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Preparation of Ciguatoxin Reference Materials from Canary Islands (Spain) and Madeira Archipelago (Portugal) Fish

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Abstract: Ciguatoxins (CTXs) are naturally occurring neurotoxins that can accumulate in fish and cause Ciguatera Poisoning (CP) in seafood consumers. Ciguatoxic fish have been detected in tropical and subtropical regions of the world including the Pacific and Indian Oceans, the Caribbean Sea, and more recently in the northeast Atlantic Ocean. The biogeographic distribution of ciguatoxic fish appears to be expanding; however, the paucity of CTX standards and reference materials limits the ability of public health authorities to monitor for these toxins in seafood supply chains. Recent reports establish that Caribbean Ciguatoxin-1 (C-CTX1) is the principal toxin responsible for CP cases and outbreaks in the northeast Atlantic Ocean and that C-CTX congener profiles in contaminated fish samples match those from the Caribbean Sea. Therefore, in this work, C-CTX reference materials were prepared from fish obtained from the northeast Atlantic Ocean. The collection of fish specimens (e.g., amberjack, grouper, or snapper) was screened for CTX-like toxicity using the in vitro sodium channel mouse neuroblastoma cytotoxicity assay (N2a cell assay). Muscle and liver tissues from toxic specimens were pooled for extraction and purified products were ultimately profiled and quantified by comparison with authentic C-CTX1 using LC-MS/MS. This work presents a detailed protocol for the preparation of purified C-CTX reference materials to enable continued research and monitoring of the ciguatera public health hazard. To carry out this work, C-CTX1 was isolated and purified from fish muscle and liver tissues obtained from the Canary Islands (Spain) and Madeira archipelago (Portugal).

Keywords: ciguatera poisoning; Caribbean Ciguatoxins; C-CTX1; reference materials; food safety

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1. Introduction

Ciguatera Poisoning (CP) is a type of seafood poisoning associated with the consumption of fish contaminated with ciguatoxins (CTXs) [1]. CP is endemic in tropical and subtropical areas of the Caribbean Sea and the Indian and Pacific Oceans [2]. Ciguatoxins are produced by dinoflagellate species of *Gambierdiscus* and *Fukuyoa*, and they are often, but not exclusively, present in large reef fish such as barracuda, amberjack, or grouper [3,4]. The factors associated with the biogeographic spread of toxic *Gambierdiscus* and *Fukuyoa* species, and the reporting of CP cases in areas such as the Canary Islands (Spain) and Madeira archipelago (Portugal) are still unknown [5]. Monitoring for these complex toxins is hampered by the limited availability of CTX standards and reference materials, which are necessary for the development and validation of detection methods. Extensive research

identified the CTX analogues responsible for CP in the Pacific Ocean [6,7]. However, very few researchers have successfully isolated Pacific ciguatoxins (P-CTXs) from natural sources (fish and dinoflagellates) for use as reference materials. Fewer still have succeeded in the chemical synthesis of P-CTX congeners [8–10]. Indian Ocean ciguatoxins (I-CTXs) are not yet structurally elucidated; however, several Caribbean Ciguatoxins (C-CTXs) have been structurally elucidated and identified as the source of CP in the Caribbean Sea, and more recently in the archipelagos of the east Atlantic Ocean [11–14]. Progress on the identification of dinoflagellate precursors and additional C-CTX congeners has been hampered by the lack of reference materials for this group of toxins [15].

Due to the trace concentrations of CTXs present in toxic fish (<1 ng/g), large amounts of fish tissue (e.g., from 4.37 kg to 125 kg), [9,11,16] including fish livers where the toxins accumulate in higher concentrations [9,17,18], are required for the preparation of reference materials and standards. Furthermore, the lipophilic nature of CTXs and the complexity of the fish matrix compounds increase the difficulty in extracting and purifying CTX congeners. The process is long and tedious. Toxin losses can occur in each step of the protocol; therefore, maximizing the efforts to ensure the efficiency of each step in the extraction and purification is essential [17,19–21].

This work was focused on the preparation of reference materials for C-CTX1, which has been identified and confirmed as the principal CTX congener in toxic fish samples from the Canary Islands and Madeira archipelagos. Fish muscle and liver tissues from species obtained from these regions were screened using the Neuro-2A cell assay (N2a) for CTX activity. The protocol includes several steps for extraction and purification. Toxin recovery was estimated using the N2a cell assay following each step in the protocol. The C-CTX1 recovered in the final product was identified, semi-quantified, and confirmed by LC-MS/MS. Previous procedures carried out by Murata et al. [22] and Lewis et al. [14] were incompletely reported for the isolation of CTXs. However, major efforts to purify the C-CTX1 analogue were carried out by R.W. Dickey (Dickey R.W., 1994 unpublished), which allowed for the successful isolation of this analogue [23]. The protocol for isolation and purification in this work is based on adaptations of the last one, and the procedure is described below.

2. Materials and Methods

2.1. Standards

CTX1B stock solution (4466 ng/mL) for the calibration curve and a mixture of P-CTXs solutions prepared from Pacific ciguatoxic fish (used only for CTXs identification) containing CTX1B, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, 49-*epi*CTX3C, CTX3C, CTX4A, and CTX4B were supplied by Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Tokyo, Japan). C-CTX1 pure standard solutions (5 ng/mL) prepared from Caribbean ciguatoxic fish were supplied by Dr. Robert Dickey (former U.S. Food and Drug Administration, Division of Seafood Science and Technology, Dauphin Island, AL, USA).

2.2. Primary Materials

The primary materials used in this work consisted of muscle and liver tissues from fish that were pre-screened for CTX activity in the N2a cell assay. The selection of specimens for extraction was limited to those with higher concentrations of C-CTX1 as previously determined in [24,25]. The muscle tissues (50 kg) were pooled from specimens of amberjack (*Seriola* sp.), dusky grouper (*Epinephelus marginatus*), and cubera snapper (*Lutjanus cyanopterus*) from the Canary Islands (Spain), and barred hogfish (*Bodianus scrofa*) from Madeira archipelago (Portugal). Fish liver tissues (6 kg) were pooled from specimens of amberjack, dusky grouper, and cubera snapper from the Canary Islands (Spain), and barred hogfish, barracuda (*Sphyraena viridensis*), zebra seabream (*Diplodus cervinus*), amberjack (*Seriola rivoliana*), and grey triggerfish (*Balistes capriscus*) from the Selvagens Islands (Madeira, Portugal).

2.3. Protocol for the Preparation of Isolated C-CTX1 RM

Fish tissues were autoclaved (45 min at 121 °C), homogenised, packed in 1 kg portions, and stored at −20 °C until extraction. The 50 × 1 kg portions of muscle tissue and 6 × 1 kg portions of liver tissue were sequentially extracted with acetone (3 L) for 24 h using a Soxhlet extraction apparatus. The acetone extracts were chilled at −20 °C overnight and cold-filtered to remove particulates. The filtered acetone extracts were then evaporated to an aqueous residue under reduced pressure using a rotary evaporator set at 45 °C (Syncore polyvap®). The aqueous residues (≈700 mL) were extracted with diethyl ether (2 × 1 L) in a separatory funnel. The diethyl ether phases were combined and evaporated to dry residues using a rotary evaporator set at 45 °C. The organic residues were reconstituted in 90% methanol (300 mL) and defatted twice with hexane (2 × 600 mL) in a separatory funnel. The hexane layer was discarded and the aqueous methanolic phase was evaporated to a dry residue using a rotary evaporator set at 45 °C.

