# OPTIMIZATION OF METHODS FOR THE EXTRACTION OF CIGUATOXINS FROM LIONFISH (*Pterois volitans*) TISSUE

A Thesis

By

BRITTNEY N. KOSAR

Bachelors of Science, University of Hawaii at Manoa, 2014

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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**August, 2016**

# ABSTRACT

## OPTIMIZATION OF METHODS FOR THE EXTRACTION OF CIGUATOXINS FROM LIONFISH (*Pterois volitans*) TISSUE

Brittney Kosar, Bachelors of Science in Marine Biology, University of Hawaii at Manoa

Chair of Advisory Committee: Dr. P.V. Zimba

Ciguatera Fish Poisoning (CFP) is a severe human illness that results from the consumption of finfish containing ciguatoxins (CTXs). To protect human health in ciguatera endemic areas, monitoring agencies must be able to differentiate between toxic and nontoxic fish. Toxin extraction is required before toxins in fish tissue can be quantified. This study addressed the critical need for optimization of extraction protocols to improve recovery of CTX from fish tissue. Invasive lionfish (*Pterois volitans*) tissue was used to assess the efficiency of various extraction techniques, optimizing Liquid-Liquid (L-L) partitioning steps and Solid Phase Extraction (SPE) steps. This research found that toxin concentrations were highest after wet tissue was processed by initial extraction steps followed by chloroform L-L solvent partitioning. Further processing with SPE cartridges reduced the concentration of toxin remaining in the extracts. This research demonstrated how lyophilization adversely affects extraction of Caribbean toxins. UPLC-TOF and the Neuro-2a bioassay were used to determine efficiency of extraction methods. The optimized method analyzed 1 g samples after chloroform L-L extraction; this method was capable of toxin detection at subnanogram concentration. The use of a highly sensitive UPLC-TOF system greatly reduces the processing time and reduces solvent cost. Results may enable future monitoring programs to be implemented using similar extraction protocols.

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# INTRODUCTION

A harmful algal bloom (HAB) occurs when microorganisms (e.g., diatoms, dinoflagellates, and cyanobacteria) cause damage by out-competing other taxa or by producing toxins. Certain species of microalgae produce toxins as secondary metabolites (Carmichael, 1992). Secondary metabolites are not required for survival, yet they can give the species an advantage over nontoxic strains (Dickey, 2008). Toxins can bio-accumulate through the food chain and affect organisms at all trophic levels. The toxin-producing organisms bloom when environmental conditions are favorable; they affect aquatic ecosystems worldwide and can be harmful to human health. Global climate change and increased nutrient loading may increase the extent and prevalence of HABs by affecting the abundance and toxicity of microorganisms and amplifying their impacts (Dickey, 2008). Marine algal toxins are responsible for over 50,000 human poisonings each year and the number of poisonings is increasing annually (Van Dolah, 2000). Algal toxins such as brevetoxins can be responsible for causing massive fish kills, while ciguatoxins accumulate and concentrate as they move up the food chain and are potentially consumed by humans (Yasumoto et al., 2001; Lewis and Holmes, 1993).

## *Ciguatoxins in the Food Chain*

Ciguatoxins (CTXs) are responsible for the majority of non-bacterial human illnesses from seafood consumption (Tester et al., 2013; Dechraoui et al., 2005). Globally, CTXs cause 50,000-500,000 human poisonings per year (Yasumoto et al., 2001; Fleming et al., 1998) with more than 20,000 poisonings per year in the United States Virgin Islands (USVI) and Puerto Rico (Lewis, 1992). This illness, referred to as

Ciguatera Fish Poisoning (CFP) can occur after humans consume finfish containing CTXs. CFP is a serious, worldwide health concern that is often under-reported and misdiagnosed (Lehane and Lewis, 2000; Tester et al., 2013). Severe neurological, gastrointestinal, and cardiovascular symptoms are associated with CFP. Ciguatoxins are commonly produced as secondary metabolites by dinoflagellates in the genus *Gambierdiscus.* These dinoflagellates can occur in the benthos or as epiphytes on macroalgae in warm, tropical waters. The process of bioaccumulation begins when herbivorous fishes consume toxic dinoflagellates and are in turn consumed by higher organisms (Lewis and Holmes, 1993).

*Structures of Ciguatoxins*

CTXs found in fish are an assembly of primary CTXs along with various structurally different toxin congeners (Lewis et al., 1991; Mak et al., 2012). The chemical structures of CTXs can change after consumption during metabolism and excretion from each consumer (Lewis and Holmes, 1993). Since toxin modifications depend on the species and its specific physiology (e.g., mitochondrial degradation enzymes and phospholipid content), each fish congener can have a different potency and concentration. Human consumption of these toxin congeners can lead to CFP. Concentrations and potency of all compounds contributing to toxicity in a single organism make up its toxin profile, which can vary greatly between organisms on the same trophic level (Pottier et al., 2002; Caillaud et al., 2010). Toxin content varies in fish of the same species from the same area (Abraham et al., 2012). There are currently around 30 known ciguatera toxin congeners (Table 1) (Meyer et al., 2015).

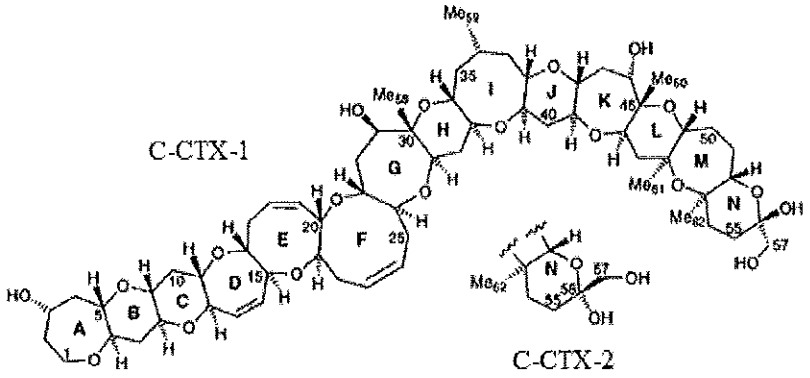


Figure 1. Structure of C-CTX-1 and C56 epimer C-CTX-2. In both cases the mass $[M+H]^+$ is 1141.6 (Vernoux and Lewis, 1997).

The primary ciguatoxin in the Caribbean, C-CTX-1 and its C56 epimer C-CTX-2 (Figure 1) were first isolated and structurally identified from a horse eyed jack (*Caranx latus*) (Lewis et al., 1998). Since then various CTX profiles have been identified in the Caribbean (Table 1); for example, a moray eel (*Lycodontis javanicus*) contained up to fourteen CTX congeners (Lehane and Lewis, 2000), a grey snapper (*Lutjanus griseus*) contained five CTX congeners, and a black jack (*Caranx lugubris*) contained seven CTX congeners (Pottier et al., 2002). There are more than 400 fish species that are potential vectors carrying CTXs (Tester et al., 2010; Lehane and Lewis, 2000). Structurally isolated toxins C-CTX-1 and C-CTX-2 (Figure 1) account for approximately 50%-60% of fish toxin profiles in the Caribbean (Vernoux and Lewis, 1997). To simplify nomenclature, CTX congeners are labeled by their primary location (Pacific, Caribbean, or Indian) and placed into groups based on their principal molecular ion mass (Table 1). Research investigating the pathways by which CTXs can bio-accumulate has been inconclusive (Lewis and Holmes, 1993).

Table 1. Known CTX compounds organized by locality of occurrence (P,C, or I indicates Pacific, Caribbean, or Indian origin) and mass to charge ratio (*m/z*) of the $[M+H]^+$ ion. Bold indicates the primary toxin in the Caribbean.

| Generic name | Location-*m/z* |
|---|---|
| 49-epi-CTX-3C; CTX-3B; CTX-3C; Ciguatoxin 3C 49-epi | P-CTX-1023 |
| 51-hydroxy CTX-3C; CTX-2C1; Ciguatoxin 3C 51-Hydroxy | P- CTX-1039 |
| M-seco-CTX-3C | P- CTX-1041 |
| Ciguatoxin 3C M-Seco Methyl acetate | P- CTX-1055 |
| 2,3-dihydroxy CTX-3C | P- CTX-1057 |
| CTX-4B;GT-4B; 52-epi-CTX-4B; CTX-4A;GT-4A;Ciguatoxin CTX 4A | P- CTX-1061 |
| P-CTX-2; CTX2A2; 52-epi-54-deoxyCTX; P-CTX-3; CTX2b2; 54-deoxyCTX; | P- CTX-1095 |
| P-CTX-1; CTX-1b | P- CTX-1111 |
| C-CTX-1127 | C- CTX-1127 |
| **C-CTX-1; C-CTX-2, 56-epi-C-CTX-1; I-CTX-1; I-CTX-2** | **C- CTX-1141**<br>**I- CTX-1141** |
| C-CTX-1143 | C- CTX-1143 |
| C-CTX-1157; I-CTX-3; I-CTX-4 | C- CTX-1157<br>I - CTX-1157 |
| C-CTX-1159 | C- CTX-1159 |

*The Threat to Human Health*

Humans consume carnivorous reef fish such as jack (*Caranx lugubris*) and barracuda (*Sphyraena barracuda*) which are at the top of the food chain and potentially contain high concentrations of CTX (Table 2). Certain areas in the tropics have been identified as high-risk areas due to their ideal conditions for *Gambierdiscus* blooms and high incidence of CFP (Tester et al., 2014). The US Virgin Islands (USVI) are considered a ciguatera hot spot with the highest incidence of CFP in the Caribbean (Tester et al., 2013). Tourist hotels and restaurants in ciguatera endemic areas such as the USVI import their seafood to avoid being associated with CFP (Dickey, 2008), while local fisheries are generally managed by word-of-mouth warnings (Tester et al., 2010). Local anglers track trends in which species are toxic, which areas are toxic, and when blooms occur (Tester

et al., 2014). An open database of fish species implicated in CFP and the location of incidents can be found online at http://www.fishbase.org/Topic/List.php?group=27. The abundance of toxic dinoflagellates, the toxicity of fish, and the incidence of CFP, all increase as water temperature increases (Tester et al., 2010). Changing environmental conditions and increased seafood-shipping capabilities (Tester et al., 2010) are causes for concern because local management may not be adequate to protect human health in these areas. The U.S Food and Drug Administration (FDA) listed unsafe fish (Table 2) that have been implicated in CFP (directly caused a human illness) and fish that have contained potentially harmful concentrations of CTX (tested to have high concentrations) in July 2013 (US Food and Drug Administration, 2014). To ensure public safety, the FDA has established guidance levels cautioning people to avoid consuming fish with C-CTX-1 concentration higher than 0.1 μg C-CTX-1 equivalents per kilogram of fish tissue (US Food and Drug Administration, 2014). Due to lack of available standards, P-CTX and brevetoxin standards are often used as surrogate standards (Hossen et al., 2015). The European Food Safety Authority established a limit of 0.01 μg P-CTX-1 equivalents per kilogram of fish tissue regardless of locality (Hossen et al., 2015). In Guadeloupe, French West Indies, analysis of fish implicated in CFP incidents from 2010-2012 found that fish with as low as 0.022 μg P-CTX-1 equivalents per kilogram of fish tissue have caused illness (Hossen et al., 2015). The development of an efficient highly sensitive analysis method is crucial for detecting all congeners at clinically relevant levels. Reliable analytical methods are necessary to monitor fish populations in ciguatera-endemic areas to protect public health.

