotoxins testing in shellfish has been well documented (Turner et al., 2013a, 2013b, 2014; Hess et al., 2007; McCarron et al., 2007). The frequency of their use is increasing due not only to increased global aquaculture production and the extra monitoring this entails (FAO, 2014) but is also coupled to the trend of monitoring laboratories moving away from animal based assays towards instrument based methods of analysis. This is necessitated by changes to EU legislation (Anon, 2006, 2011) as well as the advantages these instrumental methods give to the end user (Hess et al., 2006). Although the mouse bioassay (MBA) is still the reference method in the EU for paralytic shellfish toxins (PST) (Anon, 2005), an alternative method based on pre-column oxidation (preCOX) followed by liquid chromatography with fluorescence detection (LC-FLD) (AOAC 2005.06) was incorporated into EU legislation in 2006 (Anon, 2006). The recent approval by the AOAC of two new official methods (AOAC 2011.02 and 2011.27) for PST determination based on post-column oxidation (PCOX) followed by LC-FLD (AOAC International, 2011a) and a receptor binding assay (RBA) (AOAC International, 2011b) coupled to recent advances made in LC-MS/MS methodologies (Boundy et al., 2015) highlight the increasing need for RMs in this area.

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The increased demand for RMs puts pressure on providers, particularly producers of certified reference materials (CRMs), which are time consuming and costly to prepare and can lead to issues of limited availability which impact upon all monitoring and research laboratories (Hess et al., 2007).

Various stabilisation techniques for PST RMs have been investigated including freeze drying (Turner et al., 2013a), gamma irradiation (Turner et al., 2013b) and high pressure processing (Turner et al., 2014) in particular, significantly improving the stability of the matrix and therefore the toxins. Furthermore, gamma irradiation was used recently to prepare a commercially available CRM from the Centre for Environment Fisheries and Aquaculture Science (CEFAS) in the UK (Turner and Higman, 2012). The production of any RM requires a level of technical and organizational competence (International Organisation for Standardisation, 2014), particularly to ensure the homogeneity and stability of the materials. Certain techniques and processes may be beyond the capabilities of some laboratories given the requirements for use of certain pieces of equipment such as freeze driers, high pressure processing units or facilities for gamma irradiation (Hess et al., 2007). Simpler techniques for stabilising and ensuring the homogeneity of RMs are available however and their use would significantly improve the analytical quality control of laboratories.

This paper describes the relatively simple techniques of heat treatment and the inclusion of preserving additives during RM preparation and the significance these have on PST RM stability. The preparation techniques should be within the capabilities of most laboratories and materials prepared by these techniques have a wide array of uses such as method development and validation, quality assurance and for use in proficiency testing schemes such as those operated by QUASIMEME (Hess et al., 2006).

## 2. Materials and methods

### 2.1. Chemicals and standards

All chemicals and solvents used were of analytical or HPLC grade. The water was supplied from a reverse osmosis system (Barnstead Int., Dubuque, IA, USA). Acetic acid, hydrochloric acid, ammonium formate, ammonium acetate, sodium chloride, sodium hydroxide, hydrogen peroxide, disodium hydrogen phosphate, periodic acid, ethoxyquin and ampicillin were purchased from Sigma–Aldrich (Steinheim, Germany). Oxytetracycline and erythromycin were purchased from Fisher Scientific (Waltham, MA, USA). Acetonitrile was purchased from Labscan (Stillorgan, Ireland). Certified reference toxins (GTX-1,4, NEO, dcSTX, GTX-2,3, GTX-5, C-1,2, dcNEO, dcGTX-2,3 and STX) were obtained from the Institute of Biotoxin Metrology, National Research Council Canada (IMB, NRCC, Halifax, Nova Scotia, Canada). For preCOX LC-FLD analysis, the CRMs were first diluted in water (adjusted to pH4 ± 0.1 with 0.1 M acetic acid) to prepare primary stock solutions. Further dilutions were performed in 0.1 mM acetic acid to prepare working calibration solutions. For PCOX analysis, calibration solutions for GTX/STX analysis were prepared in 0.3 mM HCl and C1&2 standards were prepared in pH5 ± 0.1 water. All standards were stored following NRCC recommendations (Quilliam, 2007).