The dried residue from the aqueous methanol/hexane partition was dissolved in ethyl acetate (10 mL) and applied to an LC-Florisil® Solid Phase Extraction (SPE) tube (Supelclean™, bed wt. 10 g, volume 60 mL, Sigma Aldrich, St. Louis, MO, USA), 500 g fish tissue/cartridge. The LC-Florisil® SPE cartridge was pre-conditioned with 60 mL of ethyl acetate and loaded with 5 mL of the sample in ethyl acetate. The cartridge was washed with ethyl acetate (W, 100 mL) and successively eluted with ethyl acetate/methanol 9:1 (v:v) (E1, 100 mL), ethyl acetate/methanol 3:1 (v:v) (E2, 100 mL), and methanol (E3, 100 mL). All fractions obtained from this step were screened by N2a cell assay and the toxic fractions (E1 + E2, 200 mL) were combined and evaporated to dryness using a rotatory evaporator.

The toxic dried residue from LC-Florisil® SPE was further purified using Gel Permeation Chromatography (GPC). Sephadex LH-20 (10 g, 50 cm × 1 cm, 39.3 bed volume) was packed in a glass column (Tricorn 10 mm/600 mm, GE Healthcare Life Sciences, VWR, Spain) with methanol. Once packed, the flow was reduced to 0.4 mL/min and the column was conditioned for 12 h. The dried residue from LC-Florisil® was reconstituted in methanol (10 mL), and sequential 1 mL aliquots (100 g of fish tissue equivalents/GPC run) were loaded into the GPC column. The collection of the fractions was optimized to collect the toxic compounds in a single fraction. A total of 6 fractions were collected at fixed time intervals: fraction #1 (0–20 min, 8 mL), fraction #2 (20–32.5 min, 5 mL), fraction #3 (32.5–45 min, 5 mL), fraction #4 (45–57.5 min, 5 mL), fraction #5 (57.5–70 min, 5 mL), and fraction #6 (70–105 min, 14 mL). All these fractions were screened for CTX-like compounds using the Neuro-2a cell assay. Fractions with CTX-like activity were combined and evaporated under reduced pressure at 45 °C to a solid residue.

The dried residue from GPC toxic fractions was dissolved in aqueous 50% methanol (5 mL) and applied to an LC-C8® SPE tube (HyperSep Thermo, bed wt. 10 g, volume 75 mL), 500 g fish tissue/cartridge. The LC-C8® SPE cartridge was pre-conditioned with 60 mL of aqueous 50% methanol and loaded with 5 mL of the sample in 50% methanol. The cartridge was washed with 50% methanol (W, 100 mL) and successively eluted with 80% methanol (E1, 100 mL), 90% methanol (E2, 100 mL), and 100% methanol (E3, 100 mL). All the fractions obtained from this step were screened by Neuro-2a cell assay and the toxic fraction was evaporated to dryness using a rotatory evaporator set at 45 °C. The toxic residue from the LC-C8 was dissolved in methanol (1 mL) and fractionated by HPLC-UV using a C18 column, 100 g fish tissue equivalent/injection. Briefly, HPLC fractionation was performed on an Agilent 1100 G1312A binary pump with an Agilent 1260 II UV detector coupled to an Agilent 12690 II fraction collector (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was performed on a C18 column (Kinetex®, 4.6 × 250 mm, 5 µm Phenomenex). Mobile phases consisted of 5 mM ammonium formate and 0.1% formic acid in water (A) and methanol (B). The separation was performed by applying a linear gradient from 60% B to 100% of B at 80 min at a flow rate of 1 mL/min followed by a washing step for 5 min at a flow rate of 2 mL/min. The injection volume was 100 µL. C-CTX1 was collected in a single fraction from 43 min to 47 min. The fraction containing C-CTX1 was evaporated to dryness under a nitrogen stream at 50 °C and stored at −20 °C

until LC-MS analysis. The solid residue containing C-CTX1 was dissolved in methanol LC-MS grade (1 mL) and filtered through 0.22 µm (Syringe Driver Filter Unit, Millex®-CV 0.22 µm, 13 mm, Millipore, Billerica, MA, USA) before the LC-MS/MS analysis (Figure 1).

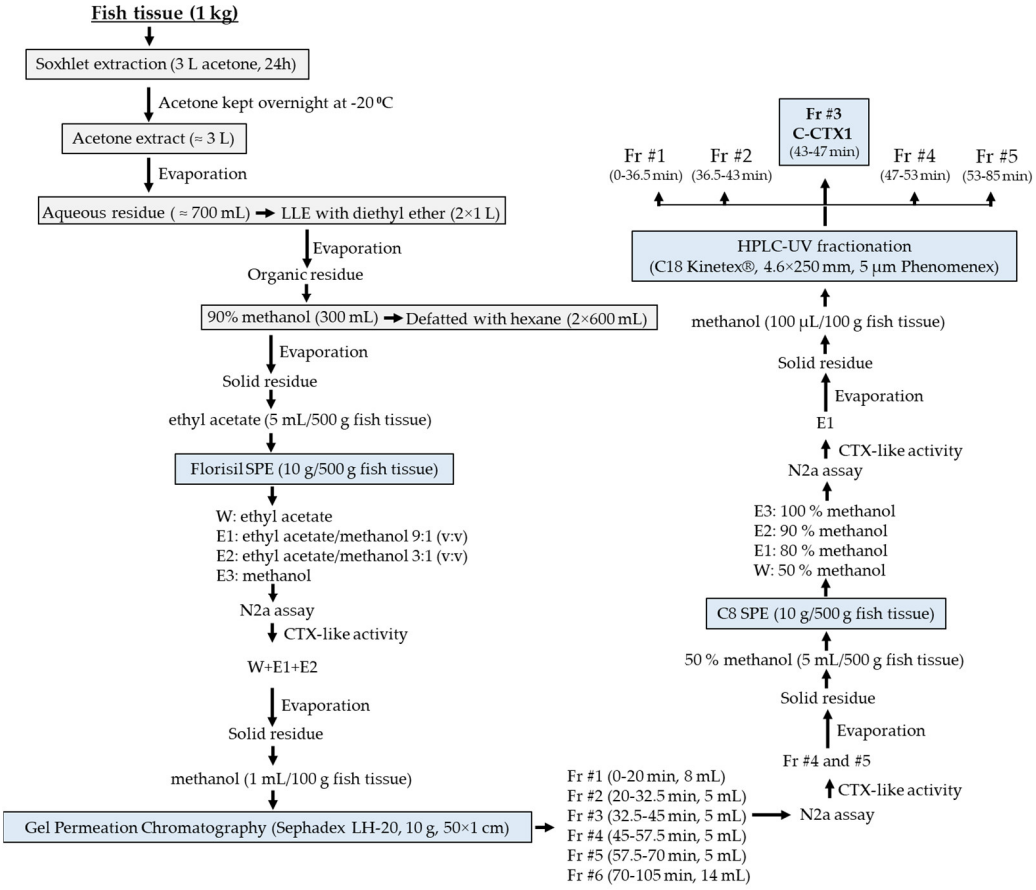

Figure 1. Scheme of the preparation of reference materials for C-CTX1 based on unpublished work by R. W. Dickey (1994).