Table 2. Fish implicated in CFP (directly caused a human illness) and fish that have contained unsafe concentrations (tested to have high concentrations) of CTX according to the US Food and Drug Administration (2014).

| Implicated in CFP | Fish with unsafe concentrations of CTXs |
|---|---|
| Barracuda (*Sphyraena* spp.) | Grouper (Family Serranidae) |
| Grouper (*Epinephelus* spp.) | Snapper (Family Lutjanidae and *Symphorus nematophorus*) |
| Gag (*Mycteroperca microlepis*) | Jacks and trevally (Family Carangidae) |
| Scamp (*Mycteroperca phenax*) | Wrasse (*Cheilinus undulatus*) |
| Amberjack (*Seriola dumerili*) | Mackerel (*Scomberomorus* spp.) |
| | Tang (Family Acanthuridae) |
| | Moray eels (Family Muraenidae) |
| | Parrotfish (*Scarus* spp.) |
| | Lionfish (*Pterois volitans* and *Pterois miles*) |

*Detection and Quantification*

As ciguatera fish poisoning is an expanding worldwide issue, various methodologies have been proposed for analysis; methods include bioassays (with animals, tissues, or cells), immunoassays, pharmacological assays, as well as chemical detection using HPLC-UV, and HPLC-MS (Caillaud et al., 2010). The N2a bioassay has been the most widely used and accepted method for analyzing ciguatoxin concentration in fish tissues (Caillaud et al., 2010). This assay was first used to analyze fish tissue for all neurotoxins, but has since been modified by the addition of Ouabain and Veratridine to be ciguatoxin-specific (Dechraoui et al., 2005). Ouabain and Veratridine (O/V) are compounds that block sodium channel pumps causing increased sensitivity to sodium channel activating toxins such as CTXs (Manger et al., 1993; Dickey et al., 1999). The N2a bioassay is not capable of differentiating between ciguatoxin congeners. HPLC-MS/MS analysis was proposed as a method for the detection and confirmation of ciguatoxin structures (Lewis et al., 1994). This method can be affected by the sample matrix and therefore requires clean up steps to reduce matrix suppression of the toxin

signal. Toxin detection in complex matrices such as fish tissue is generally difficult; toxins must be extracted from tissue prior to quantification (Orellana et al., 2015). There is no known way to reliably differentiate between toxic and nontoxic fish without chemical analysis as toxin-containing fish look, taste, and smell the same as nontoxic fish (Lehane and Lewis, 2000). The complexity and diversity of CTXs resulting from fish-specific biotransformations have limited the development of a single accepted extraction method applicable to all species.

Simplified procedures are required for screening large quantities of samples for low concentrations of CTX (Caillaud et al., 2010; Dechraoui et al., 2005). For routine monitoring purposes, a test must be able to determine if the fish sample is safe to eat. To make general precautionary guidelines based on fish species and specific area, chemical analyses must be performed to characterize the toxin profiles by identifying all toxin congeners contributing to toxicity. With C-CTX-1 and C-CTX-2 making up only 60% of the toxin profiles in the Caribbean (Lewis and Jones, 1997), analyses must be able to detect a variety of toxin congeners and degradation products. The Neuro-2a bioassay (N2a) is currently the most commonly used method for quantifying the total toxicity of a sample and High Performance Liquid Chromatography combined with Mass Spectrometry (HPLC-MS/MS) is necessary to confirm toxins present and identify congeners that are contributing to toxicity (Caillaud et al., 2010). To perform these analyses, CTXs must first be extracted from fish tissue.

*Extraction*

CTXs must be extracted from the fish tissue prior to being analyzed by the N2a bioassay and/or HPLC-MS/MS to prevent matrix effects. Matrix effects are defined as

the effects of all components within the sample tissue, other than the analyte of interest, on the analysis being performed; in many instances, matrix effects hinder analysis of ciguatoxins (Wu et al., 2011). In ciguatoxin analysis, proteins and phospholipids in the fish tissue can cause matrix effects (Meyer et al., 2015). Each extraction step is an attempt to remove interfering compounds without reducing the amount of toxin in the sample; these steps can be costly and time consuming. The variability in structure of each toxin congener makes the extraction of all CTXs difficult (Wu et al., 2011). Numerous extraction methods have been developed (Wu et al., 2011; Lewis et al., 2009; Dechraoui et al., 2005; Manger et al., 1993), yet there is currently no official AOAC (Association of Official Agricultural Chemists) method established (Tester et al., 2013). Sample extraction protocols are often chosen specifically based on matrix complexity and the grade of purity required for the quantification analyses being performed (Caillaud et al., 2010). Each protocol implements initial extraction steps followed by either Liquid-Liquid (L-L) solvent partitioning, Solid Phase Extraction (SPE) or a combination of the two techniques (Figure 2). Comparison of data obtained from differing methods is difficult. A standard procedure is needed so that analyses of CTX occurrence and concentration are both accurate and reproducible.

This study addressed the efficiency of different purification steps. Initial steps (Figure 2) consisting of centrifugation and hexane partitioning to remove unwanted fatty acids have been used by most CTX researchers (Caillaud et al., 2010). Acetone extraction is used first to extract all lipophilic compounds including CTXs from fish tissue (Caillaud et al., 2010). Hexane partitioning is then used to remove excessive fatty acids from the extract prior to additional purification steps (Figure 2).

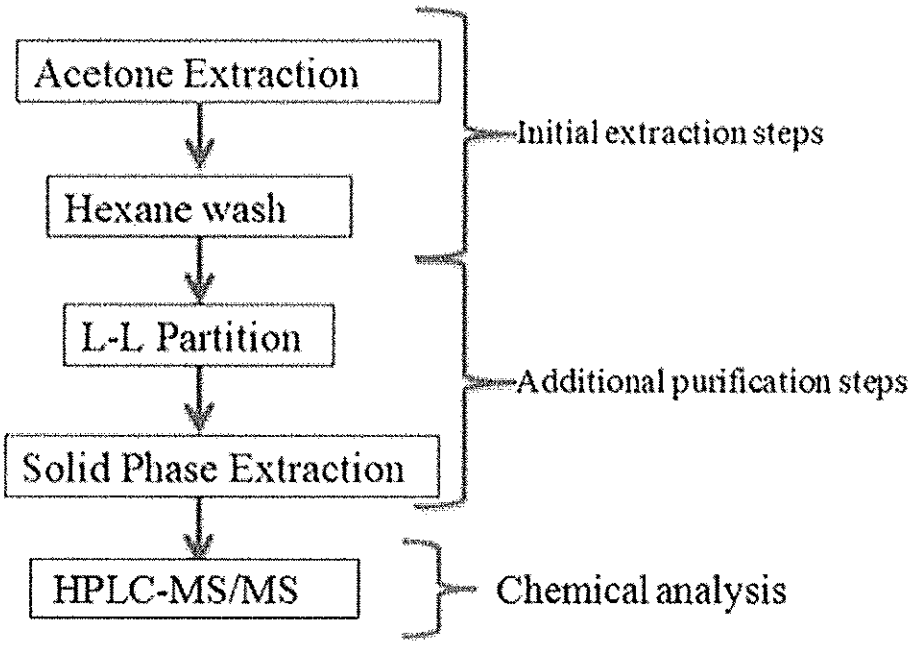


Figure 2. General steps for extraction of CTXs from fish tissue prior to HPLC-MS/MS analysis.

Additional clean up steps with L-L solvent partitioning and SPE are required for chemical analysis. To determine the most effective methods, two optimizations were analyzed. The first optimization compared solvents used in L-L partitioning and the second compared effectiveness of different SPE cartridges. This research also assessed efficiency of a new dichloromethane-only extraction method in place of both initial extraction steps and additional purification steps; this method could simplify the extraction process and reduce the amount of time required for each analysis. Different CTX extraction methods were compared and the data was used to make recommendations to improve published procedures.

*Liquid-Liquid Solvent Partitioning*

L-L partitioning relies on the principle that "like dissolves like" therefore if two

dissimilar liquids are mixed, the analyte of interest will preferentially dissolve in one liquid resulting in a more pure sample. The first optimization compared dichloromethane-only extraction to initial extraction steps followed by solvent partitioning with either chloroform, dichloromethane, or diethyl-ether, to determine which yielded the highest CTX recovery. Chloroform solvent partitioning has been previously described in CTX rapid extraction protocols (Lewis et al., 2009; Stewart et al., 2010), and has been used by the FDA for extraction of CTX from fish tissue (Robertson et al., 2014; Dickey and Plakas, 2010). Diethyl-ether partitioning has been used in recent ciguatoxin extractions in the Caribbean (Soliño et al., 2015) and dichloromethane partitioning has been used to extract toxins from various sample matrices including extracting CTX from *Gambierdiscus* cultures (Rhodes et al., 2010; Chinain et al., 2010; Bienfang et al., 2011).

*Solid Phase Extraction*

SPE is a technique used to separate and purify compounds of interest by passing a mobile liquid phase containing the analytes, over a stationary solid phase. The analytes are dissolved in a liquid and allowed to pass through the stationary phase, which selectively partitions the analytes of interest. SPE cartridges retain the CTX absorbed in the column as phospholipids and fatty acids are washed away (Dickey and Plakas, 2010). Various $C_{18}$, $NH_2$, and silica cartridges can be used; silica SPE is commonly used by the FDA to clean up sample extracts prior to quantification with HPLC-MS/MS (Abraham et al., 2012; Robertson et al., 2014). Further cleaning prior to analysis can be done with aminopropyl ($NH_2$) SPE (Soliño et al., 2015) or $C_{18}$ SPE (Lewis et al., 2009; Stewart et al., 2010; Dickey and Plakas, 2010). The addition of a HILIC SPE cartridge after

extraction with a $C_{18}$ SPE improved efficiency of extraction of P-CTX-1, 2, and 3 from fish tissue (Meyer et al., 2015).

*N2a Cell-Based Bioassay*

Cell-based bioassays are used to study the effects of toxins on cultured cells containing specific receptors affected by the toxin being tested (Cañete and Diogène, 2008). Ciguatoxins are potent sodium channel activating neurotoxins, which bind to site five of the voltage-dependent sodium channel alpha subunit receptor (Dechraoui et al., 2005). The Neuro-2a bioassay measures the cell viability of cultured mouse neuroblastoma cells after exposure to Ouabain, Veratridine (O/V), and sample extracts (Manger et al., 1993). Metabolic activity of cells is reduced by the combination of sodium channel activating toxins (such as ciguatoxins) with O/V. The N2a bioassay is known for its sensitivity and reproducibility (Dechraoui et al., 2005) and is commonly used for detecting CTX in fish samples (Dickey and Plakas, 2010; Wu et al., 2011) at subpicogram levels (Abraham et al., 2012).

*HPLC-MS/MS*

High Performance Liquid Chromatography combined with tandem Mass Spectrometry (HPLC-MS/MS) is a chemical separation technique used to separate compounds by polarity and identify compounds by their specific mass (Roeder et al., 2010). This analysis has been used to identify and quantify concentrations of CTX in fish samples at 4 ng $g^{-1}$ concentrations (Solino et al., 2015; Robertson et al., 2014; Dickey et al., 2008).

Table 3. Mass to charge ratios of CTX congeners and potential fragment ions. Compounds are arranged by source (geographic location) and by mass.

| Location $[M+H]^+$ | (m/z) | $[+H]^+$ | $[+NH4]^+$ | $[+Na]^+$ | $[+K]^+$ | $[+H-H_2O]^+$ | $[+H-2H_2O]^+$ | $[+H-3H_2O]^+$ |
|---|---|---|---|---|---|---|---|---|
| P-1023 | 1022.6 | 1023.6 | 1040.6 | 1045.6 | 1061.6 | 1005.6 | 987.6 | 969.6 |
| P-1039 | 1038.55 | 1039.55 | 1056.55 | 1061.55 | 1077.55 | 1021.5 | 1003.5 | 985.5 |
| P-1041 | 1040.6 | 1041.6 | 1058.6 | 1063.6 | 1079.6 | 1023.6 | 1005.6 | 987.6 |
| P-1055 | 1054.56 | 1055.56 | 1072.56 | 1077.56 | 1093.56 | 1023.6 | 1005.6 | 987.6 |
| P-1057 | 1056.6 | 1057.6 | 1074.6 | 1079.6 | 1095.6 | 1039.6 | 1021.6 | 1003.6 |
| P-1061 | 1060.58 | 1061.58 | 1078.576 | 1083.58 | 1099.576 | 1043.6 | 1025.6 | 1007.6 |
| P-1095 | 1094.58 | 1095.58 | 1112.58 | 1117.58 | 1133.58 | 1077.5 | 1059.5 | 1041.5 |
| P-1111 | 1110.58 | 1111.58 | 1128.61 | 1133.58 | 1149.576 | 1093.6 | 1075.6 | 1057.6 |
| C-1127 | 1126.6 | 1127.6 | 1144.6 | 1149.6 | 1165.6 | 1109.6 | 1091.6 | 1073.6 |
| C-1141 | 1140.62 | 1141.62 | 1158.62 | 1163.62 | 1179.62 | 1123.6 | 1105.6 | 1087.6 |
| C-1143 | 1142.6 | 1143.6 | 1160.6 | 1165.6 | 1181.6 | 1125.6 | 1107.6 | 1089.6 |
| C-1157 | 1156.6 | 1157.6 | 1174.6 | 1179.6 | 1195.6 | 1139.6 | 1121.6 | 1103.6 |
| C-1159 | 1158.6 | 1159.6 | 1176.6 | 1181.6 | 1197.6 | 1141.6 | 1123.6 | 1105.6 |

HPLC-MS/MS is necessary to confirm CTX's presence and identify different congener's chemical structures in fish samples (Lewis et al., 2009). HPLC-MS/MS can be used in Multiple Reaction Monitoring (MRM) mode to look for specific ions molecular weights and typical fragment ions that form from electrospray ionization. The characteristic breakdown of ciguatoxin involves the formation of an ammonium adduct ion followed by the loss of up to five waters (Table 3). The use of a more sensitive MS system is required for detection of toxins at low levels (Wu et al., 2011). UPLC-Time of Flight (TOF) offers a 10-fold higher sensitivity then MS/MS, allowing for the detection and quantification of unknown compounds (Qi et al., 2009).

