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EXTERNAL SCIENTIFIC REPORT

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Characterisation of ciguatoxins

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Summary

The present document corresponds to Deliverable No. 7: "Final Report" of the Specific Agreement 4 (SA4) "CHARACTERISATION OF CIGUATOXINS" within the Framework Partnership Agreement GP/EFSA/AFSCO/2015/03 "Risk characterization of ciguatera food poisoning in Europe". An LC-MS/MS method has been developed and optimized to be applied to the analysis of fish samples from the Canary Islands (Spain) and Madeira archipelago (Portugal). Two different LC-MS/MS approaches were proposed and developed in order to identify and quantify the ciguatoxins (CTXs) with adequate sensitivity and also to have a reliable confirmation of the CTX involved in the contamination, due to the difficulties encountered to successfully achieve the levels of sensitivity required for the confirmation in most contaminated samples obtained in this project, with low concentration levels of CTXs. The conditions for the application of LC-HRMS for the confirmation of CTXs were initially difficult to achieve but further optimizations were required to finally establish the LC-HRMS conditions that allowed the confirmation of Caribbean ciguatoxin 1 (C-CTX1) as the main responsible for the CTX contamination in this study. The lack of standards and reference materials for Caribbean CTXs has been also a key limitation for the completion of the objectives in this SG4, being necessary to develop contingency plans and optimised methodological development combining Neuroblastoma cell assay, HPLC and GPC fractionations etc, for an increased sensitivity, to be able to characterize and confirm the toxicity of the samples in particular at very low levels of contamination. C-CTX1 was confirmed as the main CTXs responsible for the contamination of the samples from the areas selected in this study. However, as mentioned above, the low concentration of C-CTX1 in the fish samples selected, close to the FDA action levels (0.1 ng/g), hampered their use for the preparation of C-CTX1 standards. Thus, it was necessary to establish a contingency to achieve this objective by developing Laboratory Reference materials containing purified C-CTX1, as well as Fish tissue Reference materials (FTRM) containing C-CTX1 as the main responsible of the contamination. Both materials will be very valuable and useful to help on the setting up of LC-MS/MS methods for the characterization of Caribbean CTXs as the typical CTXs profiles found on the EU coastal areas affected by this emerging risk. An stock of purified solutions of C-CTX evaporated to dryness as well as autoclaved fish tissue (FTRM) were prepared and evaluated for homogeneity and stability and kept in the freezer at -20ºC for further distribution to help on the LC-MS/MS implementation for the characterization of C-CTXs. The scarcity and value of these materials justifies their careful distribution. The LC-MS/MS method developed in this SG4 also allowed to characterize the profiles of the dinoflagellates samples (*Gambierdiscus* and *Fukuyoa*) provided by the SG3 in the final stage of this project. The results obtained allowed to conclude about the lack of correlation with the CTXs profiles of the contaminated fish samples found in the areas selected, since CTXs analogues were not found in these dinoflagellate samples being their toxicity attributed to the presence of several Maitotoxins (MTXs) analogues, and in particular a novel MTX analogue was found for the first time in a *G. australes* strain. Gambieric acids C and D, Gambierone, 44-methyl gambierone, gambieric acids (or their analogues) and

gambieroxide were also identified by LC-HRMS with varying degrees of confirmation in strains of *Gambierdiscus* and *Fukuyoa* from the Mediterranean Sea and North East Atlantic Ocean.

Key words: Contaminated fish tissue reference materials, Ciguatera toxins, Purification, Fractionation

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Abstract

The primary objective of this specific grant was to characterize the risk associated to Ciguatera Poisoning (CP) in the EU by developing a sensitive methodology of LC-MS/MS. The preparation of reference materials including the main ciguatoxins (CTXs) responsible for the contamination was considered the secondary objective to facilitate the implementation of the LC-MS/MS methods in the EU laboratories to characterize this emerging risk. C-CTX1 has been identified by LC-MS/MS, and further confirmed by LC-HRMS, as the main responsible for the CTX toxicity in the samples from the areas selected for this study. The low concentrations levels of CTXs found on the samples evaluated has been a key limitation for the completion of the objectives of this specific grant (SG), being necessary to establish contingency plans, not only to overcome the problems of sensitivity that might compromise the confirmation of the toxic profiles, but also to accomplish the task of preparing reference materials. The contingency plans involved the development of two complementary LC-MS/MS approaches, as well as a methodological approach combining LC-MS/MS, Neuroblastoma cell assay and chromatographic fractionations (HPLC and GPC) to characterize the toxins present in the contaminated extracts. This approach has been also used for the preparation of reference materials in order to confirm the presence of the C-CTX1 in both pure solutions of C-CTX1 and Fish Tissue Reference materials (FTRM) containing C-CTX1. The LC-MS/MS analysis of Dinoflagellates samples (*Gambierdiscus* and *Fukuyoa* sp) allowed to confirm the lack of correlation between the CTXs contamination and the fish samples from the areas were these dinoflagellates were collected. In these samples, the toxicity was attributed to several Maitotoxins analogues as well as gambieric acids C and D, Gambierone, 44-methyl gambierone, and gambieroxide that were identified by LC-HRMS with varying degrees of confirmation in strains of *Gambierdiscus* and *Fukuyoa* from the Mediterranean Sea and North East Atlantic Ocean.

Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

This grant was awarded by EFSA to: University of Vigo (UVIGO)

Beneficiary: University of Vigo (UVIGO), L´Institut Français de Recherche pour l`Exploitation de la Mer (IFREMER)

Grant title: Risk characterization of ciguatera food poisoning in Europe

SPECIFIC AGREEMENT 4: CHARACTERIZATION OF CIGUATOXINS

Grant number: Framework Partnership Agreement GP/EFSA/AFSCO/2015/03

1.2. Interpretation of the Terms of Reference

The present document corresponds to Deliverable No. 7: "Final Report (data analysis and report)" of the Specific Agreement 4 (SA4)

This deliverable was due on 30 November 2020.

1.3. Additional information

The main goal of this Specific Agreement is to provide an analytical methodology for the analysis of ciguatoxins (CTXs) in fishery products as well as to analyze samples suspected of containing CTXs from different origins in European Union waters, and to select material for the preparation of reference material according to the objectives of the Framework Partnership Agreement GP/EFSA/AFSCO/2015/03 "Risk characterization of ciguatera food poisoning in Europe".

2. Methodologies and Data

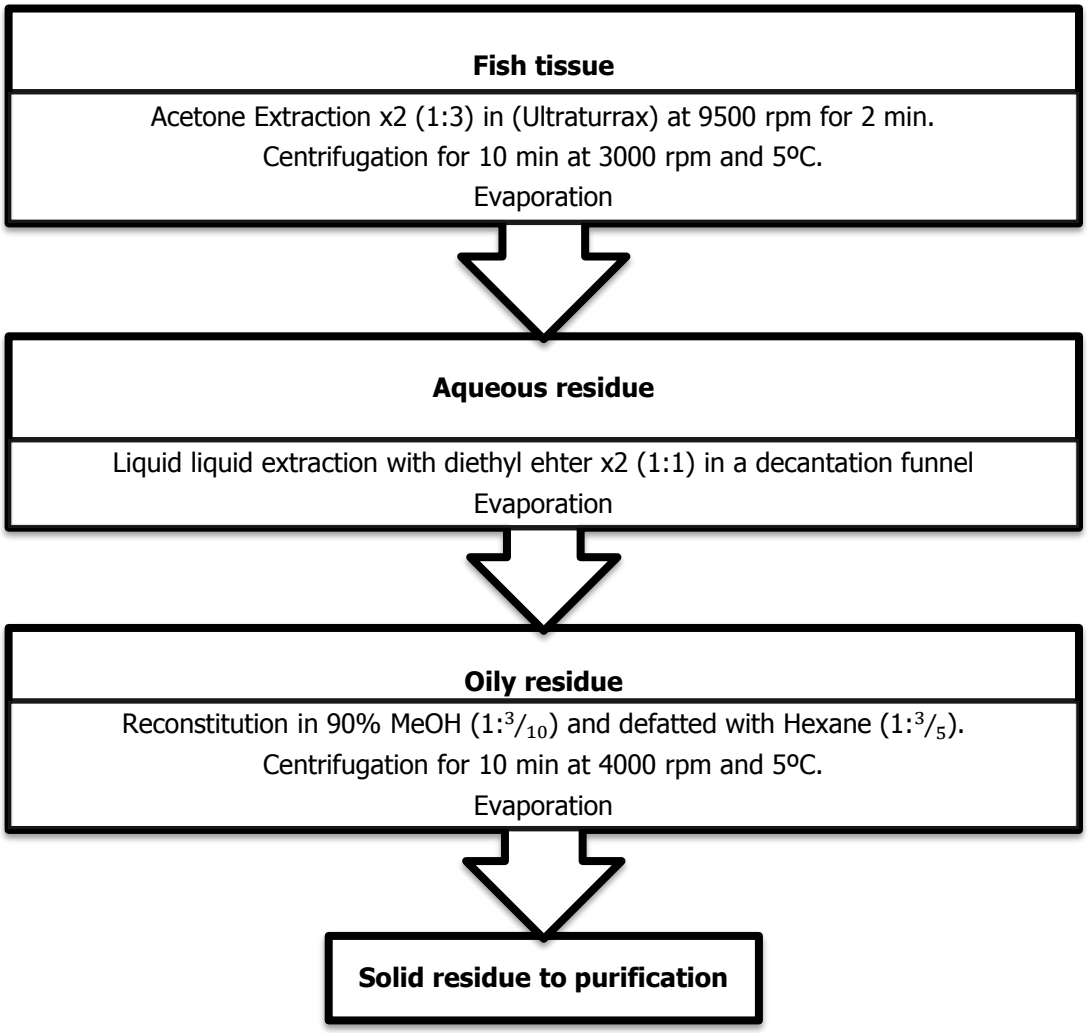
The methods used for the analysis and confirmation of CTXs in the fish and dinoflagellates samples evaluated in this project are LC-MS/MS and LC-HRMS. These methods are based on previously described methods [1–3] and were optimized in order to be applied to the samples which were studied [4–6].