2.4. N2a Analysis

2.4.1. Mouse Neuroblastoma Neuro-2a (N2a) Cell Line and Culture Maintenance

Neuro-2a (N2a) cell line (ATCC® CCL-131) was purchased at American Type Culture Collection (LGC standards S.L.U., Barcelona, Spain). N2a cells were cultured in T-75 flasks with 30 mL of RPMI-1640 medium (Sigma, Irvine, UK) supplemented with 1% (*v*/*v*) 100 mM sodium pyruvate solution (Sigma, Irvine, UK), 1% (*v*/*v*), penicillin (5000 units) and streptomycin (5 mg·mL^{-1}) solution (Sigma, St. Louis, MO, USA), 1% (*v*/*v*), 200 mM L-glutamine solution (Sigma, Irvine, UK), and 10% (*v*/*v*) of Fetal Bovine Serum (FBS, Sigma, St. Louis, MO, USA) to obtain a complete growth media (RPMI-10). T-75 flasks were maintained in a water-jacked incubator (Forma® 3100/3200 Series) at 37 °C with a humidified atmosphere (95% humidity) enriched with 5% CO_2. N2a cells were routinely passaged as previously described by Castro et al., 2020 [26].

2.4.2. N2a-MTT Assay

Screening for CTX-like sodium channel activity in fish extracts and chromatographic fractions obtained throughout the purification process was performed by the N2a-MTT assay after the methods of Castro et al., 2020 [26]. Briefly, 40,000 cells in 0.2 mL of complete growth medium (RPMI-5) supplemented with 5% (*v*/*v*) FBS were seeded into the wells of 96-well microplates (Corning, NY, USA). Assay plates were incubated for 22–24 h at 37 °C in a humidified atmosphere enriched with 5% CO_2 to ensure 80% confluent monolayers of cells. After the incubation period, plates were divided into sensitized and non-sensitized

sections. Cells of the sensitized section were exposed to 20 µL/well of a mixture of Ouabain (O3125, Sigma, St. Louis, MO, USA) and Veratridine (V5754, Sigma, St. Louis, MO, USA) (+OV section) prepared from the 10 mM ouabain and 1 mM veratridine stock solutions in RPMI-C (growth medium without FBS), allowing a reduction of 20% of cell viability. A volume of 20 µL of PBS was added to the wells of the control non-sensitized cells (−OV section).

Aliquots from each step of the purification procedure were evaporated to dryness under an N_2 stream at 40 °C and reconstituted in MeOH at a concentration of 20 mg tissue equivalents (TE)/µL. Ten-fold (2 mg TE/µL) and twenty-fold (1 mg TE/µL) dilutions were prepared in RPMI-C (growth medium without FBS) from these stock solutions. +OV sensitized, and −OV non-sensitized cells were exposed in triplicate to 10µL of each dilution, reaching final concentrations per well of 87 and 44 mg TE·mL^{-1}, respectively.

Following a 16 h exposure of N2a cells, cell viability was assessed using the colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St. Louis, MO, USA) [26]. Absorbance values were read on a multi-well scanning spectrophotometer (Thermo Fisher Scientific Oy, Ratastie, Finland) by a double reading method using a testing wavelength of 570 nm and a reference wavelength of 630 nm, and data were processed using SigmaPlot v.12.0 software (Systat Software GmbH, Frankfurt, Germany). Cell viability of both sensitized and non-sensitized cells was expressed as a percentage in relation to the respective cell controls.

2.4.3. Dose-Response Curves of C-CTX1 Standard

A calibration of N2a cells for detection of CTX-like compounds was produced by an analysis of the reference C-CTX1 standard in the same conditions as for the extracts obtained from the purification and isolation procedure. A total of ten two-fold serial dilutions were prepared in RPMI-C from the C-CTX1 stock standard stored dry. Sensitized cells were exposed 16 h to each concentration level in replicas of four wells, ranging from 22 to 0.22 fg C-CTX1·µL^{-1}, after that absorbance values were assessed using the MTT assay.

Absorbance values were expressed as the percentage of cell viability relative to the cell controls and dose-response curves were obtained using a sigmoidal four-parameter logistic function with Hill slope (4PL) using SigmaPlot v.12.0 software (Systat Software GmbH, Frankfurt, Germany).

2.5. LC-MS/MS Analysis

LC-MS/MS analyses for the characterization of C-CTX1 reference materials were performed following the method described by Estevez et al. [27]. LC-MS/MS analyses were performed using an Agilent 1290 Infinity Liquid Chromatography system coupled to an Agilent 6495 triple quad (Agilent Technologies, Santa Clara, CA, USA). The method combines two different approaches for the characterization of the reference materials, which are briefly described:

The first approach was used to identify and quantify C-CTX1. CTXs were separated in a C18 column (Poroshell 120-EC-C18, 3.0 × 50 mm, 2.7 µm, Agilent, Santa Clara, CA, USA) set at 40 °C. Mobile phases for the chromatographic separation were (A) 0.1% formic acid and 5 mM of ammonium formate in water and (B) methanol. The flow rate was set at 0.4 mL/min and the injection volume was 1 µL. The column was eluted using a linear gradient of 78% B to 88% B at 10 min, holding at 88% B for 5 min, increasing to 100% B at 15.01 to 18.01 min before returning to 78%% B, with 4 min equilibration before the next injection. The LC-MS/MS instrument operated in positive ionization mode monitoring in the Multiple Reaction Monitoring (MRM) and the CTXs $[M+Na]^+$ as a precursor and product ion using a Collision Energy (CE) of 40 eV and a Collision Acceleration Voltage (CAV) of 4 eV as follows: CTX1B (m/z 1133.6 -> m/z 1133.6), C-CTX1 (m/z 1163.7 -> m/z 1163.7), C-CTX1-Me (m/z 1177.6 -> m/z 1177.6), 52-epi-54-deoxyCTX1B/54-deoxyCTX1B (m/z 1117.6 -> m/z 1117.6), 49-epiCTX3C/CTX3C (m/z 1045.6 -> m/z 1045.6), and CTX4A/CTX4B (m/z 1083.6 -> m/z 1083.6).

The ion source and interface parameters were: Gas flow of 15 L/min at 290 °C, sheath gas flow of 12 L/min at 400 °C, nebulizer pressure at 50 psi, capillary voltage at 5000 V, nozzle voltage at 300 V, and fragmentor potential at 380 V.