*Fish Selection*

Invasive lionfish (*Pterois volitans)* were chosen for this research because of their abundance in an area previously known as a high-risk area for ciguatera. Lionfish were introduced off the Florida coast in the 1980s (Green et al., 2012) and have rapidly spread

throughout the Caribbean with devastating effects on biodiversity (Arias-González et al., 2011). These invasive fish are quickly replacing meso-predators in many reef fish communities. Lionfish have an extremely diverse diet and consume many invertebrate and fish species on the reef (Green et al., 2012), potentially resulting in a diverse assembly of CTX congeners in their tissue (Lewis and Holmes, 1993). About 40% of lionfish in the USVI contained detectable levels of CTX with 12% above FDA guidance levels of 0.1 ppb C-CTX equivalents (Robertson et al., 2014).

# MATERIALS AND METHODS

A total of 26 lionfish (*Pterois volitans*) were collected by spear fishing from reefs surrounding the USVI. Fish sizes, sector locations and collection depths were recorded (Table 4). Muscle filets were removed and sent frozen to Texas A&M University Corpus Christi, where they were kept at -20 °C or lower until analysis. A fillet of channel catfish was obtained from a local supermarket to serve as a negative control.

*Preliminary Toxin Quantification (Wet Tissue Samples from Individual Fish)*

Ten-gram subsamples from each fish were extracted according to a commonly used method. This method was previously used for extraction of Caribbean CTX from lionfish tissue (Solino et al., 2015) and is the original method for this research (Figure 3). Tissue subsamples (10 g) were heated in a water bath at 70 $^{0}$C for 10 minutes to denature interfering fish proteins. Samples were then homogenized in acetone (2 ml $g^{-1}$ Tissue Extract [TE]) using mortar and pestle, and centrifuged at 3000 x g for 10 minutes. The supernatant was removed and the sample pellet was re-extracted with acetone (2 ml $g^{-1}$ TE). Supernatants were pooled, filtered through a 0.7 µm syringe filter (Whatman International Ltd, Maidstone UK), and evaporated under nitrogen; the remaining tissue pellet was discarded. The dried residue was resuspended in MeOH: $H_2O$ (9:1 v/v) and washed twice with n-hexane at 0.5 ml $g^{-1}$ TE. The hyperphases (n-hexane layers) were removed and discarded. The MeOH fractions were pooled, evaporated under nitrogen, reconstituted in EtOH: $H_2O$ (1:3 v/v) (0.5 ml $g^{-1}$ TE), and partitioned twice with diethyl-ether (0.5 ml $g^{-1}$ TE).

Table 4. Summary of site locations (latitude and longitude in decimal degrees), number of fish samples collected, depth ranges (m), and total fish length ranges of lionfish collected from the US Virgin Islands.

| Site location | # of fish | Depth (m) | Fish Length (cm) |
|---|---|---|---|
| 18° 20.769' N, 64° 47.39' W | 4 | 11.8-24 | 11-19 |
| 18° 14.622' N, 64° 50.52' W | 1 | 25.3 | 32 |
| 18° 17.986' N, 64° 54.76' W | 2 | 15.2-19.8 | 11-33 |
| 18° 18.222' N, 65° 0.082' W | 1 | 4.5 | 16 |
| 18° 16.649' N, 64° 53.90' W | 8 | 16.8-21 | 19-35 |
| 18° 21.806' N, 64° 52.25' W | 6 | 16.8 | 25-36 |
| 18° 18.365' N, 64° 57.38' W | 2 | 10.7 | 21-23 |
| 18° 24.008' N, 64° 41.48' W | 3 | 13.1 | 21-24 |

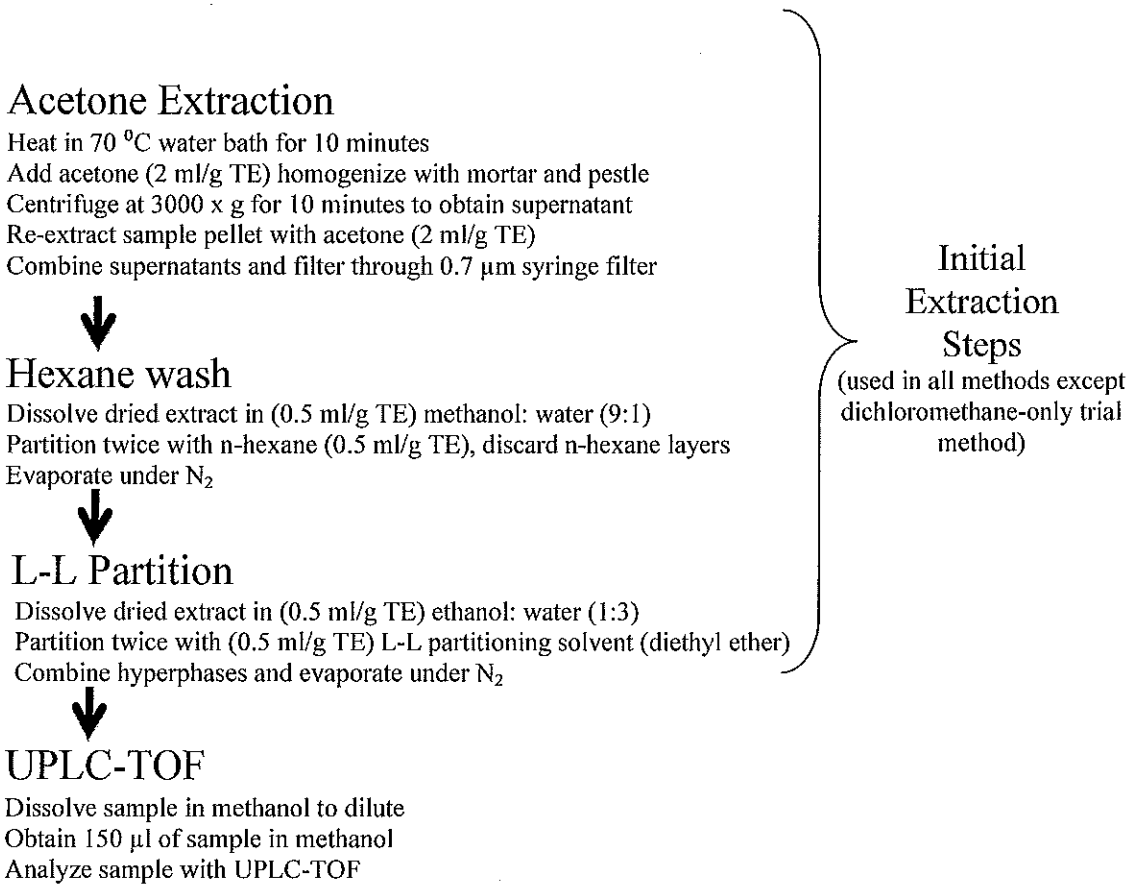


Figure 3. Flowchart detailing steps of original method used for preliminary quantification of 26 individual fish.

The hyperphases containing diethyl-ether were collected, pooled, and dried under nitrogen gas. The cleaned extracts were then reconstituted in 4 ml MeOH and stored at -20 °C until analysis.

*UPLC-TOF*

Methanol aliquots (0.5 ml) from each extracted individual fish sample were filtered through a 0.2 µm syringe filter (Whatman International Ltd, Maidstone UK) into HPLC vials. An Agilent 6200 UPLC-TOF (Agilent Technologies, Wilmington DE, USA) was used for identification of peak areas associated with CTX. A Zorbax RRHD Eclipse Plus $C_{18}$ (2.1 x 100, 1.8 µm) column (Agilent Technologies, Wilmington DE, USA) was used with a flow rate set at 0.4 ml $min^{-1}$, a 10 µl injection volume, and a 15-min linear gradient. Solvent A consisted of deionized, filtered water, mobile phase B consisted of acetonitrile: water (95:5 v/v), and both solvents contained 2 mM ammonium acetate + 0.01 % acetic acid (LC/MS grade). The linear gradient was initially 30% B, increasing to 95% B over 8 minutes and then was held at 95% B for 7 minutes before returning to 30% B. Stop time was set at 15.1 minutes. There was a 4-minute equilibration time between samples.

The mass spectrometer system was set to total ion acquisition mode to detect all CTX molecular weights. A database was created with all structurally identified ciguatoxins and the Agilent Mass Hunter Data Acquisition program (version B.04) was used to obtain qualitative and quantitative information about the samples. Ciguatoxin congeners were identified by their retention time and mass-to-charge ratio (*m/z*). Relative toxin concentration was estimated by combined toxin peak areas obtained from all

ciguatoxins detected by the TOF mass spectrometer. Toxicity ranking (1-10) was determined for each individual fish by taking the sum of all the CTX peak areas and dividing it by 10000.

*L-L Solvent Partitioning Optimization (Wet Tissue Aggregate)*

For method optimization, 10 g muscle strips were removed from each of the 10 fish fillets having greatest CTX area counts determined by UPLC-TOF. Strips were pooled and homogenized in a blender to produce an aggregate sample (100 g). Twenty-four (1 g) replicates were removed from the aggregate sample for the first optimization; the remaining aggregate sample was frozen for later use. Six replicates were used for the dichloromethane-only trial method (Figure 4) and 18 replicates underwent initial extraction steps (Figure 3) before being partitioned with one of the L-L solvents being tested (Figure 5).

*Dichloromethane-only Extraction (Trial Method)*

Replicate 1 g samples (n=6) were homogenized in dichloromethane (0.5 ml $g^{-1}$ TE) using mortar and pestle and placed into individual scintillation vials. The mortar and pestle was rinsed twice with 0.5 ml dichloromethane between samples and the rinse solvent was added to each vial. Homogenized tissue was left in a capped vial to extract for 24 hours at room temperature. Samples were then centrifuged at 3000 x g for 10 minutes, the supernatant was transferred to a new scintillation vial and allowed to evaporate for 24 hours. The tissue pellet was discarded.

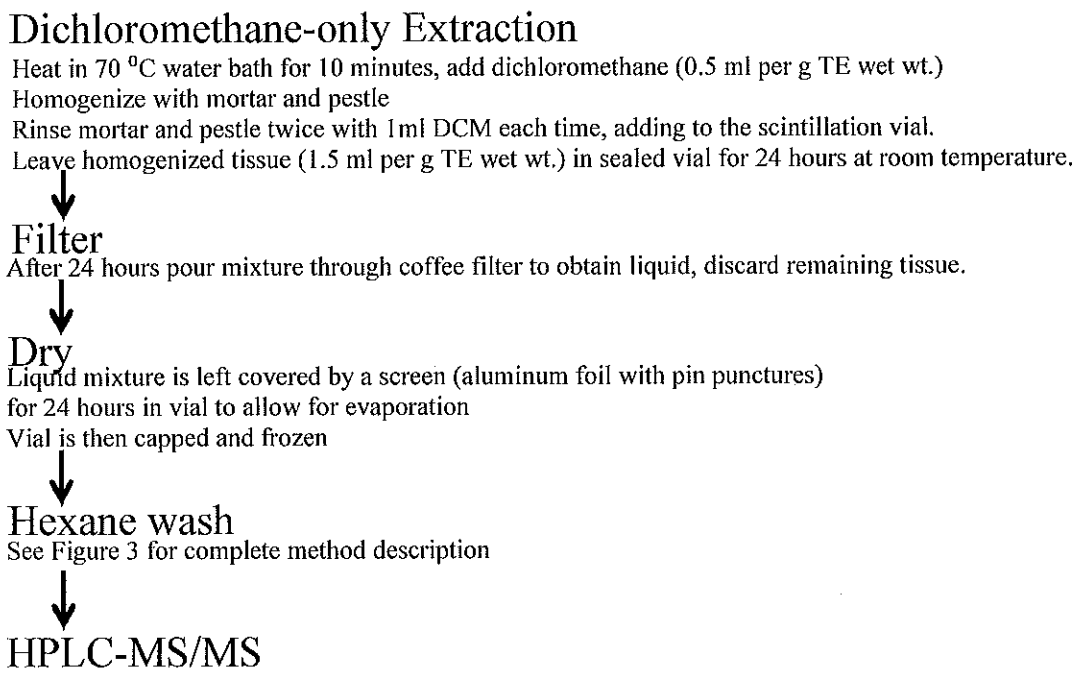


Figure 4. Flowchart detailing dichloromethane-only trial method.