2.1. Development optimization and validation of LC-MS/MS and LC-HRMS

The development, optimization and validation of the LC-MS/MS and LC-HRMS methods were the initial tasks in this SG. These methods were used, not only for the confirmation of the CTXs toxicity in the selected samples, but also for the selection of contaminated samples to be used as candidates for the preparation of fish tissue reference material (FTRM) and its further characterization.

2.1.1. Extraction and purification

Sample pretreatment, including extraction and purification steps, was carried out following the conditions initially proposed in [1] with optimization described in [4]. Briefly, fish tissue samples (15 g) were extracted twice by homogenizing in 45 mL of acetone during 2 min at 9000 rpm (Ultra Turrax® T25 basic). The combined extracts were concentrated to an aqueous residue and extracted twice with 15 mL diethyl ether (Et_2O) and evaporated to dryness. The solid residue was dissolved in 4.5 mL 90% methanol (MeOH). The aqueous MeOH solution was defatted with hexane (9 mL) and evaporated under light nitrogen gas stream (Figure 1).



In brackets mass/volume ratio

Figure 1: Extraction conditions

The solid residue was dissolved in 2 mL ethyl acetate (AcOEt) and interfering matrix constituents were removed by using normal- and then reversed-phase (SPE) cleanup. The normal-phase SPE (Florisil), removed polar interferences from the extract. Reverse-phase SPE (C18) removed non-polar and semipolar interfering matrix constituents. The elution process used for the Florisil cleanup was carried out as follows (Table 1): the sample extract in 2 mL AcOEt was passed through a Florisil cartridge (J. Baker, 500 mg) previously conditioned with 3 mL of AcOEt, and eluted in three consecutive steps, with 3 mL of AcOEt, 5 mL of AcOEt-MeOH (9:1) and AcOEt-MeOH (3:1). The toxin, eluting in AcOEt-MeOH (9:1) fraction, was dried under nitrogen stream at 50°C [7]. The residue from Florisil clean-up was dissolved in 2 mL of 60% MeOH-H_2O and applied to a C18 cartridge (SUPELCLEAN, Supelco, 500 mg) previously conditioned with 3 mL of 60% MeOH-H_2O (Table 1). The cartridge was washed with 3 mL of 60% MeOH-H_2O and the retained toxins were eluted with 5 mL of 90% MeOH-H_2O. The eluate was dried, dissolved in 0.5 mL of MeOH and filtered through a 0.22 µm PVDF filter (Syringe Driver filter Unit, Millex®-CV 0.22 µm, 13 mm) prior to LC-MS/MS analysis.

Table 1: Optimal conditions for the clean-up by solid phase extraction

Optimal CLEAN-UP conditions	
Florisil SPE Bakerbond™ SPE, J.T.Baker ® , 500 mg, 3 mL	**C18 SPE** SupelClean™, Supelco 500 mg, 3 mL
Condition: 3 mL AcOEt	**Condition:** 3 mL MeOH/H_2O (60%)
Load: 2 mL sample extract in AcOEt	**Load:** 2 mL sample extract in MeOH/H_2O (60%)
Wash: 3 mL AcOEt	
Elution: 5 mL AcOEt:MeOH (9:1)	**Wash:** 3 mL MeOH:H_2O (60%)
Second elution: 5 mL AcOEt:MeOH (3:1)	**Elution:** 5 mL MeOH:H_2O (90%)

2.1.2. Analysis by LC-MS/MS and LC-HRMS

2.1.2.1. LC-MS/MS analysis

The LC-MS/MS analyses were carried out using the optimized conditions developed in the laboratory, using the LC-MS/MS System described as follows: Agilent 1290 Infinity LC system coupled to an Agilent 6495 Triple Quadrupole LC-MS (Agilent Technologies, CA) equipped with an Agilent Jet Stream electrospray ionization source (iFunnel). The new configuration iFunnel on the electrospray ionization allowed an increased sensitivity. The LC-MS/MS analyses were carried out following two different methods as described by [4]; the first one, focused on the sensitive identification and quantification of the CTXs selecting the $[M+Na]^+$ ion; whilst the second method was focused on the confirmation of specific CTXs by selecting water losses $[M+H-nH_2O]^+$ ion and specific fragments.

Method for the identification and quantification of CTXs

Liquid Chromatography (LC) conditions

LC separation was achieved using a Poroshell 120 EC-C18 column (3.0 × 50 mm, 2.7 µm, Agilent USA) at 40°C using a linear gradient of mobile phases: 5 mM ammonium formate and 0.1% formic acid in water (A) and MeOH (B). The concentration of solution B was increased from 78% to 88% in 10 min and held for 5 min. The flow rate was 0.4 mL/min, and the injection volume was 1 µL. The conditions used for LC-separation are summarized in Table 2.