The second approach was used to confirm the presence identification of C-CTX1 in naturally contaminated samples by assessing its fragmentation pattern. CTXs were separated in a C18 column (Poroshell 120-EC-C18, 2.1 × 100 mm, 2.7 µm, Agilent, Santa Clara, CA, USA) set at 40 °C. Mobile phases for the chromatographic separation were (A) 0.1% formic acid and 5 mM of ammonium formate in water and (B) acetonitrile. The flow rate was set at 0.4 mL/min and the injection volume was 5 µL. The gradient of the mobile phase started at 35% of B for 2 min increasing to 80% of B in 15 min, and increasing to 95% of B in 1 min, keeping for 5 min at 95% of B until decreasing in 21.01 min to 35% of B, equilibrating the column for 5 min at 35% of B before the next injection.

The LC-MS/MS instrument was operated in positive ionization mode using Multiple Reaction Monitoring (MRM) of C-CTX1 water losses $[M+H-nH_2O]^+$ and two specific fragments as a precursor and product ion. The MRM ion transitions for C-CTX1 are summarized in Table 1.

Table 1. MRM ion transitions monitored by LC-MS/MS for C-CTX1. **FP**: Finger Prints (Specific fragments selected as product ions, where FP1 corresponds to m/z 191.1 and FP2 corresponds to m/z 108.9).

Compound	MRM Transitions Q1/Q3 (m/z)		CE (eV)	CAV (eV)
C-CTX1	$[M+H-H_2O]^+/[M+H-2H_2O]^+$	1123.6/1105.6	25	1
	$[M+H-H_2O]^+/[M+H-3H_2O]^+$	1123.6/1087.6	29	2
	$[M+H-H_2O]^+/[M+H-4H_2O]^+$	1123.6/1069.6	37	2
	$[M+H-H_2O]^+$/FP1	1123.6/191.1	41	2
	$[M+H-H_2O]^+$/FP2	1123.6/108.9	52	1

2.6. Relative Molar Response (RMR)

The limited amount of pure C-CTX1 standard precludes full calibration studies necessary to carry out an adequate quantitation of the reference materials. The common approach used in these cases is to select a standard from the same class of compounds to perform this calibration. This approach is typically used for the quantitation of marine biotoxins analogues when standards are not available, for example in the LC-MS/MS reference method for the analysis of lipophilic toxins in shellfish [28]. This approach can be used once the Relative Molar Responses (RMR) of the different analogues had been evaluated. The evaluation assessment of the RMR under different LC and MS parameters was recently carried out for some regulated toxins, including both Diarrhetic Shellfish Toxins (DST) and Paralytic Shellfish Toxins (PST) [29–31]. However, the RMR for CP toxins has never been evaluated due to the lack of reference materials. In the case of C-CTX1, the unavailability of enough standards to perform the full calibration justified the use of other CTXs analogues available in sufficient amount. RMR for C-CTX1 pure standard (4.4 fM) was evaluated by selecting the most potent toxin congener as reference compound (RMR = 1), and in this case CTX1B standard (4466 ng/mL) kindly provided by Prof. Yasumoto. CTX3C (4.9 fM) prepared by dilution of a stock solution of synthetic CTX3C standard (100 ng/mL), commercially available from Wako, has also been used for the calculation of RMR. The RMR of the investigated compound (i) was determined using equation 1, in which the ratio of response of the investigated compound (i) to that of the reference compound (j) is shown as A, the peak area and C, the concentration [29].

$$RMR_i = \frac{A_i \cdot C_j}{A_j \cdot C_i} \quad (1)$$

A proper evaluation of the RMR should be carried out using certified reference materials working in triplicate and on different days. However, the limited amount of reference materials for CTXs makes a thorough evaluation unfeasible.

As indicated above, the method used for the determination of RMR is described by Estevez et al. [24]

3. Results and Discussion

3.1. Isolation and Purification of C-CTX1

P-CTXs such as CTX3C or CTX4A/4B can be extracted and purified from algae, which is a less complex matrix compared to fish tissue. However, not all CTX congeners related to CP are present in algae and, therefore, these toxins must, at present, be obtained from fish. The isolation of CTXs from fish muscle tissue is a long arduous process with multiple steps including, liquid/liquid partitions, solid phase extractions (SPE), gel permeation chromatography (GPC), HPLC fractionations, and numerous solvent removal (evaporation) steps. CTX losses throughout the multi-step process are inevitable. Furthermore, the concentration of CTXs in fish tissue from the NE Atlantic is generally very low (<1 μg/kg), which together lead to poor recoveries. These limitations resulted in the decision to include parallel extractions of fish livers, in which the concentration of the CTXs is much higher [24], to increase CTX yield. However, the higher complexity of the liver matrix required additional purification steps for the isolation of CTXs.

The initial step was to autoclave fish tissues to improve homogenization and denature proteins for removal following initial extraction [16,17]. Generally, in the extraction of lipophilic constituents from biological tissues, amphipathic acetone is commonly used to maximize yields. In the preparation of CTXs reference materials, acetone was used for the initial extraction to avoid the loss of CTXs [9,11,16,18]. Standard addition experiments to determine the efficiency of CTX recovery were not performed due to insufficient amounts of CTX quantitative standards. Consequently, in this work, exhaustive Soxhlet extraction with acetone for 24 h was employed. This step was followed by cold precipitation of denatured proteins in the acetone extract at −20 °C overnight [9,18]. After the removal of the precipitate by cold filtration, acetone was removed by evaporation under reduced pressure to yield an aqueous suspension. Two liquid-liquid extractions (LLE) were carried out to remove non-toxic constituents in two consecutive steps (Figure 1).

Further purification of C-CTX analogues was performed in four subsequent chromatographic steps in which all eluate fractions were screened for toxicity using the N2a cell assay. The LLE product was fractionated by Florisil SPE using a step-wise gradient elution of increasing polarity, which enabled the removal of residual polar constituents. C-CTX analogues eluted in consecutive fractions W (ethyl acetate), E1 (ethyl acetate:methanol 9:1 (v:v)), and also E2 (ethyl acetate:methanol 3:1 (v:v)) (Figure 2). Non-specific toxic effects (i.e., not sodium channel-specific) were observed in fraction W when N2a cells were exposed at a concentration of 87 mg TE·mL^{-1} (Figure 2(A.2,B.2)). The non-specific activity appeared to be due to matrix effects, which was confirmed by a 2-fold dose dilution (44 mg TE·mL^{-1}). The negative N2a cell assay response to the final eluate fraction with the highest elutropic strength, E3 (methanol), indicated the full extent of recovery for C-CTX analogues from Florisil SPE, excluding potentially irreversible adsorptive losses.