The dried residue was resuspended in 1 ml MeOH: $H_2O$ (8:2 v/v), and then partitioned twice with 1 ml n-hexane. The hyperphase (n-hexane layer) was discarded and the CTX in MeOH: $H_2O$ was stored at -20 °C until analysis.

*Initial Extraction Steps (Acetone and Hexane)*

Initial steps consisting of heating, extracting in acetone, and washing with hexane were repeated for all methods tested. Replicate 1 g samples (n=18) were processed as previously described (Figure 3).

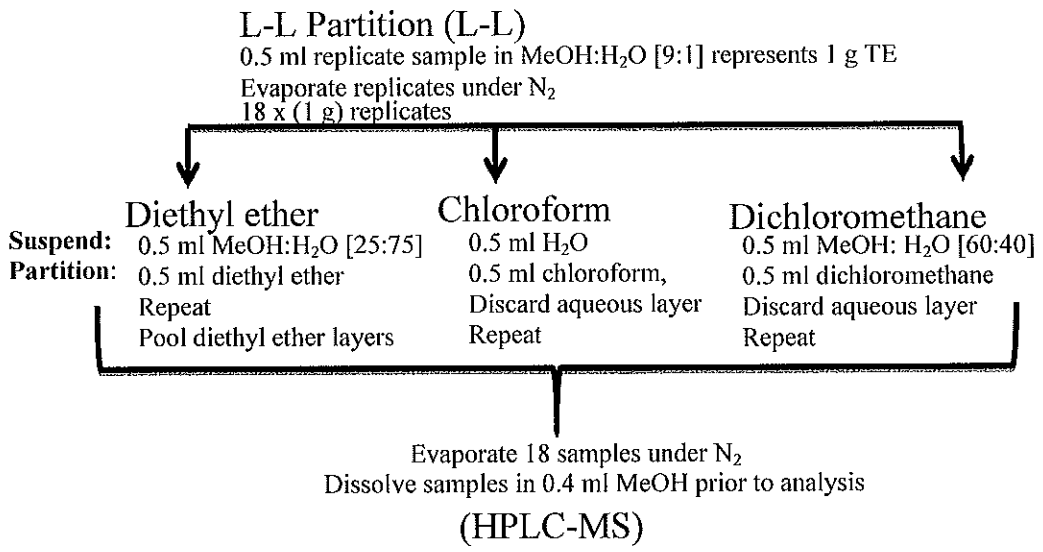


Figure 5. Flowchart detailing solvent optimization.

*Solvent Optimization (Chloroform, Dichloromethane, Diethyl-ether)*

For the L-L optimization, replicates (n=18) from 1 g TE were partitioned with one of the following solvents: diethyl-ether, chloroform, or dichloromethane (Figure 5), steps 1, 2, and 3 were performed simultaneously.

(1) Diethyl-ether samples (n=6) were suspended in 0.5 ml EtOH: $H_2O$ (1:3 v/v) and partitioned twice with 0.5 ml diethyl-ether. Hyperphases (diethyl-ether layers) were collected and pooled in new 15 ml centrifuge tube and then evaporated under nitrogen gas. The lower aqueous layer was discarded.

(2) Chloroform samples (n=6) were suspended in 0.5 ml water and partitioned twice with 0.5 ml chloroform. Aqueous hyperphases were discarded and the remaining chloroform layer was collected.

(3) Dichloromethane samples (n=6) were suspended in (0.5 ml $g^{-1}$ TE) MeOH: $H_2O$ (2:3 v/v) and partitioned twice with (0.5 ml $g^{-1}$ TE) dichloromethane. Aqueous

hyperphases were discarded and the remaining dichloromethane layer was collected.

*UPLC-TOF*

All sample extracts (each representing 1 g TE) from dichloromethane-only extraction (n=6), diethyl-ether L-L partitioning (n=6), chloroform L-L partitioning (n=6), and dichloromethane L-L partitioning (n=6) were dried under nitrogen, reconstituted in 0.4 ml of MeOH, and filtered through a 0.2 μm syringe filter into HPLC vials. A proprietary solvent modifier additive combination is currently being developed in this lab and was used for all further analysis. Solvent A consisted of deionized, filtered water, mobile phase B consisted of acetonitrile: water (95:5 v/v), and both solvents contained proprietary solvent modifier additive combination NaRc-A. The linear gradient was initially 30% B, increasing to 95% B over 8 minutes and then was held at 95% B for 2 minutes before returning to initial conditions. There was a 4-minute post-time run in between each sample. UPLC-TOF analysis was performed in positive ion mode as previously described. Ciguatoxin congeners were identified by their retention time (Table 5) and mass to charge ratio (*m/z*). Caribbean CTX area counts were quantified in aggregate samples by combining peak areas of C-CTX-2, C-CTX-1, and up to three water loss ions obtained from the UPLC-TOF data acquisition software.

*P-CTX-1 Standards*

C-CTX standards are not currently available and a P-CTX-1 standard was used as a surrogate.

Table 5. Retention times of Caribbean ciguatoxin ions (C-CTX-1, C-CTX-2 and up to three water loss ions) using UPLC-TOF.

| Ion | Retention time |
|---|---|
| Parent ion | 5.5-5.8 |
| 1 water loss | 5.3-5.5 |
| 2 water loss | 5.1-5.3 |
| 3 water loss | 3.1-3.3 |

Standards are necessary to quantify concentrations of toxin to convert from peak area (UPLC-TOF) or increased LDH activity (N2a bioassay) to toxin concentration. P-CTX-1 standards were obtained from R. Lewis at the University of Queensland, Australia. P-CTX-1 calibration standards ranging in concentrations from 0.1-2 ng $ml^{-1}$ were injected directly into the UPLC-TOF to generate a linear standard curve from peak area measurements. Calibration standards were stored at -20 °C until used in the N2a bioassay.

*L-L Solvent Partitioning Optimization (Dry Tissue Aggregate)*

In an attempt to improve the homogeneity of the aggregate sample, an additional 5 g aliquot was removed from each of the 10 fish identified as containing highest relative toxin concentrations. This tissue was added to the toxin aggregate to create a new toxin aggregate sample. The new aggregate was homogenized in a blender, freeze dried, homogenized with mortar and pestle, and then ground in a coffee grinder to ensure homogeneity of the sample. Wet weight tissue dried to approximately 0.2 g of lyophilized tissue per gram wet weight. Eighty 0.2 g dry replicates were weighed out and placed into individual 15 ml centrifuge tubes. Fifteen of these replicates were extracted by previously described initial extraction steps (Figure 3) followed by L-L solvent partitioning (Figure 5) with one of the three solvents being tested (chloroform, diethyl-ether, or

dichloromethane). The remaining 65 replicates underwent initial extraction steps and then were stored at -80 $^{o}$C until the SPE optimization. Five replicates were extracted with each L-L partitioning solvent and analyzed by UPLC-TOF as previously described.

*SPE Optimization (Dry Tissue Aggregate)*

Replicate (0.2 g dry wt.) samples (n=65) were used for the SPE cartridge optimization. Protocols for SPE were determined by following previously published CTX extraction methods (Agilent products) or the manufacturer's recommendations (Phenomenex and Waters products). A total of five replicate analyses were made for each SPE method and all analyses were done concurrently (Figure 6).

For all Phenomenex cartridges (Phenomenex LLC, Torrance, CA, USA) 33 μm, 30 mg / 1 mL, cartridges (Strata™-X Polymeric Reversed Phase and Strata™-X-CW Polymeric Weak Cation), the sample was homogenized in Milli-Q water (2 ml $g^{-1}$ TE), the cartridge was conditioned with 0.5 ml of acetonitrile and equilibrated with 0.5 ml of Milli-Q water prior to loading of the sample. The cartridge was then washed with 0.5 ml of Milli-Q water and 0.5 ml MeOH and dried under vacuum for 3 minutes before the sample was eluted with 0.5 ml of formic acid: acetonitrile (5:95 v/v). The condition, wash and elution volumes were doubled for all Phenomenex 60 mg cartridges.

Manufacturer's recommended protocols were followed for all Waters cartridges (Waters Corporation, Milford, MA, USA). For the Oasis HLB 3cc cartridges, the sample was dissolved in 1 ml of water, the cartridge was conditioned with 1 ml of MeOH and equilibrated with 1 ml of Milli-Q water before the sample was loaded. The cartridge was then washed with 1 ml MeOH: $H_2O$ (5:95 v/v) before being eluted with 1 ml of MeOH.

For the Waters Sep Pak $C_{18}$, the sample was dissolved in 6 ml of MeOH: $H_2O$ (6:4 v/v). The cartridge was conditioned with 6 ml of MeOH and equilibrated with 6 ml of MeOH: $H_2O$ (1:1 v/v) before the sample was loaded. The cartridge was then washed with 6 ml MeOH: $H_2O$ (65:35 v/v) and eluted with 12 ml MeOH: $H_2O$ (8:2 v/v). The sample was then evaporated under nitrogen and resuspended in 100 µl MeOH and a 50 µl aliquot was removed prior to the next SPE. The extract remaining after the $C_{18}$ SPE underwent an additional SPE with either Water Si Sep Pak or Thermo Scientific HILIC (Figure 6).

Agilent cartridges (Agilent Technologies, Wilmington DE, USA) have been used previously in CTX extractions to further purify extracts. These samples were processed following published protocols (Dechraoui et al., 2005; Dickey, 2008; Abraham et al., 2012; Robertson et al., 2014; Soliño et al., 2015). The samples were extracted with chloroform L-L partitioning as described previously (Figure 5) before being further processed using an Agilent 500 mg 3ml Bond Elut 40 µm Straight Barrel Cartridge ($NH_2$ or SI)

The two $NH_2$ cartridges (Waters Sep Pak and Agilent Bond Elut) were processed the same; the sample was dissolved in 100 µl of chloroform, the cartridge was conditioned with one column volume of chloroform, and the sample was loaded. The sample container was then rinsed 3 times with 100 µl of chloroform. The column was then washed with one column volume of chloroform before being eluted with one column volume of chloroform: isopropanol (2:1 v/v).

Two of the silica cartridges (Waters Sep Pak Silica Plus and Agilent Bond Elut SI) were processed the same; first, the sample was dissolved in 2 ml of chloroform. The cartridge was conditioned with one column volume MeOH: $H_2O$ (95:5 v/v) and one

column volume of MeOH before being equilibrated with one column volume of chloroform. The sample was then loaded and the column was washed with five column volumes of chloroform before the sample was eluted with 10 column volumes of 10% MeOH in chloroform.

The HILIC Silica cartridge (Thermo Fisher Scientific, Waltham, MA, USA) was processed according to protocols described for previous CTX analysis (Meyer et al., 2015). The sample was dissolved in 1 ml of acetone with 0.1% formic acid. The cartridge was conditioned with 2 ml of acetone and 4 ml of 5 mM ammonium acetate in water: acetone (5:95 v/v). A collection vial was then placed under the cartridge and the sample was loaded and eluted with 8 ml of 5 mM ammonium acetate in water: acetone (5:95 v/v).