Table 2: Gradient of mobile phase for the LC-MS/MS analysis

Time (min)	% A	% B	Flow (mL min^{-1})
0.00	22.00	78.00	0.40
10.00	12.00	88.00	0.40
15.00	12.00	88.00	0.40
15.01	0.00	100.00	0.40
18.00	0.00	100.00	0.40

A: 5 mM ammonium formate and 0.1% formic acid in water; B: MeOH.

Mass spectrometry conditions

The mass spectrometer was operated in positive ionisation mode in order to monitor sodium adduct ions ($[M+Na]^+$). The optimized conditions were as follows: collision energy 40 eV and the $[M+Na]^+$ ions were used as precursor ions and product ions. Source and interface conditions were optimized for the

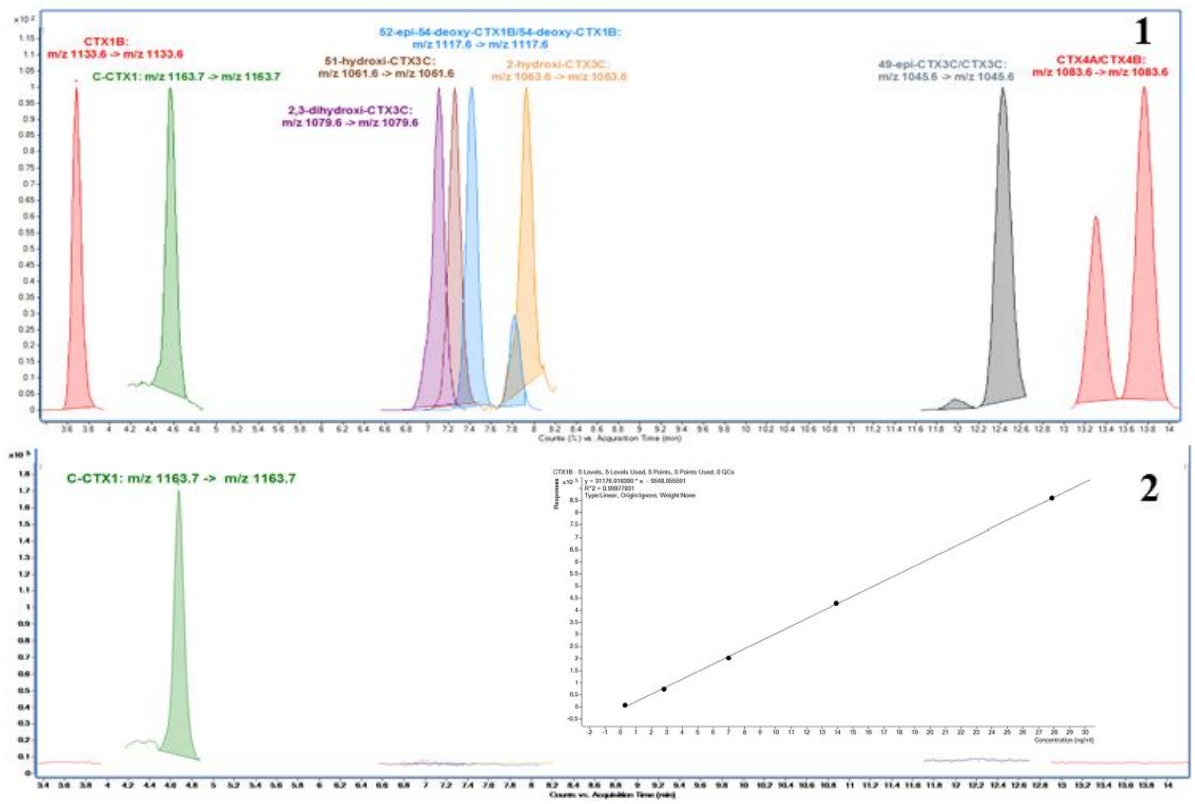
analysis of CTXs in positive ionization mode and were adjusted to achieve the optimal sensitivity for the selected ions.

The instrumental parameters were set as follows: Drying gas, 15 L min^{-1} of N^2 at 290 °C; sheath gas flow, 12 L min^{-1} of N^2 at 400°C; nebulizer gas, N2 at 50 psi; capillary voltage, 5000 V; nozzle voltage: 300 V; fragmentor energy 380 V. This method monitors ions from all CTXs with known structures and all standards available that were kindly provided by Prof. Yasumoto (Mix Pacific Ciguatoxins standard contains: CTX1B, 2,3-dihydroxiCTX3C, 51-hydroxiCTX3C, 52-epi-54-deoxyCTX1B/54-deoxyCTX1B, 49-epi-CTX3C, CTX3C, CTX4A and CTX4B) and Dr. Ronald Manger and Dr. Robert Dickey (Caribbean Ciguatoxin-1, (C-CTX1)). The optimal MS conditions are detailed in Table 3.