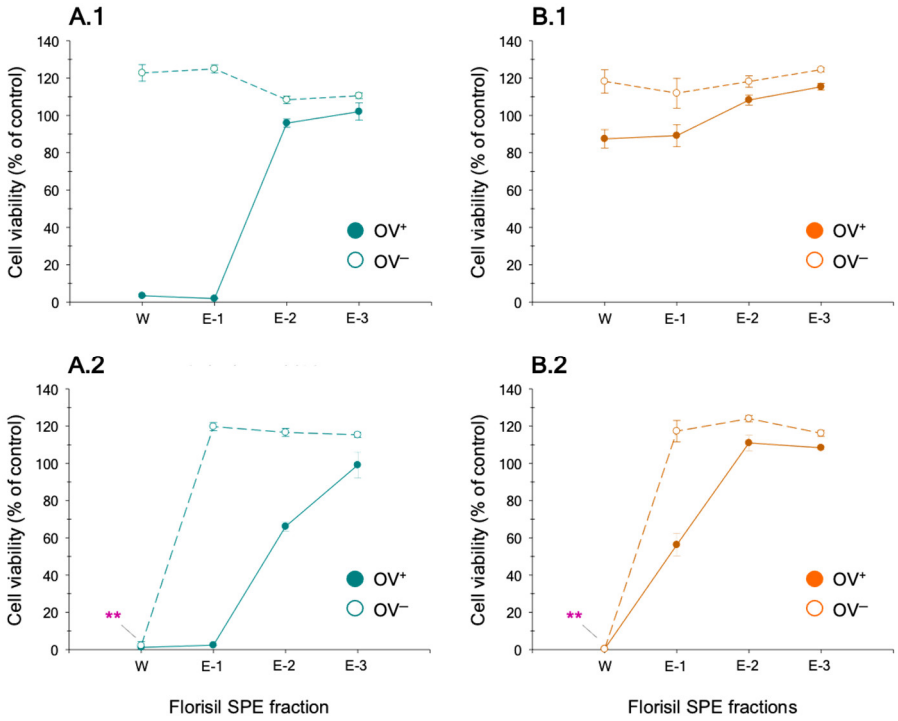

Figure 2. Example of the N2a toxicity profile of the fractions obtained from the Florisil SPE for a highly contaminated (**left**) and for a low toxic sample (**right**). Each fraction was analyzed at two concentration levels: (**A.1**,**B.1**): 44 mg TE·mL^{-1} (10 mg TE·well^{-1}) and (**A.2**,**B.2**): 87 mg TE·mL^{-1} (20 mg TE·well^{-1}). OV^{+}: Sensitized cells; OV^{-}: Non-sensitized cells. ** Florisil SPE fractions showing non-specific toxic effects.

Gel permeation chromatography (GPC), also referred to as size exclusion chromatography (SEC), was an indispensable step in the preparation of CTXs reference materials. Sephadex LH-20, hydroxypropylated cross-linked dextran, is a common GPC stationary phase for lipophilic compounds. In this work, Sephadex LH-20 was suspended in methanol and packed in a glass column with a bed height of 50 cm and a diameter of 1 cm. The column bed was stabilized overnight by gravity flow at approximately 0.4 mL/min before the Florisil SPE product was loaded and eluant flow resumed. Fractions were collected at different times as indicated in Figure 1, and screened by the N2a cell assay for CTX-like toxicity. Fractions #4 and #5 were positive for sodium channel specific activity, consistent with ciguatoxins mode of action (Figure 3).

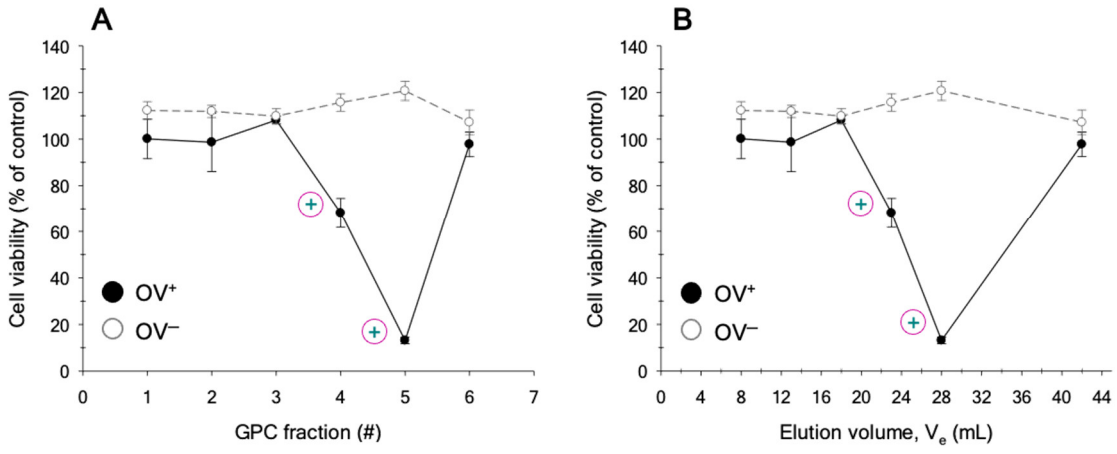

Figure 3. Example of the N2a toxicity profile after GPC at a concentration of 44 mg TE·mL^{-1}, corresponding to 10 mg TE·well^{-1}. Fraction (**A**) and elution volume (V_e) (**B**) vs. cell viability.

Octadecylsilane (C18) stationary phase columns are commonly used in environmental sciences to separate and analyze components of chemical mixtures. In a preparative application for this work, C18 columns were considered for isolating C-CTX analogues from other non-target components of the mixture. However, in the case of C-CTXs and I-CTXs, considerable losses of CTX on the C18 stationary phase have been reported [11]. Therefore, to avoid losses at this stage of extract purification, an octylsilane (C8) SPE cartridge was selected. The four fractions obtained from the C8 SPE step were screened by the N2a cell assay and CTX-like activity was detected only in fraction E1 (80% methanol). The negative N2a responses to fractions W (50% methanol), E2 (90% methanol), and E3 (100% methanol) indicated the full extent of recovery for C-CTX analogues from the C8 SPE step, excluding the possibility of irreversible adsorptive losses.

The final step in the isolation of C-CTX analogues was to fractionate the C8 SPE product using C18 HPLC with UV detection at 215 nm. HPLC conditions followed the method of Estevez et al. [32,33]. However, prior to committing the C8 SPE product to fractionation, the authentic C-CTX1 standard was profiled using the same system and conditions. Forty-nine (49) fractions were collected and screened by N2a cell assay and LC-MS/MS. C-CTX1 activity was detected by the N2a cell assay, and confirmed by LC-MS/MS, from fractions 24 to 28 (43–47 min; Figure 4A,B). Accordingly, fraction collection parameters for fractionation of the C8 SPE product were modified to collect a single fraction between 43 and 47 min.