The SPE cartridges designed to remove excess lipids (Phenomenex Phree Phospholipid removal 1 ml and Agilent Bond elut QuEChERS dSPE EMR-Lipid Enhanced Matrix Removal) were used following manufacturers recommendations. For QuEChERS replicates, the sample was dissolved in 5 ml acetonitrile with 1% acetic acid. Five milliliters of water was added to the EMR-lipid dSPE tube and then the sample was added and vortexed for 2 minutes before being centrifuged at 3000 rpm for 3 minutes. The supernatant was then transferred to a 15 ml EMR-Lipid polish tube and it was vortexed for an additional 1 minutes before being centrifuged at 3000 rpm for 3 minutes. The upper acetonitrile layer was then collected. For Phree Phospholipid removal replicates, the sample was dissolved in 200 µl of water and then was loaded and eluted with 0.8 ml MeOH with 0.1% formic acid.

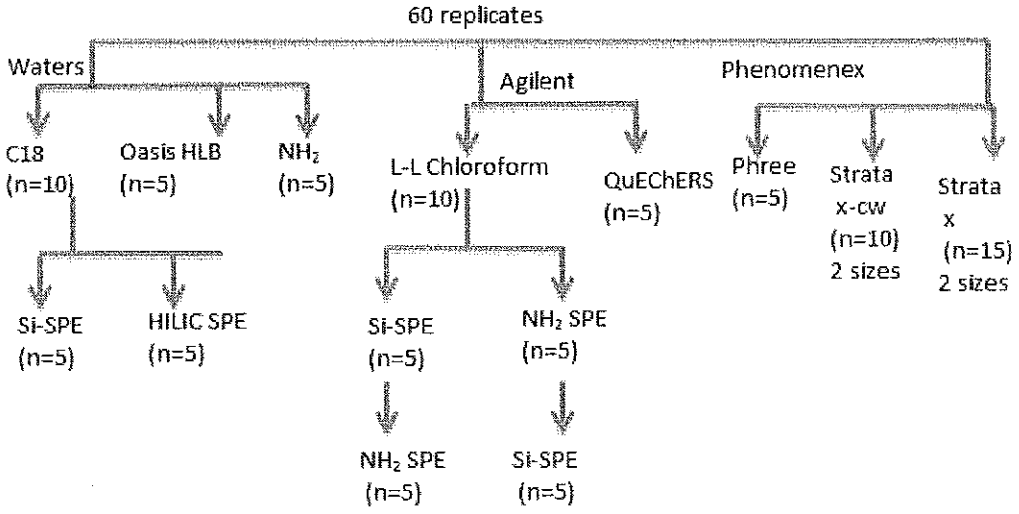


Figure 6. Flowchart detailing sequence for SPE cartridge optimization.

All sample extracts from SPE cartridge optimization were dried under nitrogen, and reconstituted in 0.4 ml of 50 % methanol. Samples were analyzed by UPLC-TOF as previously described. Agilent Mass hunter workstation software was used to determine which SPE cartridge method yielded the highest CTX area counts. The method that yielded greatest area counts was selected as the new optimized method.

*Method Validation and Correlation (Wet Tissue Samples from Individual Fish)*

The optimized sequence of extraction steps was performed on 1 g wet weight samples from each of the 26 individual fish. Aliquots were taken after each extraction step and analyzed with UPLC-TOF. Samples from each individual fish were also extracted with the original extraction method (diethyl-ether L-L solvent partitioning). LDH activity (N2a assay) and peak area (UPLC-TOF) was quantified for each individual fish extracted after new optimized method and standard extraction method.

*N2a Cell Based Bioassay*

The cell-based bioassay was used to correlate toxin concentrations calculated from biological activity (LDH activity) with those calculated from chemical analysis (peak area) after the optimized extraction method was performed on the 26 individual fish. N2a (American Type Culture Collection [ATCC] CCL-131) cells were cultured following ATCC guidelines using 5% $CO_2$ at 37 $^0$C. Samples extracted by both the original method (diethyl-ether L-L partitioning) and the new optimized method were assayed in triplicate following previously described protocols (Manger et al., 1993; Dickey et al., 1999). Four calibration standards ranging in concentration from 0.1-2 ng $ml^{-1}$ were assayed and the linear relationship of increased LDH activity was determined. Neuroblastoma cell cultures were grown in T-75 sterile culture flasks with 10 ml of DMEM culture growth media (ATCC) containing 4 mM L-glutamine, 4500 mg $L^{-1}$ glucose, 1 mM sodium pyruvate, and 1500 mg $L^{-1}$ sodium bicarbonate. Media was supplemented with 10% heat inactivated fetal bovine serum (FBS) prior to use. When cultures reached 85%-90% confluence, cells were seeded into the inner 60 wells (200 µl $well^{-1}$) of each 96 well culture plate at a density of ~10,000 cells per well (Dickey et al., 2008). Equal volumes (200 µl) of PBS were pipetted into the outer 36 wells of every plate to prevent edge effects. Plates were then incubated in the same conditions as culture growth for 24 hours. The following day, half of the interior wells were treated with Ouabain and Veratridine (O/V) (Sigma-Aldrich, USA) at concentrations of 1 and 0.1 mM respectively. Samples were diluted in DMEM media to final concentrations of 20 mg $ml^{-1}$ and 10 µl was added to each well (O/V+ and O/V-) in triplicate. Cell viability was measured by the LDH cytotoxicity test. Twenty four hours after treatment, all the media

was removed from the wells by vacuum pipetting and 50 µl of LDH reaction mix was added to each test well and allowed to react for 30 minutes at room temperature in the dark. After the reaction, a color change was visible and 50 µl of LDH stop solution was added to each well. The LDH assay measures cytotoxicity based on the presence of LDH, which is released when the cell membrane breaks down. Cytotoxicity was determined by spectrophotometer readings of absorbance at 490 nm and 680 nm. Positive (P-CTX-1 standard) and negative controls (toxin-free fish extract) were assayed in parallel with samples (Fotakis and Timbrell, 2006).

*Statistics and Data analysis*

Relative toxin concentrations of each individual fish obtained from the preliminary quantification were compared to the fish sizes recorded to determine if a relationship was present. A one way analysis of variance was performed followed by a Tukeys HSD test with a Westfall adjustment (Westfall, 1997). This analysis compared CTX peak areas from aggregate replicates after differing extraction steps to determine if significant differences were present between methods. A Westfall adjustment was used with an alpha of 0.1; if the p.-value was below 0.1, the variation was significant. A paired t-test was used to determine if significant differences were present between the optimized method and the standard method by both analysis (N2a and UPLC-TOF). A paired t-test was chosen because each method (original and optimized) and each analysis (UPLC-TOF and N2a) was performed on all of the 26 individual fish. Pearson's Ordinary correlation analysis was used to determine if CTX values estimated from the N2a bioassay were correlated to analytically derived CTX area counts determined from UPLC-TOF analysis.

Correlations were determined between CTX values obtained after original extraction method and optimized extraction method for both the UPLC-TOF and the N2a bioassay. All statistical analyses were run using R 3.1.3. statistical software. Since at least one factor in each correlation analysis was non-normally distributed, a Spearman's rho rank correlation test and a Kendall's tau correlation test was performed in parallel. A correlation matrix was used to visualize the Pearson's correlation analysis.

# RESULTS

UPLC-TOF analysis of the preliminary toxin extracts from individual fish after diethyl ether L-L partitioning identified detectable peak areas corresponding to CTX in 20 out of the 26 fish. The fish containing the highest relative CTX concentrations were 1, 9, 19, 18, 15, 3, 10, 14, 22, and 5 respectively (Table 6). The channel catfish fillet contained no detected CTX, but did contain several other toxins in low abundance (including microcystins). None of the recorded factors (fish weight, fish length, or depth) correlated with the relative fish toxicity at $p \leq 0.05$.

*L-L Solvent Partitioning Optimization (Wet Tissue Aggregate)*

Combined peak areas for Caribbean ciguatoxins ranged from 0-6000 milliabsorbance units (Figure 7). There were no significant differences in mean CTX area counts between L-L partitioning solvents being tested (Figure 8A). The mean CTX area count from replicates extracted with the dichloromethane-only trial protocol was significantly lower than all other mean CTX area counts ($p.= 0.072$ see Figure 8A).

*P-CTX-1 Standards*

P-CTX-1 calibration standards had a linear relationship for peak area (using UPLC-TOF) and for LDH activity within the concentration range tested (0.2-2 ng $ml^{-1}$) with $R^2$ values of 0.97 and 0.99, respectively (Figure 9). The characteristic ionization of ciguatoxin involves the formation of an ammonium adduct ion followed by the loss of up to five waters; this pattern was detected in the P-CTX-1 standard analyzed with the UPLC-TOF (Figure 10).

Table 6. Location, depth (m), fish length (cm), sample weight (g), and toxicity rank from 26 individual fish. Toxicity is based on UPLC-TOF peak area counts from diethyl-ether extracts. Mean peak areas of known ciguatoxins were combined and divided by 1000 to estimate toxicity rank.

| Fish ID | Location | Depth (m) | Fish length(cm) | Sample weight (g) | Toxicity rank |
|---|---|---|---|---|---|
| 1 | SE | 25.3 | 32 | 158 | 10 |
| 9 | SW | 21.3 | 33 | 138 | 9 |
| 19 | SE | 10.7 | 23 | 68 | 6 |
| 18 | NE | 16.8 | 30 | 95 | 5 |
| 15 | NE | 16.8 | 25 | 70 | 5 |
| 3 | SE | 19.8 | 33 | 129 | 5 |
| 10 | SW | 21.3 | 22 | 63 | 5 |
| 14 | NE | 16.8 | 28 | 86 | 4 |
| 22 | SE | 13.1 | 24 | 61 | 3 |
| 5 | SW | 21.3 | 21 | 61 | 3 |
| 16 | NE | 16.8 | 28 | 83 | 2 |
| 11 | SW | 21.3 | 26 | 63 | 2 |
| 23 | NW | 13.1 | 21 | 60 | 2 |
| 24 | NW | 13.7 | 19 | 79 | 2 |
| 7 | SW | 21.3 | 19 | 53 | 2 |
| 25 | NW | 13.7 | 19 | 79 | 1 |
| 17 | NE | 16.8 | 36 | 147 | 1 |
| 26 | NW | 13.7 | 18 | 36 | 1 |
| 12 | SW | 21.3 | 23 | 49 | 1 |
| 8 | SW | 21.3 | 35 | 127 | 1 |
| 13 | NE | 16.8 | 27 | 140 | 1 |
| 21 | SE | 3.9 | 22 | 61 | 0 |
| 20 | SE | 10.7 | 21 | 65 | 0 |
| 6 | SW | 21.3 | 27 | 60 | 0 |
| 2 | NW | 15.24 | 12 | 30 | 0 |
| 4 | NW | 13.7 | 11 | 22 | 0 |
| C | Grocery store | | 35 | 50 | 0 |

*L-L Solvent Partitioning Optimization (Dry Tissue Aggregate)*

When an analysis of variance was performed on the dry tissue replicates, there were no significant differences in mean CTX area counts between L-L partitioning solvents being tested (Figure 8B). Of the 20 dry replicates analyzed after either no L-L extraction (extracts after initial clean-up) or L-L extraction with one of the solvents being tested (diethyl-ether, chloroform, and dichloromethane), Caribbean ciguatoxins were only detected in 3 of 5 of the dichloromethane replicates.

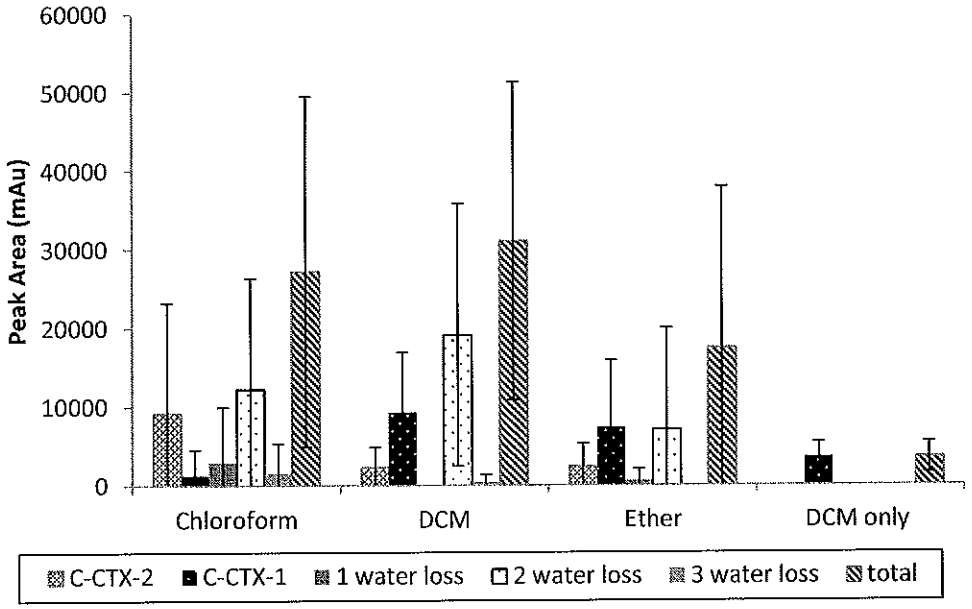


Figure 7. UPLC-TOF analysis of wet aggregate replicates after extraction with either a L-L solvent or dichloromethane-only. Mean peak areas of C-CTX-1, C-CTX-2, and up to three water losses are shown individually and as pooled values. Error bars represent standard deviation.