Table 3: Mass spectrometry conditions for CTXs

Toxin	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (eV)	Cell Acc (eV)
CTX1B	1133.6	1133.6	380.0	40.0	6
C-CTX1	1163.7	1163.7	380.0	40.0	6
2,3-dihydroxi CTX3C	1079.6	1079.6	380.0	40.0	6
51-hydroxi CTX3C	1061.6	1061.6	380.0	40.0	6
52-*epi*-54-deoxy CTX1B/54-deoxyCTX1B	1117.6	1117.6	380.0	40.0	6
49-*epi*-CTX3C/CTX3C	1045.6	1045.6	380.0	40.0	6
CTX4A/CTX4B	1083.6	1083.6	380.0	40.0	6

An example of the LC-MS/MS analysis carried out for the standard mix for Pacific CTXs and C-CTX1 under the optimal conditions is shown in Figure 2.



1) Mixture of P-CTXs and C-CTX1; 2) C-CTX1 detected in a naturally contaminated sample.

Figure 2: LC-MS/MS chromatogram selecting sodium adduct [M+Na]+ as precursor and product ion

Method for the confirmation of specific CTXs

Liquid Chromatography (LC) conditions

LC separation was achieved using a Poroshell 120 ECC18 (2.1 x 100 mm, 2.7 µm, Agilent USA) at a column temperature of 40°C. LC mobile phase was 5 mM ammonium formate and 0.1% formic acid in H_2O (A) and acetonitrile (ACN) (B). The gradient used was 35% B for 1 min, linear gradient to 80% B at 15 min, 95% B at 16 min, hold for 5 min and return gradient to 35% B at 24 min (Table 4). The flow rate was 0.4 mL/min, and the injection volume was 5 µL.

Table 4: Gradient of mobile phase for LC-MS/MS analysis

Time (min)	%A	%B	Flow (mL min^{-1})
0.00	65.00	35.00	0.40
1.00	65.00	35.00	0.40
15.00	20.00	80.00	0.40
16.00	5.00	95.00	0.40
21.00	5.00	95.00	0.40
24.00	65.00	35.00	0.40

A: 5 mM ammonium formate and 0.1% formic acid in water; B: ACN.

