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An updated ciguatoxin extraction method and silica cleanup for use with HPLC-MS/MS for the analysis of P-CTX-1, PCTX-2 and P-CTX-3

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ABSTRACT

Ciguatera fish poisoning is a debilitating human neuro-intoxication caused by consumption of tropical marine organisms, contaminated with bioaccumulated ciguatoxins (CTXs). The growing number of cases coupled with the high toxicity of CTXs makes their reliable detection and quantification of paramount importance. Three commonly occurring ciguatoxins, P-CTX-1, 2 and 3 from five different ciguatoxic Spanish mackerel (*Scomberomorus commerson*), were used to assess the effectiveness of different extraction techniques: homogenization (high powered blending vs. ultrasonication); C-18 column sizes (500 mg vs. 900 mg); and a novel HILIC SPE cleanup. Despite minor differences, blending and sonication proved equally effective. Larger 900 mg columns offered a greater extraction efficiency, increasing detected P-CTX-1 by 37% ($P < 0.001$). The newly adapted cleanup was highly effective at reducing co-eluting phospholipids thereby reducing matrix effects and increasing detectable CTXs by HPLC-MS/MS. Silica cleanup extraction efficiencies were also compared between the highly effective and validated ciguatoxin rapid extraction method (CREM) and current best practice extraction method employed by Queensland Health (QH). Overall, the QH protocol proved more effective, especially when paired with the newly adapted cleanup, as this increased the amount of extracted P-CTX-1 by 46% ($P < 0.01$), P-CTX-2 by 10% and P-CTX-3 by 71% ($P = 0.001$). This study suggests the QH protocol utilizing a 900 mg C-18 column and newly adapted HILIC SPE cleanup was most effective at extracting P-CTX-1, -2, -3. Specifically P-CTX-1, the primary ciguatoxin congener of concern due to its extremely high potency and an ability to cause CFP at 0.1 μg/kg following consumption of carnivorous fish flesh. Despite being more time intensive (an additional 85 min per batch of 12 samples), this will be especially effective for assessing lower toxin burdens, which may be near the limit of detection.

1. Introduction

Ciguatera fish poisoning (CFP) is a debilitating human neuro-intoxication caused by consumption of tropical marine organisms, contaminated with bioaccumulated ciguatoxins (CTXs) (Donati, 2006). Global estimates suggest that CFP affects between 50,000 and 500,000 people annually (Fleming et al., 1998) with more than 400 fish species implicated in these poisonings (Tester et al., 2010). Within Australia, >90% of the population consumes seafood, with consumption rising from 13.6 kg to 25 kg per person per year between 1975 and 2010. 2013 estimates have valued the industry at $2.2 billion domestically with an export value at $1.2 billion (DAFF, 2013). Unfortunately, ciguatoxic fish are indistinguishable from uncontaminated fish with regard to taste, smell and appearance. Their distribution is sporadic and unpredictable (Lehane and Lewis, 2000). These dangerous characteristics coupled with a remarkable diversity (30 different congeners from three distinct regions, the Pacific and Indian oceans and the Caribbean sea) and the high toxicity of CTXs makes their reliable detection and quantification of paramount importance (Cailluad et al., 2010).

The growing number of CFP incidents around the world has spurred the development of a range of CTX detection methodologies with numerous approaches (e.g., toxicological symptoms, antibody recognition, cell lines, mass spectrometry, etc.) (Cailluad et al., 2010). The current, most widespread protocol is the Ciguatoxin Rapid Extraction Method (CREM), developed in 2009 (Lewis et al., 2009), which has formed the basis of a multitude of working methodologies employed today. This method allows for small

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quantities (2.0 g of fish tissue, instead of 50–100 g with other methods) to be efficiently tested for CTXs at clinically relevant levels above 0.1 ppb (Lewis et al., 2009). This method provides comparatively high recovery-rates for three predominate Pacific CTXs (P-CTX-1, -2, -3), a low limit of detection (0.03 ng g^{-1}, Stewart et al., 2010), and has shown excellent reproducibility across a range of fish species (Lewis et al., 2009; Stewart et al., 2010), making it ideal for rapid assessment of background levels of CTXs, which may be below the detection limit of the other methodologies (Stewart et al., 2010; Cailluad et al., 2010).

Despite the simplification and increased sensitivity of the CREM method, there remains the ubiquitous hurdle of extraction efficiency and matrix interferences in a range of tissue matrices. This was addressed in conjunction with a 2010 study using Lewis's CREM accordingly adjusted for use at Queensland Health's facilities (Stewart et al., 2010). Spike and recovery using the most common Pacific specific CTX (P-CTX-1), prior to extraction ($n = 10$) yielded a 53.3 ± 15.3% recovery rate. Although this method improved reporting limits from 0.1 μg/kg to 0.07 μg/kg P-CTX-1, the variety of fish tissue matrices being tested (e.g. oily vs. less oily fish) means improvements in cleanup processes need to be explored. Improved efficiency and analytical capacity is becoming increasingly important as research groups worldwide seek to reveal the enigmatic nature of CTX bioaccumulation below clinically relevant concentrations. The efficiency of the CREM relies on: a) effective toxin extraction from tissue matrices; and b) appropriate cleanup procedures for analytical detection (Lewis et al., 2009; Stewart et al., 2010; Wu et al., 2011). Thus, it is these two areas this study addressed in order to increase the efficiency of the current CTX extraction method.