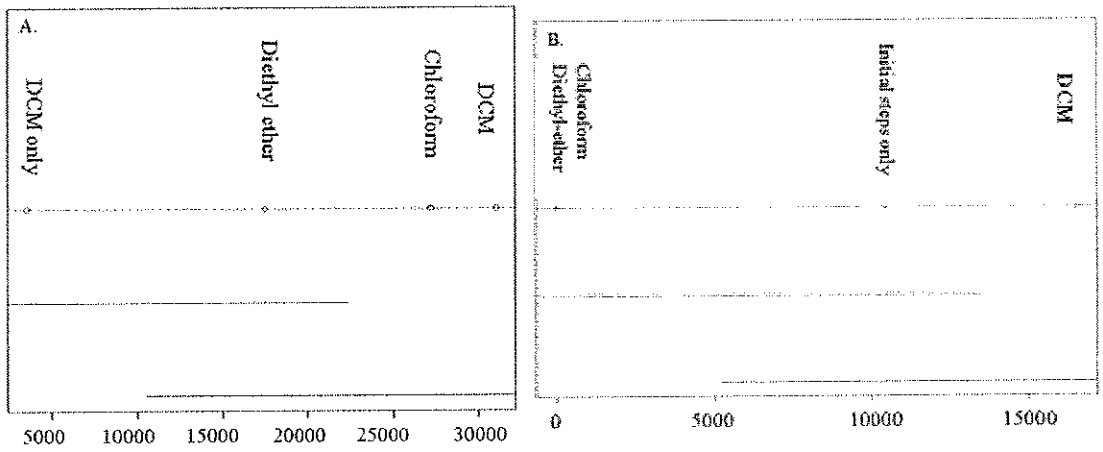


Figure 8. Westfall summary of a one way analysis of variance for peak areas of aggregate replicates extracted with different L-L solvents and analyzed by UPLC-TOF. Means connected by lines are not significantly different. A. Wet aggregate B. Dry aggregate.

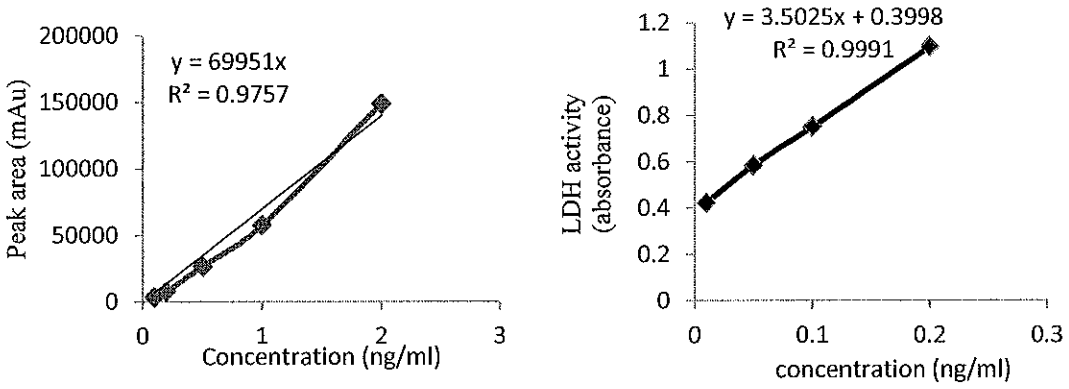


Figure 9. P-CTX-1 calibration standards ranging in concentration from 0.2-2 ng $ml^{-1}$. Linear relationship equation and $R^2$ are on graph.
A. UPLC-TOF peak areas vs. injected toxin concentrations.
B. LDH activity vs. toxin concentration.

Dry replicates extracted by L-L partitioning with either chloroform or diethyl-ether had no detectable ciguatoxin, however those extracted with dichloromethane contained slightly lower areas than the wet tissue replicates (Table 7). During the preliminary individual fish toxin quantification, Caribbean ciguatoxin (C-CTX) peaks were identified in samples by their mass-to-charge ratio (*m/z*) and retention time (Table 5). A representative sample (Fish 1) with clearly defined peaks for C-CTX 2 and C-CTX-1 (Figure 11A) was selected as a reference for further analyses. The EIC chromatogram (Figure 11) from fish 1 was used to identify and confirm ionization adducts in fish extracts that were suspected as C-CTX by the Agilent Mass Hunter Qualitative analysis program but had a score less than 70. Ciguatoxins ionize in a characteristic pattern and the ammonium adduct ion is typically the most abundant ion formed. Presence of the ammonium adduct ion was used as additional confirmation of C-CTX presence. The ammonium adduct ion for C-CTX-1 and C-CTX-2 (both in mass class C-CTX-1141) has a mass-to-charge ratio (*m/z*) of 1158.65 (Figure 11B).

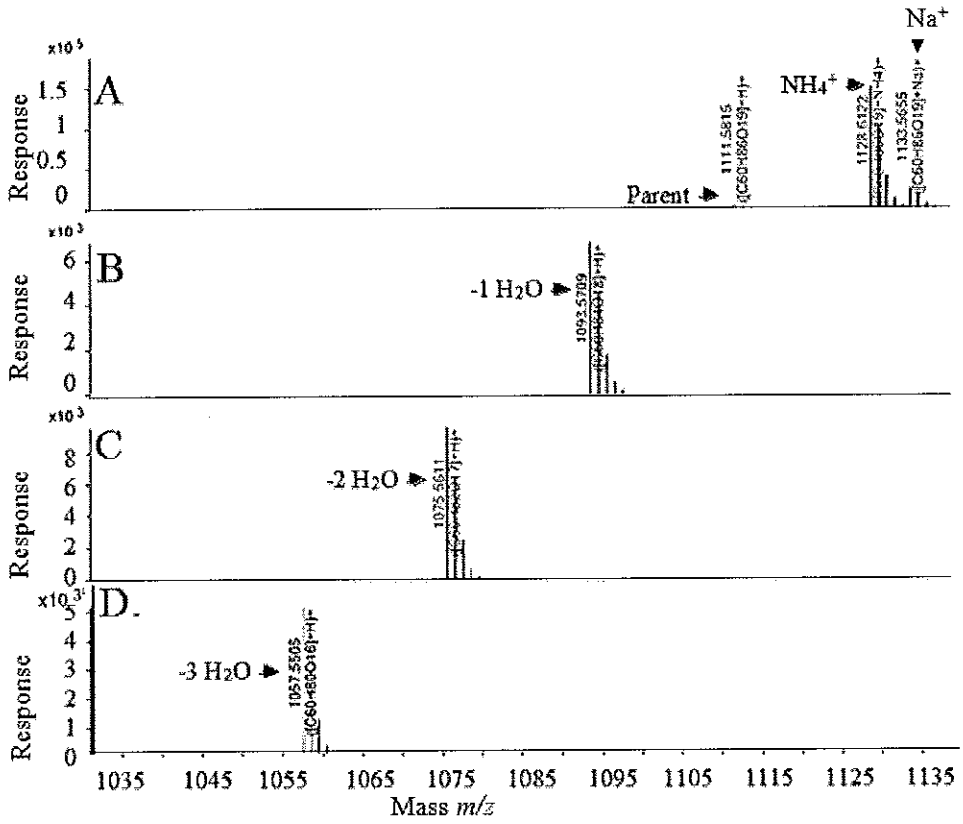


Figure 10. EIC chromatogram showing typical ionization degradation pattern in P-CTX-1.
A.) Parent ion P-CTX-1 *m/z* 1111.58, ammonium adduct ion *m/z* 1128.61, and sodium adduct ion *m/z* 1133.56
B.) Loss of one water *m/z* 1093.57
C.) Loss of two waters *m/z* 1075.56
D.) Loss of three waters *m/z* 1057.

*SPE Optimization (Dry Tissue Aggregate)*

For dry aggregate replicates analyses, L-L solvent (diethyl-ether, and chloroform) extracts did not have detectable areas of CTX without further clean up (Table 7). Further processing of chloroform extracts with amino $NH_2$ SPE cartridge significantly increased detectable CTX area counts. DCM removed CTX equally well in both wet and dry samples.

Table 7. UPLC-TOF analysis of mean peak area for wet and dry aggregate replicates extracted with each L-L solvent for C-CTX-1, C-CTX-2, and up to three water losses (n=5).

| Solvent | Wet | Dry |
|---|---|---|
| Chloroform | 22,302 | none detected = 0 |
| DCM | 20,196 | 16488 |
| Diethyl-ether | 20,466 | none detected = 0 |

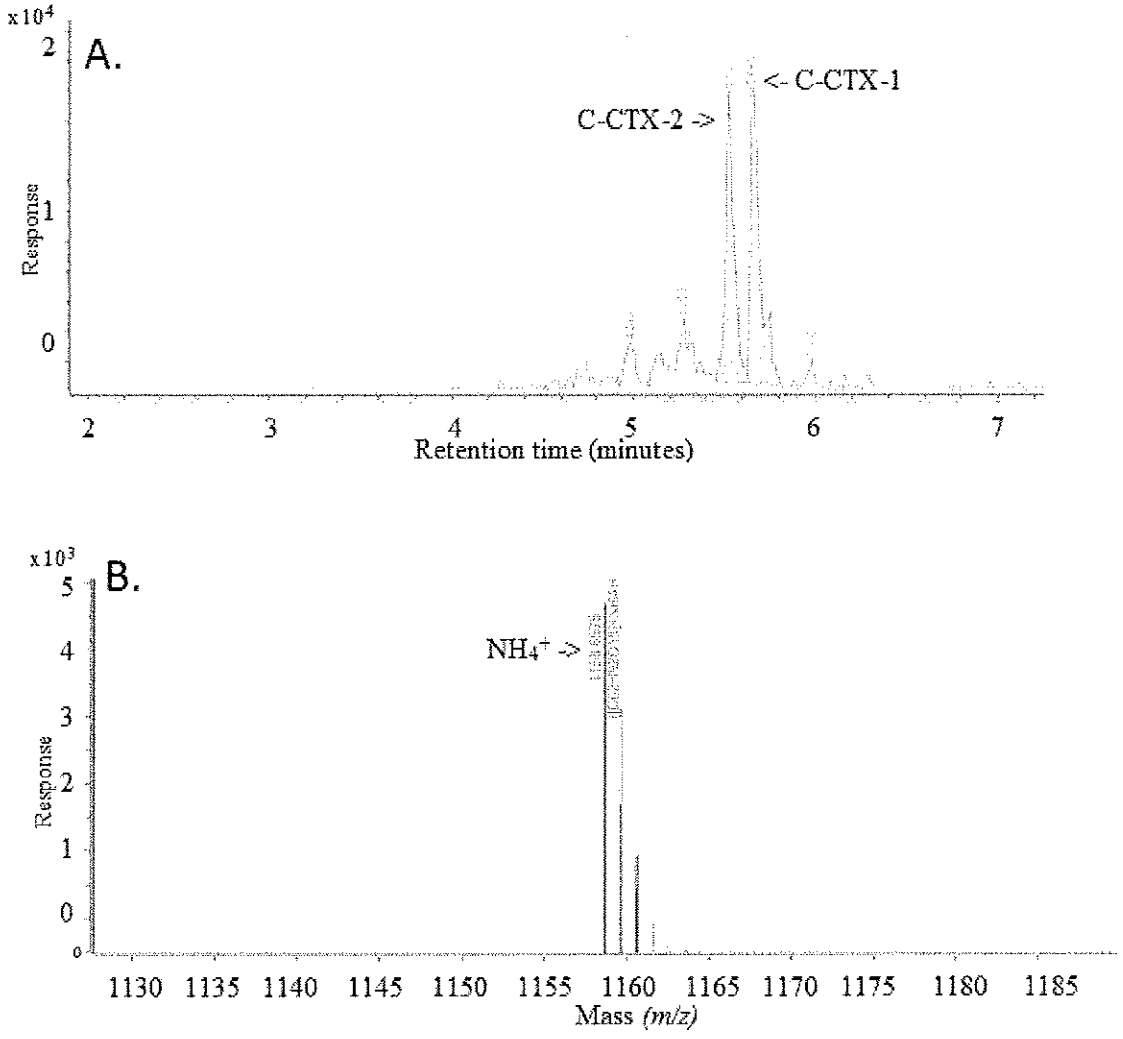


Figure 11. A.UPLC-TOF EIC chromatogram of C-CTX-2 and C-CTX-1 from the highest toxin containing USVI fish (Fish #1) analyzed. B. The primary ion formed by ciguatoxins is the ammonium adduct ion, for toxins in C-CTX-1141 class, the ammonium adduct has a mass-to-charge ratio of *m/z* 1158.65 (shown from fish 1).