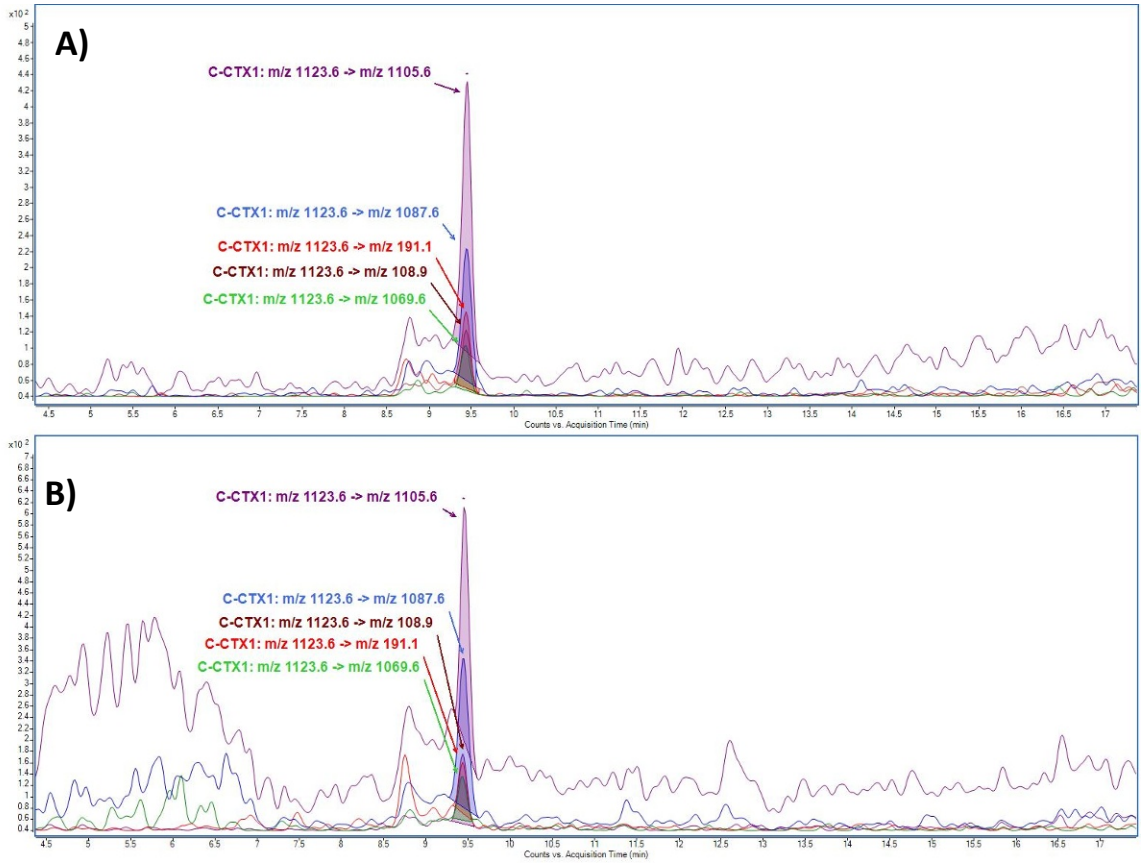
Mass spectrometry conditions

The mass spectrometer was operated in positive ionisation mode in order to monitor water loss ions ($[M+H-nH_2O]^+$) and C-CTX1 fragments by Multiple Reaction Monitoring (MRM) mode. The first water loss, *m/z* 1123.6 ($[M+H-H_2O]^+$), was selected as precursor ion and the following ions were monitored: three water loss ions, *m/z* 1105.6 ($[M+H-2H_2O]^+$), *m/z* 1087.6 ($[M+H-3H_2O]^+$) and *m/z* 1069.6 ($[M+H-4H_2O]^+$); and two C-CTX1 fragments, *m/z* 191.1 and *m/z* 108.9. The collision energy for each precursor/product transition pair were 21 eV for 1123.6/1105.6 and 1123.6/1087.6, 29 eV for 1123.6/1069.6, 40 eV for 1123.6/108.9 and 45 eV for 1123.6/191.1 (Table 5). The instrumental parameters were set as follows: Drying gas, 16 L min^{-1} of N_2 at 250°C; sheath gas flow, 12 L min^{-1} of N_2 at 400°C; nebulizer gas, N_2 at 15 psi; capillary voltage, 4500 V; nozzle voltage: 400 V; fragmentor potential 380 V.

Table 5: MS/MS conditions for the confirmation of specific C-CTXs

Compound	Precursor Ion (Q1)	Product Ion (Q3)	CE (eV)
C-CTX1	$[M+H-H_2O]^+$ *m/z* 1123.6	$[M+H-2H_2O]^+$ *m/z* 1105.6	21
	$[M+H-H_2O]^+$ *m/z* 1123.6	$[M+H-3H_2O]^+$ *m/z* 1087.6	21
	$[M+H-H_2O]^+$ *m/z* 1123.6	$[M+H-4H_2O]^+$ *m/z* 1069.6	29
	$[M+H-H_2O]^+$ *m/z* 1123.6	*m/z* 191.1	45
	$[M+H-H_2O]^+$ *m/z* 1123.6	*m/z* 108.9	40

An example of the LC-MS/MS confirmatory analysis carried out for C-CTX1 standard and C-CTX1 in a naturally contaminated sample is shown in Figure 3.



A) C-CTX1 standard; B) C-CTX1 detected in *Bodianus scrofa* from the Selvagens Islands.

Figure 3: Example of an LC-MS/MS chromatogram for the confirmatory analysis

2.1.2.2. LC-HRMS analysis

Ciguatera Fish Poisoning (CFP) is primarily caused by consumption of tropical and sub-tropical fish contaminated by CTXs. These lipid-soluble, polyether neurotoxins are produced by dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa*. While there is no regulatory level in Europe for CTXs, the European Food Safety Authority (EFSA) endorsed the United States guidance level of 0.01 µg P-CTX1B eq.kg−1 of fish. This limit is extremely low and requires significant improvement in the detection of CTXs. In this study, we compared analytical protocols based on LC coupled to tandem low- or high-resolution mass spectrometry (LC-LRMS or HRMS) to find the best conditions for sensitivity and/or selectivity. Different approaches such as modifying LC conditions, ion choice and acquisition modes, were evaluated to detect the Pacific-ciguatoxins (P-CTXs) on a triple quadrupole (API4000 Qtrap, Sciex) or a quadrupole time of flight (QTOF 6550, Agilent Technologies) spectrometer. Moreover, matrix effects were calculated using matrix-matched calibration solutions of P-CTX1B and P-CTX3C prepared in purified fish extract. Subsequently, the method performance was assessed on naturally-contaminated samples of seafood and phytoplankton.

With LRMS, the ammoniated adduct ion used as a precursor ion showed an advantage for selectivity through confirmatory transitions, without affecting signal-to-noise ratios, and hence limits of detection (LODs). As also reported by some studies in the literature, methanol-based mobile phase gave better selectivity and sensitivity for the detection of P-CTXs. While the LOD for P-CTX1B and P-CTX3C met the EFSA recommendation level when using LRMS, the findings suggested careful evaluation of instrumental parameters for determination of CTXs. LODs were significantly higher for HRMS, which currently results in the need for a significantly higher sample intake. Nevertheless, HRMS allowed for the identification of artefacts and may allow for improved confirmation of the identity of P-CTXs analogues. Consequently, LRMS and HRMS are considered complementary to ensure adequate quantification and identification of P-CTXs.

The results of this part of the study are fully described in [6].