### 2.2. Preparation of study materials

The study materials consisted of mussel (*Mytilus edulis*) tissues from Norway and Iceland naturally contaminated with a range of PSP toxins and diluted with *M. edulis* whole flesh tissue from Ireland, shown to be PST-free following preCOX LC-FLD analysis. These tissues were combined and homogenised in a large scale Waring blender (Hartford, CT, USA) for 5.0 min. The homogenates were transferred to polypropylene containers, sealed and stored at −20 °C until further use. The moisture content (MC) of each homogenate was determined using a loss on drying rotary vacuum method before storage.

Each of the study materials were prepared similarly, with the homogenate being defrosted at room temperature overnight prior to preparation. Each of the materials was adjusted to an 85% MC to reflect that of a natural mussel matrix with the additives being introduced during this initial step. Tissue A was spiked with an antioxidant (ethoxyquin) and three antibiotics (ampicillin, oxytetracycline and erythromycin). Each of the additives was added at a concentration of 0.02% w/w and dissolved in ethanol (1% of total tissue weight) to improve homogeneity. Tissue B was autoclaved using a Systec VE-100 (Focus Scientific Solutions, Meath, Ireland), at 121 °C for 15.0 min and Tissue C was left untreated as a control apart from MC adjustment. The tissues were transferred gravimetrically to Waring blenders (Hartford, CT, USA), the dissolved additives solution was poured into Tissue A before making up to a final weight using deionised water. Each material was blended for 5.0 min and transferred to a PP 3.0 L beaker before being homogenised further using a Polytron with coarse and fine head attachments for 50.0 min.

The materials were continually mixed while being dispensed as 5.3 g aliquots using a calibrated peristaltic pump (Bernant, IL, USA) into 5.0 ml PP tubes (Teklab Ltd., Durham, UK). The tubes were hermetically sealed with aluminium lids under a stream of argon using a manual heat sealer (MK 1, Seal-it-Systems, Lancashire, UK), before being fitted with wadded screw caps. A total of 125 aliquots of each, Tissue A, B and C were dispensed and all materials were stored at −80 °C until further use.

### 2.3. Stability and homogeneity testing

The between-bottle homogeneity of each material was assessed through the intra-batch analysis of 10 aliquots selected by stratified random sampling of the entire fill series. This involved the division of the population into smaller groups known as strata, from which simple random sampling or systematic sampling is applied to each stratum.

All materials were stability tested over a short term (ca. 30 day) and long term (ca. 12 month) period following a reverse isochronous experimental design (Lamberty et al., 1998). The short term study was conducted with triplicate samples consisting of five time points (0, 4, 6, 16 and 30 day for Tissue A, 0, 4, 8, 15 and 28 days for Tissue B and 0, 4, 8, 15 and 32 days for Tissue C) and three temperature conditions (−20 °C, +4 °C and +40 °C). The time points used in the study of each material were slightly different as it was necessary to alter the time models slightly to ensure analysis was completed within the required time frame. The long term study was conducted with triplicate samples consisting of five time points (0, 3, 6, 9 and 12 month) and three temperature conditions (−20 °C, +4 °C and +20 °C). The reference temperature used in both studies was −80 °C. All samples were stored in the dark throughout the duration of both the short and long-term stability studies.

At the end time point all samples were removed from storage, allowed to equilibrate to room temperature before being extracted and analysed by preCOX LC-FLD closely following AOAC 2005.06 (AOAC International, 2005). PCOX LC-FLD analysis was additionally performed on all samples (AOAC International, 2011a) to specifically investigate the extent of epimerisation, if any, of GTX2&3 in each material. The epimerisation of GTX1&4 was not determined as the concentration of these toxins in each tissue was below the limit of detection (<LOD) of the PCOX LC-FLD method.