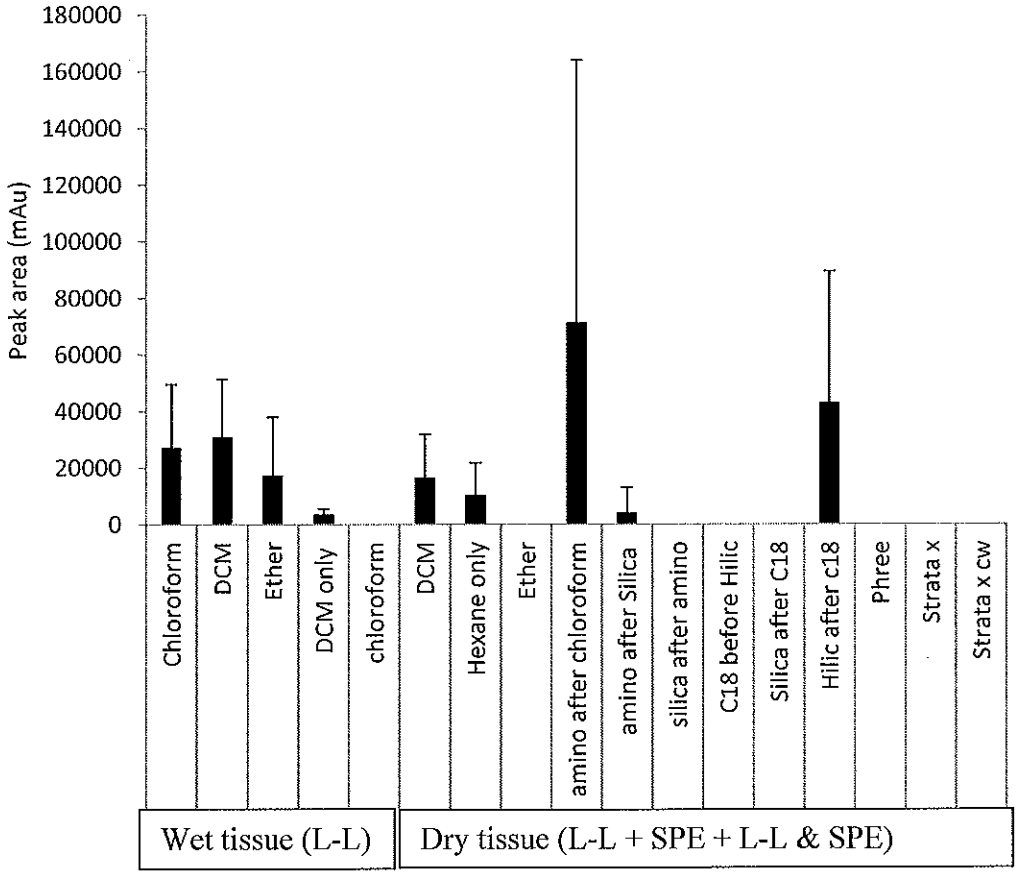


Figure 12. UPLC-TOF analysis of Caribbean CTX mean area counts from replicates after processing with differing extraction methods (L-L, SPE, or a combination of both). Areas represent C-CTX-2, C-CTX-1, ammonium adduct ion, and up to three water loss peaks combined. Error bars represent standard deviation.

Caribbean ciguatoxin area counts were highest in replicates extracted in chloroform prior to SPE with an Agilent 500 mg 3 ml Bond Elut $NH_2$ Straight Barrel Cartridges (Figure 12). For all the dry aggregate replicates tested, Caribbean CTX was only detected in five different extraction treatments sporadically (Figure 13). CTX area counts were detected after the following treatments: DCM L-L partitioning, hexane only initial extraction, amino ($NH_2$) SPE after chloroform L-L partitioning, amino ($NH_2$) SPE after silica (Si) SPE, and HILIC silica SPE after $C_{18}$ SPE. No statistics were computed due to the high incidence of zeros (no detectable CTX peaks) and poor replication.

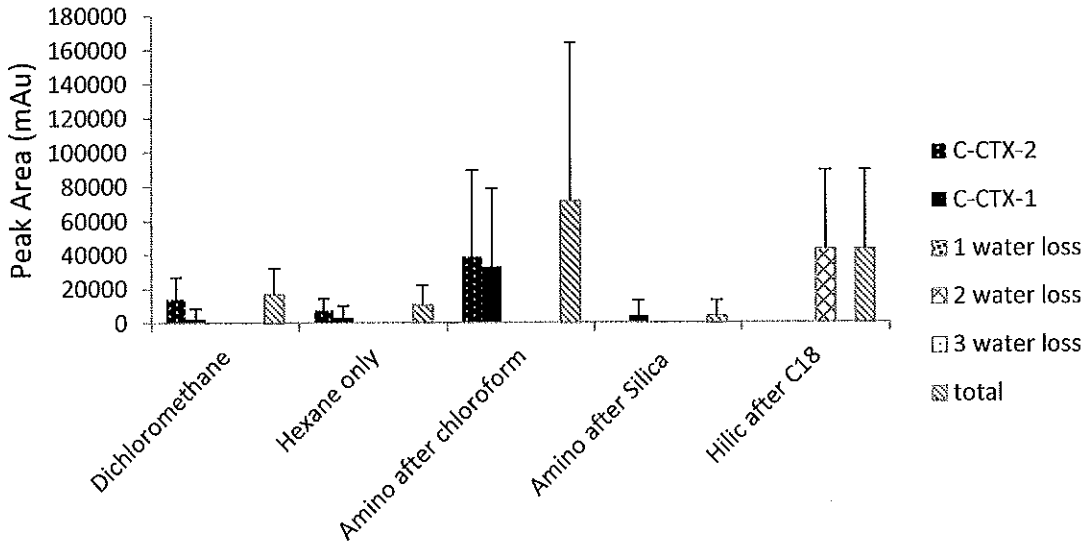


Figure 13. UPLC-TOF area counts of Caribbean ciguatoxin in dry aggregate replicates after different extraction steps. Mean peak areas of C-CTX-1, C-CTX-2, and up to 3 water losses are shown individually and as pooled values. Error bars represent standard deviation.

Table 8. UPLC-TOF mean area counts (standard deviation) of Caribbean ciguatoxin in dry aggregate replicates after different extraction steps.

| | **C-CTX-2** | **C-CTX-1** | **1 water loss** | **2 water loss** | **3 water loss** | **total** |
|---|---|---|---|---|---|---|
| Dichloromethane | 13948 (12853) | 2540 (5679) | 0 | 0 | 0 | 16488(15437) |
| Hexane only | 7342 (6841) | 3076.6(6879) | 0 | 0 | 0 | 10419(11380) |
| Amino after chloroform | 38568.4 (50809) | 32743(46016) | 0 | 0 | 0 | 71311(92658) |
| Amino after Silica | 0 | 4034(9020) | 0 | 0 | 0 | 4034(9021) |
| Hilic after $C_{18}$ | 0 | 0 | 0 | 43027(46264) | 0 | 43027(46264) |

Water loss ions were only present in large concentrations when dry aggregate replicates were processed with $C_{18}$ prior to HILIC silica SPE (Figure 13 and Table 8). Parent ions were not present and only the ions of the ammonium adduct after two water losses were detected in these samples so confirmation of C-CTX was not possible.

*Method Validation and Correlation (Wet Tissue Samples from Individual Fish)*

Caribbean ciguatoxin area counts from UPLC-TOF analyses decreased in all wet individual fish samples after additional processing with SPE (Figure 14). Paired t-tests determined significant differences present in CTX from individual fish samples extracted with chloroform and samples extracted with diethyl-ether. Chloroform extracts had significantly higher CTX than diethyl-ether extracts analyzed by both UPLC-TOF ($p.=0.0016$) and N2a bioassay ($p.=1.467e^{-8}$ ). L-L solvent partitioning with chloroform was the best extraction method for wet samples as it was the most effective at cleaning samples without decreasing CTX area counts (Figure 14).

One problem throughout these experiments was the lack of Caribbean standards. Without a standard present, the TOF was used to scan for masses of interest and peak area was used for quantification. The goal was to express data as ciguatoxin concentrations, but in order to do this, the data needed to be converted from peak area (UPLC-TOF) or increased LDH activity (N2a bioassay) to toxin concentration. The data from 26 individual fish was normalized to the P-CTX standard curve; however, no significant relationship was determined. When area counts and LDH activity values were analyzed (i.e. non-normalized to P-CTX concentration), a significant correlation was present (Table 9 and Figure 15).

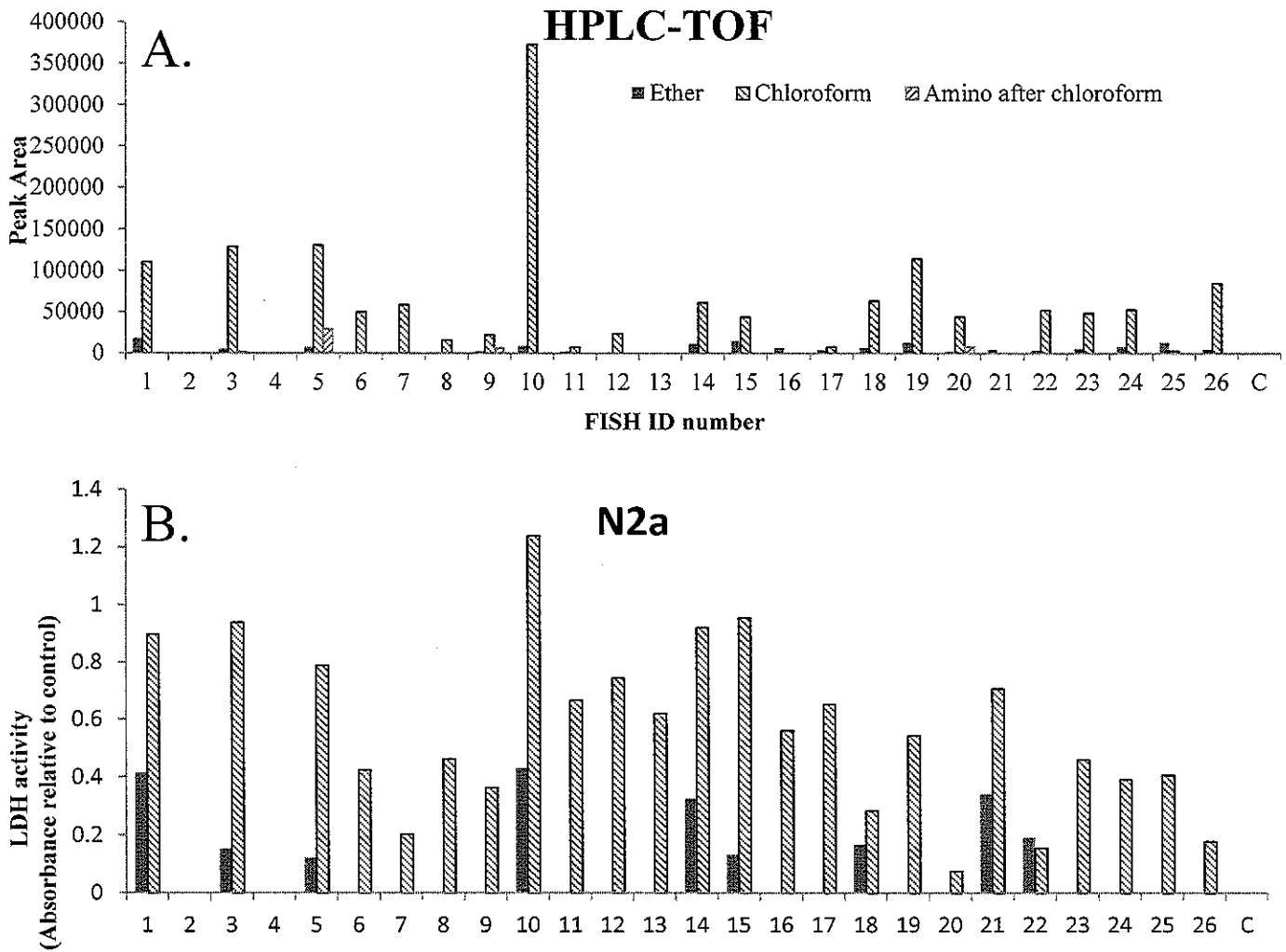


Figure 14. A. UPLC-TOF (peak area) and B. N2a bioassay (LDH activity) analyses of ciguatoxin concentrations in individual fish extracted in chloroform and diethyl-ether. Nontoxic fish (C) was tested as a negative control. Error bars represent standard deviation.