- Confirmation of C-CTX1 in a reference material and fish from the North East Atlantic

Our studies showed that Caribbean ciguatoxin-1 (C-CTX1) is the main toxin causing CP through fish caught in the Northeast Atlantic, e.g. Canary Islands (Spain) and Madeira (Portugal). The use of LC-MS/MS combined with neuroblastoma cell assay (N2a) allowed the initial confirmation of the presence of C-CTX1 in contaminated fish samples from the abovementioned areas. Nevertheless, the lack of commercially available reference materials for these particular CTX analogues has been a major limitation to progress on the research. The EuroCigua project allowed the preparation of C-CTX1 laboratory reference material (LRM) from fish species (*Seriola fasciata*) from the Madeira archipelago (Portugal). This reference material was used to implement an LC-HRMS method for the detection of C-CTX1, acquisition of full-scan as well as collision-induced mass spectra of this particular analogue. Fragmentation pathways were proposed based on fragments obtained. The optimized LC-HRMS method was then applied to confirm C-CTX1 in fish (*Bodianus scrofa*) caught in the Selvagens Islands (Portugal).

The results of this part of the study are fully described in [5].

2.2. Analysis of *Gambierdiscus* and *Fukuyoa* extracts by LC-MS/MS

The results of the characterization of *Gambierdiscus* and *Fukuyoa* extracts are presented and discussed as follows.

The *Gambierdiscus* and *Fukuyoa* extracts provided by SG3 (IRTA) consisted of MeOH extracts from the *Gambierdiscus* cells. Information is included in Table A1. These extracts were filtered through a 0.22 µm PVDF and analysed by LC-MS/MS using the method developed in this SG4 [1]. The CTXs monitored were the ones for which standards were available, i.e. P-CTXs and C-CTX1. The results of the LC-MS/MS analysis confirmed that none of the CTXs for which standards were available were initially present on the *Gambierdiscus* and *Fukuyoa* extracts analysed in this SG4.

2.2.1. Characterization of *Gambierdiscus* sp (HPLC/N2a/LC-MS/MS)

In order to confirm the toxicity of these samples, the approach developed in this SG4 consisting in combining HPLC fractionation with N2a as published in [8], was used for characterization. This approach allowed to focus on toxic fractions to facilitate the further confirmation by LC-MS/MS. On the other hand, the evaluation of the N2a results also allowed to suspect that the toxicity could be associated to toxic compounds other than the CTX-like toxic ones.

2.2.2. Sample fractionation by HPLC-UV

The *Gambierdiscus* extract with higher CTX-like activity (*G. australes* ref: IRTA-SMN-17-271) from Balearic Islands (Spain) was fractionated following the conditions described in [8]. The extracts were subject to HPLC-C18 fractionation using the conditions described in [8]. An Agilent 1100 G1312A LC system with an Agilent 1260 II automatic fraction collector, coupled to an Agilent 1260 II UV detector (Agilent Technologies, Waldbronn, Germany) was used for the HPLC fractionation. The separation was performed on a Kinetex® LC-C18 column (4.6 x 250 mm, 5 µm, 100 Å, Phenomenex) and the mobile phases consisted in: Mobile Phase A water with 5 mM of ammonium formate and 0.1% of formic acid and Mobile Phase B methanol. The gradient conditions started from 60% to 100%for mobile phase B in 85 min at a flow rate of 1mL/min being the injection volume of 100 µL. A total of 49 fractions were collected and evaporated to dryness under N2 at 50ºC, and reconstituted to a final volume of 1 mL of MeOH.

2.2.3. LC-MS/MS analysis

The fractions for which toxicity was confirmed by N2a were analysed by LC-MS/MS, monitoring water losses $[M+H-nH_2O]^+$ and specific fragments for CTXs. The specific ions characteristic for suspected Maitotoxins (MTXs), such as hydrogen-sulphate *m/z* 96.5 $[HOSO_3]^-$, were also monitored by LC-MS/MS. Further confirmation by LC-HRMS was achieved at IFREMER.

2.2.4. Confirmation by LC-HRMS

Over the last decade, knowledge has significantly increased on the taxonomic identity and distribution of dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa*. Additionally, a number of hitherto unknown bioactive metabolites have been reported, while the role of these compounds in ciguatera poisoning (CP) remains to be clarified. CTXs and maitotoxins (MTXs) are very toxic compounds produced by these dinoflagellates and have been described since the 1980s. CTXs are generally described as the main contributors to this food intoxication. Recent reports of CP in temperate waters of the Canary Islands (Spain) and the Madeira archipelago (Portugal) triggered the need for isolation and cultivation of dinoflagellates from these areas, and their taxonomic and toxicological characterization. Among MTXs, specifically maitotoxin-4 has been described as one of the most toxic compounds produced by these dinoflagellates (e.g. *G. excentricus*) in the Canary Islands. Thus, characterization of toxin profiles of *Gambierdiscus* species from adjacent regions appears critical. The combination of LC coupled to either LRMS or HRMS allowed for characterization of several strains of *Gambierdiscus* and *Fukuyoa* from the Mediterranean Sea and the Canary Islands. Maitotoxin-3, two analogues tentatively identified as gambieric acid C and D, a putative gambierone analogue and a putative gambieroxide were detected in all *G. australes* strains from Menorca and Mallorca (Balearic Islands, Spain), while only maitotoxin-3 was present in an *F. paulensis* strain of the same region. An unidentified *Gambierdiscus* species (*Gambierdiscus* sp.2) from Crete (Greece) showed a different toxin profile, including both maitotoxin-3 and gambierone, while the availability of a *G. excentricus* strain from the Canary Islands (Spain) confirmed the presence of maitotoxin-4 in this species. Overall, this study showed that toxin profiles not only appear to be species-specific but probably also specific to larger geographic regions.