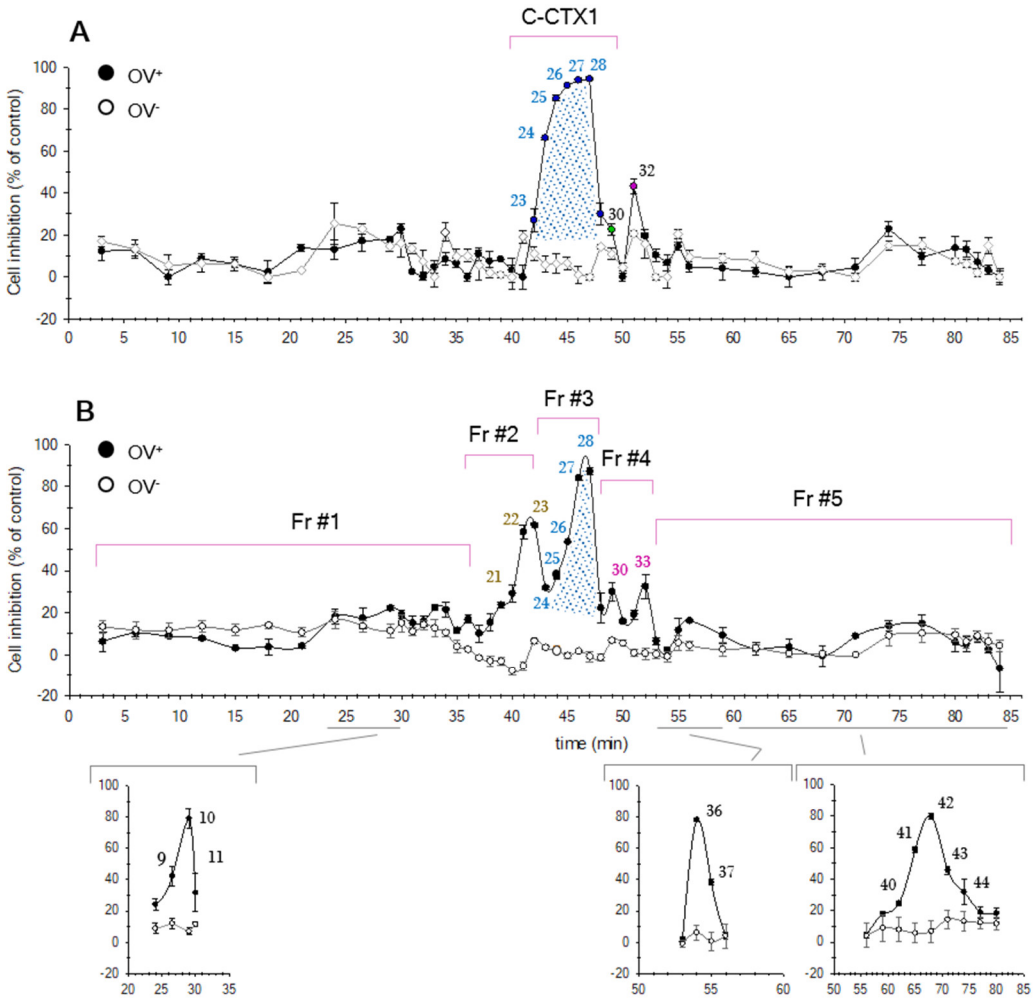

Figure 4. Example of the N2a toxicity profile after the HPLC fractionation of (**A**) Authentic C-CTX standard; (**B**) Naturally contaminated fish sample highlighting the main toxic regions. CTX-like compounds were collected in a total of five fractions: Fr #1, Fr #2, Fr #3, Fr #4, and Fr #5.

C18 HPLC fractionation and N2a cell assays of the C8 SPE product matched the C-CTX1 standard profile. However, fractions before and after C-CTX1 elution also exhibited CTX-like activity between 38–43 min and 47–56 min. Subsequently, five pooled fractions were collected (Figure 4B): Fr #1 (0–36.5 min), Fr #2 (36.5–43 min), Fr #3 (43–47 min), Fr #4 (47–53 min), and Fr #5 (53–85 min) (Figure 4A,B).

The N2a cell assay profile obtained after the HPLC fractionation of the C8 SPE product showed that Fr. #3 (C-CTX1 range) exhibited the highest activity followed by Fr. #2 and #4. No CTX-like toxicity was detected in Fr. #1 and #5 (Figure 5A). The C-CTX1 isolated from the C8 SPE product, following the procedure described in this work, was compared with the N2a cell assay activity of authentic C-CTX1 standard, showing similar responses in both cases (Figure 5B,C).

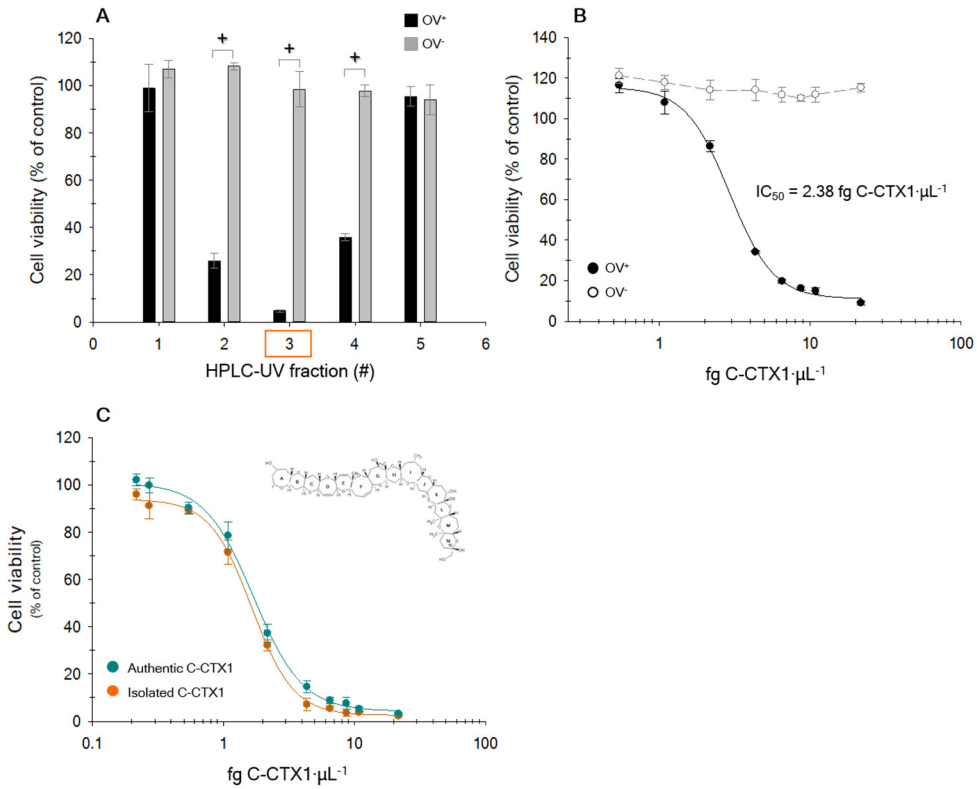

Figure 5. (**A**) N2a toxicity profile after the HPLC fractionation. C-CTX1 was collected individually in Fr. #3. + symbol: fractions showing CTX-like activity; (**B**) Toxic response of sensitized (●) and non-sensitized (○) cells obtained from the analysis of Fr. #3 by N2a-MTT assay and (**C**) comparative dose-response curve of authentic C-CTX1 and C-CTX1 isolated in this work.