Table 9. Pearson's, Spearman's, and Kendall's correlation analysis. Constant is the factor that is the same and "Between" are the factors that are correlated.

| Constant | Between | Pearson's | p value | Spearman's | p value | Kendall's | p value |
|---|---|---|---|---|---|---|---|
| Diethyl-ether | analyses | 0.5291 | 0.0045 | 0.5229 | 0.0051 | 0.4110 | 0.0078 |
| Chloroform | analyses | 0.5420 | 0.0035 | 0.3504 | 0.0732 | 0.2339 | 0.0931 |
| N2a | solvents | 0.6098 | 0.0007 | 0.5430 | 0.0034 | 0.4190 | 0.0060 |
| UPLC-TOF | solvents | 0.3752 | 0.0538 | 0.5376 | 0.0038 | 0.3750 | 0.0078 |

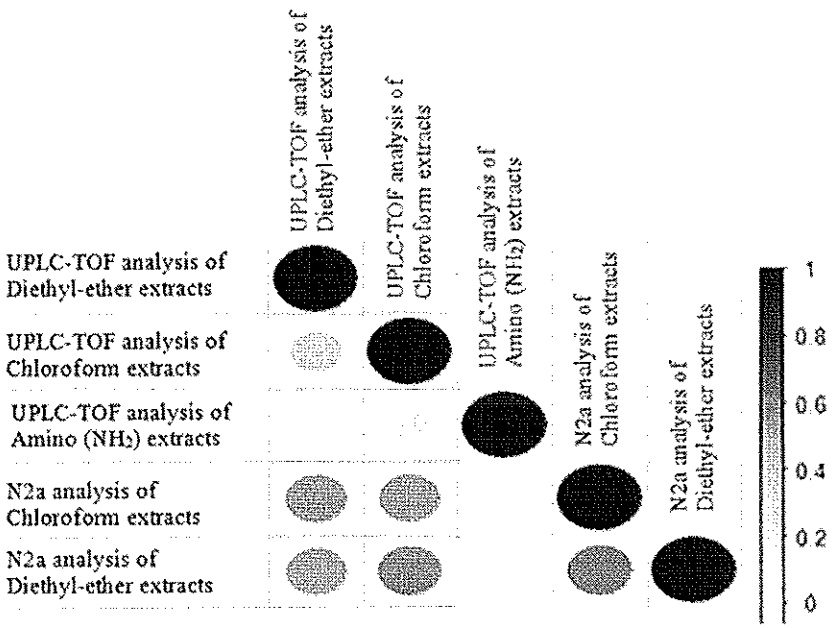


Figure 15. Correlation matrix between N2a bioassay and UPLC-TOF analyses and between original method (diethyl ether L-L partitioning) and optimized method (chloroform L-L partitioning). Size and shade indicate level of correlation.

# DISCUSSION

The following processing steps are required for optimal CTX assessment in fish (Figure 2):

1. Homogenization of tissue
2. Initial extraction steps (acetone and hexane)
3. Additional purification steps (L-L solvent partitioning and SPE)
4. Chemical analysis (HPLC-MS/MS).

This project established an optimized method for analyzing ciguatera toxins in lionfish tissue (Figure 16). Conclusions from this optimization and method recommendations (Table 10) are discussed below.

*Homogenization*

Homogenization of wet tissue was difficult and improved homogenization methods for wet tissue need to be explored in future studies. There was concern about the homogeneity of the wet aggregate sample after high variation was observed within sample replicates. Previous work with Pacific ciguatoxins reported that lyophilization improved homogeneity without affecting extraction efficiency (Meyer et al., 2015). Aggregate tissue was lyophilized in an attempt to improve homogeneity, but lyophilization reduced toxin recovery in all replicates analyzed. This step should be fully evaluated andadditional studies are needed to explore the effects of different extraction sequences on both wet and dry tissue to better understand the effects of lyophilization on extraction efficiency.

Table 10. Recommendations for method development based on results from this research. (+) represents a method step that was showed to be positive/necessary in this research, (x) represents a method step that was inconclusive, and (-) represents a method step that is not recommended for use in future methods.

| Method Step | Recommendation |
|---|---|
| Homogenization | |
| - Lyophilization | (-) do not dry |
| Initial Extraction Steps | (+) |
| - DCM-only | (-) |
| Additional Purification Steps | |
| - L-L solvent partitioning | |
| - SPE (dry tissue) | (+) chloroform |
| - SPE wet | |
| | (x) inconclusive |
| | (-) |
| HPLC-MS/MS | |
| - HPLC-QQQ | (x) need C-CTX standard |
| - UPLC-TOF | (+) |
| - N2a | (+) |

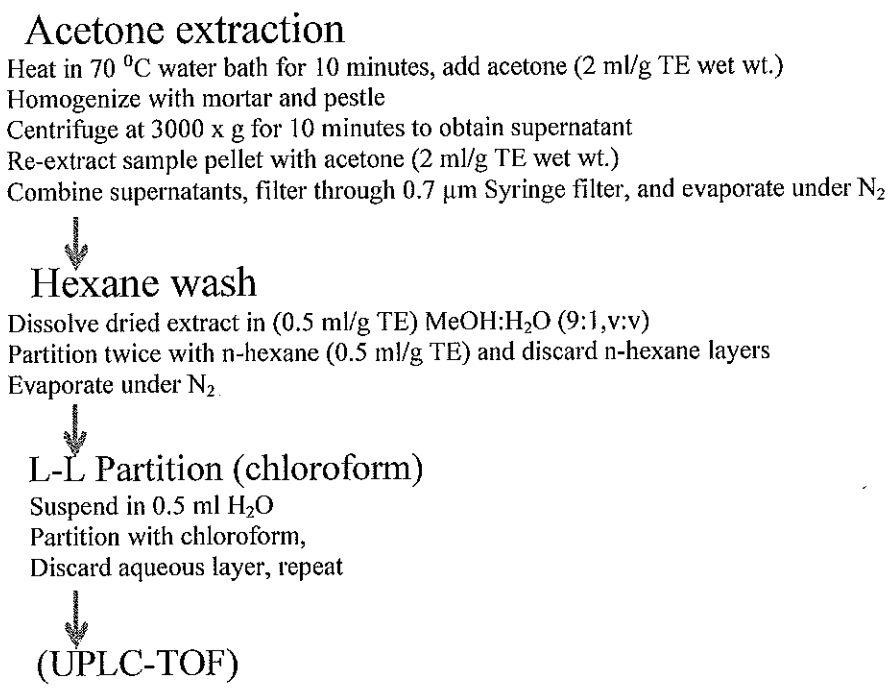


Figure 16. Flowchart detailing optimized method for extraction of ciguatoxins from wet tissue samples prior to HPLC-MS/MS analysis.

*Initial Extraction Steps*

The initial steps, which consist of acetone extraction followed by two hexane washes, were not evaluated; however, these steps are essential to remove excessive fatty acids. The dichloromethane-only trial method was assessed, and this trial method was not efficient at extracting ciguatoxins from tissue. The initial extraction steps are necessary to avoid matrix suppression and are recommended for future method development.

*Additional Purification Steps*

L-L partitioning solvents and SPE cartridges were evaluated and an optimized method was developed. When only the dry aggregate replicates were compared, amino ($NH_2$) SPE after chloroform L-L solvent partitioning yielded the highest mean CTX peak area, however, there were not enough replicates to definitively say that this is the best method for dry tissue analysis. This research needs to be expanded with increased sample and replicate numbers to make definitive conclusions about an optimized method for dry tissue.

When considering additional purification steps individually, L-L solvent partitioning was more critical than SPE; the L-L extracts contained more CTX than SPE extracts. L-L solvent partitioning was an essential step to extracting ciguatoxins from lionfish tissue, as further processing with an amino ($NH_2$) SPE decreased toxin signal in all individual fish samples. It is unclear why the amino ($NH_2$) SPE improved recovery of CTX in dry aggregate samples but reduced recovery of CTX in wet individual fish samples. Chloroform L-L partitioning was efficient at extracting CTX from wet tissue without further processing. This was verified by the UPLC-TOF and N2a analyses of 26

individual fish samples; there was consistently more CTX remaining in sample extracts processed by this optimized method.

*HPLC-MS/MS*

HPLC-MS/MS analysis was first attempted using an Agilent HPLC 1200 series system with an Agilent triple quadruple (QQQ) 6410 LC/MS system (Agilent Technologies, Wilmington DE, USA), but quantification of toxin concentration was not possible without a Caribbean standard for comparison. HPLC-QQQ was unable to resolve CTX peaks and we were unable to duplicate previous research utilizing water loss transition ions to confirm identification. As a C-CTX standard was not available, a surrogate standard (P-CTX-1) standard was used. The use of a surrogate standard is helpful in estimating concentrations, yet with a large amount of variability in toxin congeners and degradation products present, accurate quantification is not possible. Accuracy can be improved by the use of a highly sensitive UPLC-TOF system. The development of a highly sensitive UPLC-MS/MS quantification method greatly reduces the time required for processing and reduces solvent cost but ultimately still requires standards for quantitation.

*Comparison to Previous Work*

In recent studies, 13% of lionfish in Guadeloupe (Solino et al., 2015) and 12% of lionfish in the USVI (Robertson et al., 2014) were determined to have toxin concentrations exceeding the FDA recommended limit of 0.1 ppb as measured by the N2a bioassay. My analysis reduced the amount of tissue per sample by ten times and

toxin concentrations greater than the FDA limit were measured in nearly twice as many fish (23%) as reported by Robertson et al. (2014). Future research can benefit from the reduction in sample size that still was sufficient for reliable quantification. Previous studies used diethyl-ether partitioning (Solino et al., 2015) or chloroform partitioning followed by silica SPE (Robertson et al., 2014). Reported differences in toxicity are clearly affected by extraction method utilized. Additional sources of variation include locality, fish size, toxicity of fish's diet, and/or fat content. Both Solino et al. (2015) and Robertson et al. (2014) used HPLC-MS/MS for confirmation of toxins present, however they did not use peak area to quantify toxin concentrations. Herein, LDH activity values measured by the N2a bioassay were significantly correlated to CTX area counts obtained from the UPLC-TOF analysis. This supports previous research emphasizing that the N2a assay is satisfactory when a HPLC-MS/MS system is not available and no other sodium channel toxins/poisons are present.

## SUMMARY

This research demonstrated how lyophilization adversely affects extraction of C-CTX-1 and C-CTX-2 from lionfish. After evaluating several methods of toxin extraction, toxin concentrations were highest in lionfish extracts after wet tissue was processed with initial extraction steps followed by chloroform L-L solvent partitioning. Further processing with an SPE cartridge reduced the concentration of toxin remaining in the extracts. L-L partitioning was more critical than solid phase extraction for CTX recovery. N2a bioassay results were highly correlated with UPLC-TOF analyses. The optimized method, assessing 1 g samples after chloroform L-L extraction, was assessed using UPLC-TOF and was capable of toxin detection at subnanogram concentrations.

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