2. Materials and methods

2.1. Methods

The method analyzed in this study is based on the original Ciguatoxin Rapid Extraction Method (CREM) (Lewis et al., 2009), with updates based on the findings of Stewart et al. (2010). Since 2010, the method has undergone a series of further amendments, improving both the analytical and practical capacity of the protocol (outlined in Table 1).

Most notably, the current QHP uses freeze dried tissue in lieu of cooked tissue. Although previous studies identified the benefits of cooking tissue in order to denature interfering proteins, the C18 cleanup and chloroform back extraction included in the method was shown to adequately remove these interfering proteins (unpub. data), rendering the cooking step unnecessary. Additionally, the primary interference of concern comes from co-eluting phospholipids which are present in both cooked and freeze dried fish flesh. This, along with the other developed amendments, have been previously addressed (unpub. Data) and are outlined here, however are out of the scope of this study.

The aim of this study was therefore to maximize the extraction efficiency of the current protocol (Table 1) by trailing sonication and larger C-18 columns and while reducing the problematic phospholipids with the addition of an HILIC SPE cleanup.

2.2. Chemicals and reagents

Pacific CTX-1, -2 and -3 toxin standards (purity ≥ 95%) were obtained from Prof. Richard Lewis, Institute of Molecular Biology, University of Queensland (Lewis et al., 1991). Analytical grade methanol, n-hexane, chloroform and sodium chloride, formic acid, acetone and Millex-Q water were used for all extractions.

2.3. Tissue acquisition

Muscle samples from five Spanish mackerel (*Scomberomorus commerson*) were acquired from Queensland Health's Forensic and Scientific Services department. All fish had been implicated in poisoning incidents and had previously tested positive for P-CTX-1, P −2 and −3. Raw tissue had been frozen at −80 °C, freeze-dried (−60 °C at 0.05 bar) then ground into powder using an automated mortar and pestle, then stored at −20 °C until use. Each of the five fish were partitioned into 16 replicates of 2.0 g of dried tissue in 50 ml Falcon tubes in preparation for solvent extraction. These 16 replicates would then undergo CTX extraction using a combination of three factors (Fig. 1), each combination of which would be tested in duplicates and across all five fish. Note that 13 of the planned 80 samples were not analyzed due to inadequate tissue quantity and minor laboratory issues resulting in 67 testable samples (Fig. 1).

2.4. Solvent extraction

As outlined in Fig. 2, samples underwent homogenization (2 min) with methanol (8.0 ml) and n-hexane (3.0 ml) with either high-powered blending (IKA Ultra Turrax T25) or ultra-sonication (Branson Sonifier 450). Sample vials were suspended in an ice bath during homogenization to avoid methanol evaporation, as the following extraction procedure hinges upon a specific methanol dilution. The homogenate was centrifuged at 2600 rpm for 10 min at 12 °C, followed by decanting into a 100 ml separation funnel. This was repeated a second time, and extracts were combined. The remaining tissue was washed with 4 ml methanol, 2 min vortex mixing and centrifuged then added to the separation funnel (providing a total solvent volume of 20 ml:6 ml methanol:hexane). To facilitate solvent separation, 15 ml of Millipore water was added, followed by the removal of the lower aqueous methanol layer, which was then centrifuged and any remaining n-hexane was removed and discarded.

2.5. Solid phase extraction of the crude extract

The crude extract underwent solid phase extraction (SPE cleanup) utilizing either 900 mg or 500 mg C18 SPE cartridges (Alltech Prevail Maxi-Clean) connected to a vacuum manifold and in the case of the 900 mg columns retrofitted with 12 ml syringes. Cartridges were preconditioned with 6.0 ml of methanol, followed by 6.0 ml of 50% aqueous methanol. The aqueous methanol extraction solvent was passed through the cartridge at a rate of 1 drop s^{-1}, after which the vacuum suction was briefly increased, to allow slight drying to the cartridge. The cartridge was washed with

Table 1
Protocol amendments developed at Queensland Health.

Amendment	Current protocol	CREM (Lewis et al., 2009)	QHP (Stewart et al., 2010)	Change to extraction output (unpub data)
Tissue preparation	Freeze-dried	Cooked	Cooked	No difference
Chloroform extract	Twice	Once	Once	Improved extraction
Filtration	Centrifuged Not filtered	Filtered	Filtered	No difference