3.2. LC-MS/MS Analyses of C-CTX1

The fractions obtained from the final C18 HPLC step were analysed by LC-MS/MS following methods described by Estevez et al. [27]. Two different methods were used to characterize these reference materials. The first method was used to identify and quantify C-CTX1 by monitoring its sodium adduct $[M+Na]^+$ as a precursor and product ion. C-CTX1 present in the fractions was compared to the authentic C-CTX1 standard. Retention time and ion transition m/z 1163.6 $[M+Na]^+$ -> m/z 1163.6 $[M+Na]^+$ were consistent with authentic C-CTX1 standard. An additional LC-MS/MS method monitoring C-CTX1 water loss molecules and specific fragments m/z 191.1 and m/z 108.9 in MRM mode was used to confirm the presence of C-CTX1 in the purified fractions. Retention time and ion ratios were consistent with standard C-CTX1 (Figure 6). Traces of C-CTX1 methylated congener (C-CTX1-Me) were also detected in some of the purified fractions as a consequence of slight acidification in methanolic solution during fraction collection and sample processing for LC-MS/MS analyses [34].

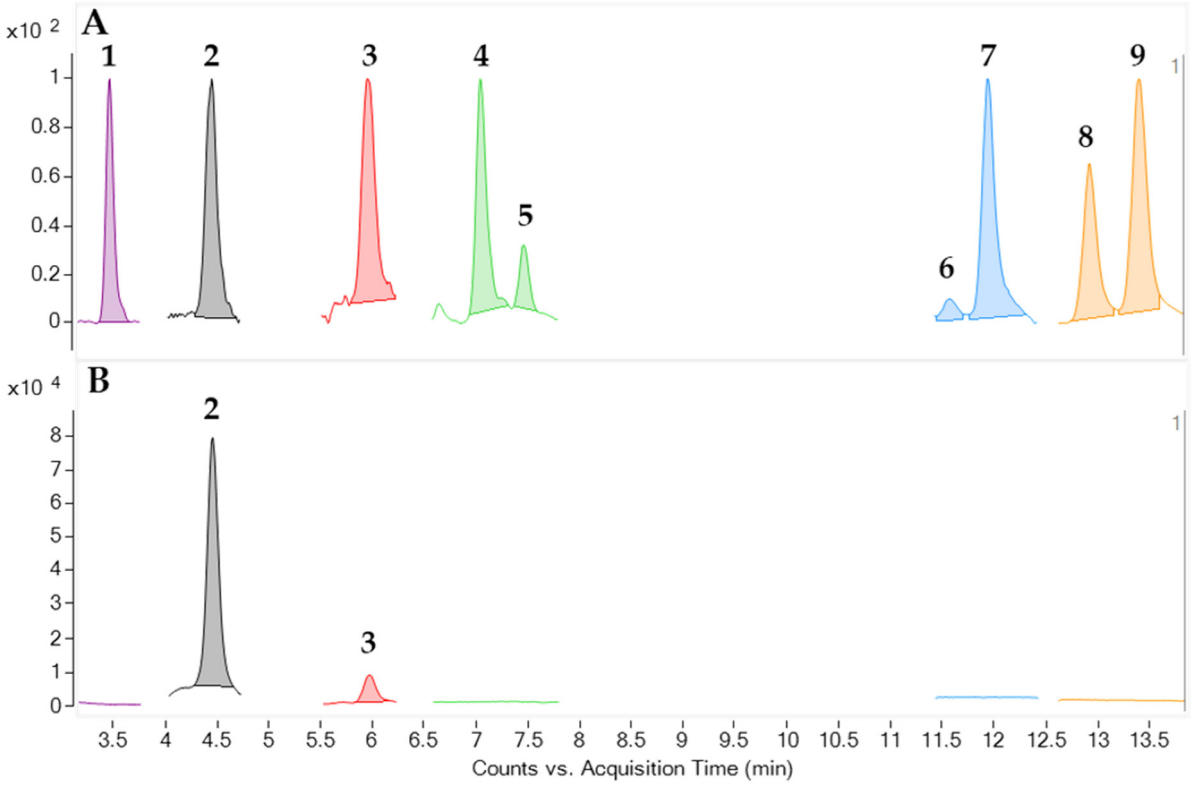

Figure 6. LC-MS/MS chromatogram of: (**A**) Reference materials of CTXs: CTX1B (1) (m/z 1133.6 -> m/z 1133.6), C-CTX1 (2) (m/z 1163.7 -> m/z 1163.7), C-CTX1-Me (3) (m/z 1177.6 -> m/z 1177.6), 52-epi-54-deoxyCTX1B/54-deoxyCTX1B (4 & 5) (m/z 1117.6 -> m/z 1117.6), 49-epiCTX3C/CTX3C (6 & 7) (m/z 1045.6 -> m/z 1045.6), CTX4A/CTX4B (8 & 9) (m/z 1083.6 -> m/z 1083.6); (**B**) C-CTX1 (2) (56.9 ng/mL) and traces of C-CTX1-Me (3) detected in the reference material.

C-CTX1 in the reference materials was also characterized by performing product ion experiments at different collision energies selecting C-CTX1 first water loss molecule m/z 1123.6 $[M+H-H_2O]^+$ as a precursor ion. C-CTX1 water losses at m/z 1105.6 $[M+H-2H_2O]^+$ and m/z 1087.6 $[M+H-3H_2O]^+$ were detected at low energies (25 & 30 eV), while a higher CE was needed to detect a fourth water loss m/z 1069.6 $[M+H-4H_2O]^+$ (Figure 6). On the other hand, most of the characteristic fragments m/z 253.1, m/z 209.1, m/z 191.1, and m/z 181.1 were detected at high collision energies (40, 35 & 50 eV) (Figure 7). These results are in agreement with those reported in [15,35] where most of the fragments resulted from the fragmentation at the termini of the molecule (Figure 7).

The same RMR value was obtained for CTX1B and CTX3C. The mobile phase composition, which is considerably different at the compound's retention time, does not affect the RMR under these conditions. C-CTX1 showed a different RMR compared to CTX1B and CTX3C (Table 2). This difference ($RMR_{C\text{-}CTX1}$ = 0.5) may be due to structural differences mostly related to the presence of an extra ring (N) with an hemiketal group in contrast with CTX1B and CTX3C. Therefore, to quantitate C-CTX1 using CTX1B or CTX3C calibration a correction factor must be applied. The quantitation of the reference materials of C-CTX1 was carried out as follows: C-CTX1 pure standard was expressed in CTX1B equivalents by interpolation in the calibration curve (1 ng C-CTX1/mL = 0.5 ng CTX1B/mL). C-CTX1 detected in the sample was quantified as CTX1B in the calibration curve (0.450–44.660 ng CTX1B/mL) and converted to C-CTX1 equivalents with the correction factor previously obtained with C-CTX1 pure standard. Limit of detection (S/N = 3) and quantitation (S/N = 10) were calculated in matrix-matched samples spiked with CTX1B and were of 0.004 ng of CTX1B/g fish tissue and 0.015 ng of CTX1B/g fish tissue [27].

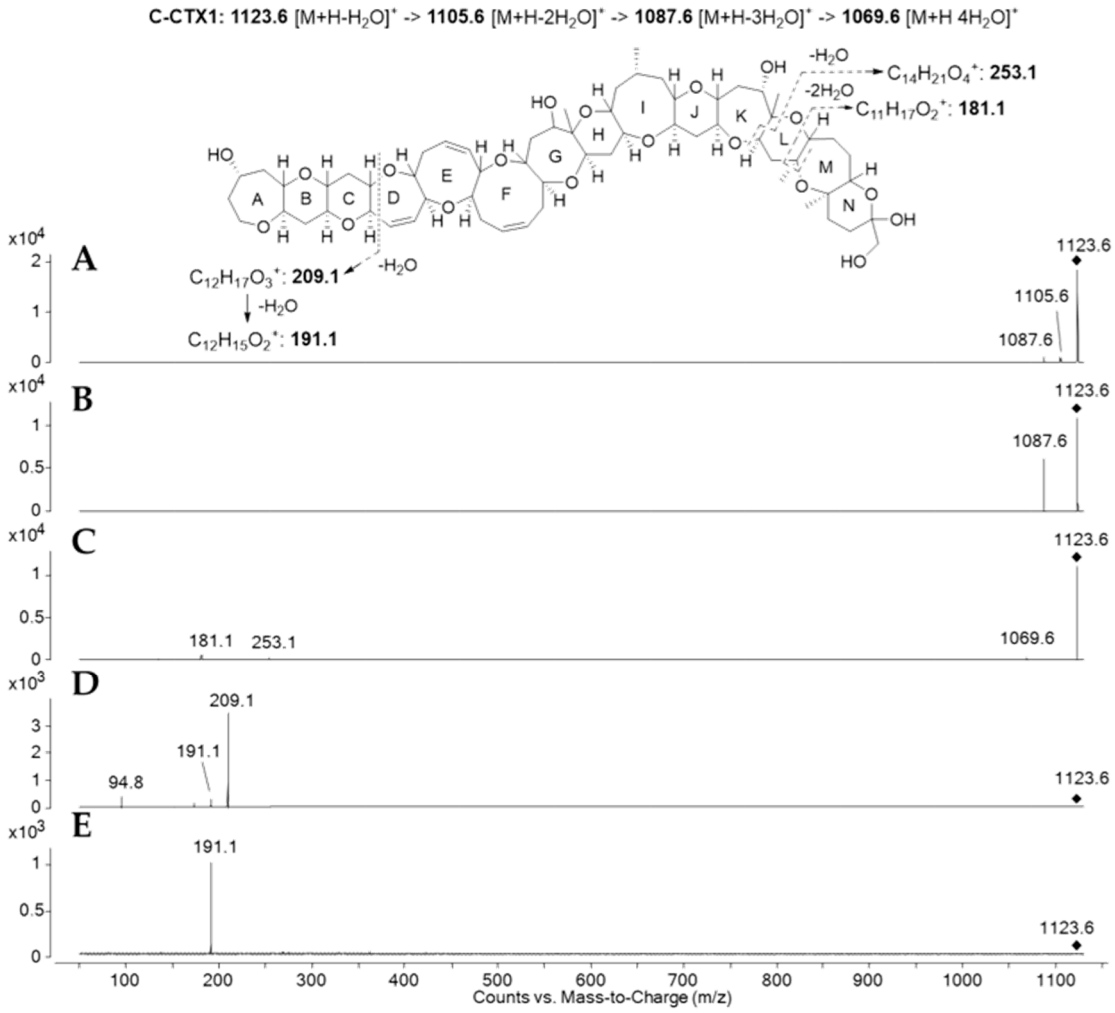

Figure 7. MS/MS spectra of C-CTX1 purified reference material selecting m/z 1123.6 $[M+H-H_2O]^+$ as precursor ion and fragmented at different collision energies: (**A**) 25 eV; (**B**) 30 eV; (**C**) 40 eV; (**D**) 45 eV; (**E**) 50 eV.

Table 2. Relative Molar Response (RMR) and concentration (fM) of the CTXs evaluated in this work.

	CTX1B	C-CTX1	CTX3C
Concentration (fM)	4.5	4.4	4.9
Peak area	21.375	10.116	23.614
RMR	1.0	0.5	1.0

Overall, 48 fractions of purified C-CTX1 were obtained from the extraction of 19 kg of autoclaved and homogenized fish muscle (equivalent to 50 kg of fish). The concentration of C-CTX1 ranged from 0.4 to 40.5 ng/mL. An additional 6 kg of fish liver from contaminated fish containing C-CTX1 was selected to also produce reference materials of C-CTX1. The use of livers has been included in order to increase the C-CTX1 concentration taking into account that the lipophilic character of CTXs increases their potential for absorption in the liver. In total, 41 fractions containing C-CTX1 were obtained with concentrations ranging from 0.4 to 56.9 ng/mL. From the data obtained in this work, the concentration of C-CTX1 in the liver was 2.9 times higher than in the fish muscle tissue. This ratio is lower than the value reported by Ramos-Sosa et al. [24] where the CTX concentrations in fish muscle tissue and liver from species from the Canary Islands (Spain) were compared by an N2a cell assay, albeit both works confirmed a considerably higher amount of CTXs in the fish liver.

However, the complexity of the liver matrix in which lipophilic endogenous compounds are absorbed required additional steps in the purification of the reference materials.

The toxin solutions of C-CTX1 were characterized by LC-MS/MS and, after that, evaporated to dryness and were kept at −20 °C for future use.

The main limitation of the isolation process of C-CTX1 from naturally contaminated samples from the Canary Islands (Spain) and Madeira archipelago (Portugal) was the generally low concentration of the toxin present in the fish (≈0.1 ng/g) [36]. The multiple steps required for purification and isolation have been challenging and compromised the final yield of the process. Improved sampling strategies with a focus on hotspot areas may improve the acquisition of specimens with higher CTXs concentrations, which would increase the efficiency and CTX yield of the isolation and purification protocols.

4. Conclusions

The protocol for the preparation of ciguatoxin reference materials described here has been successfully applied to produce a critical resource for ciguatera research and public health protection. The main impediment to the production of larger quantities of pure C-CTX1, which is the principal toxin responsible for ciguatera poisoning in the Macaronesia Archipelagos, is the ultra-trace quantities of ciguatoxins found in contaminated fish. Yet, the ultra-trace quantities of this potent toxin in fish are sufficient to cause ciguatera poisoning in seafood consumers. The selection of fish specimens with higher CTX concentrations, yet still trace by definition, and the inclusion of fish livers were necessary to increase CTX yields as much as possible. The low levels of CTXs in the fish samples used as primary materials for the toxin isolation and purification have been the key limitation, not only to performing a more exhaustive purification process, but also to complete the characterization of the reference materials produced through additional NMR studies. Further, steps in the protocol were updated to reduce CTX adsorptive losses and improve efficiencies, e.g., continuous Soxhlet extraction and the removal of interfering matrix components. The integration of the mouse neuroblastoma N2a cell assay throughout the protocol for screening extracts, partition products, and chromatographic fractions was a critical tool for tracking protocol efficiency. Due to the lack of certified CTX standards, the cell assay also served as a proxy for traditional chemical methods, e.g., standard addition to assess and optimize analyte recovery.

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