The results of this part of the study are fully described in [9].

2.3. Preparation of Fish Tissue Reference Material (FTRM)

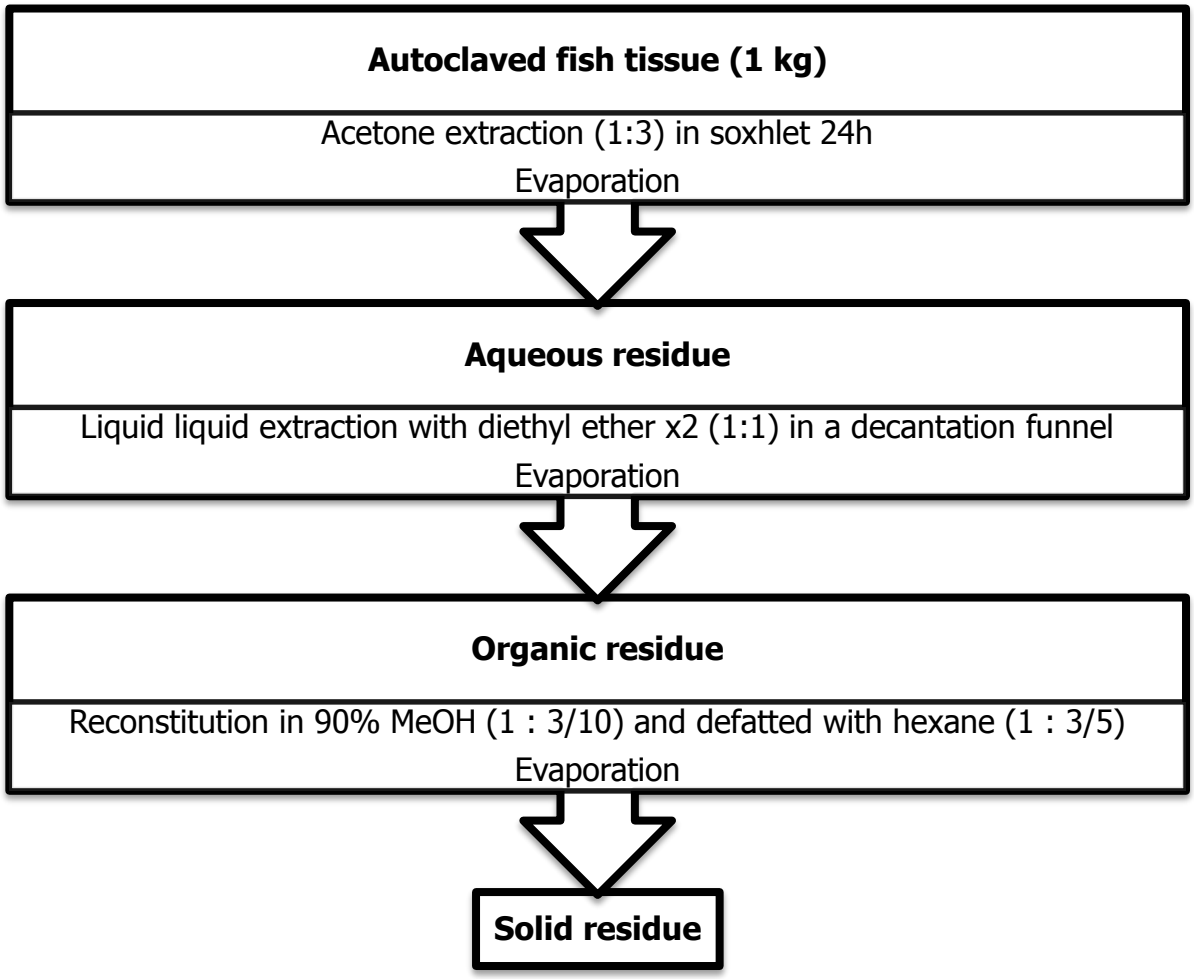
Reference materials were prepared by selecting fish samples in which maximum concentration of C-CTXs had been determined by the LC-MS/MS method developed in this SG4 [4]. A total of 600 Kg of contaminated fish, also including livers, was provided by SG3 (IUSA). Two different types of reference materials were prepared, (i) pure solutions of C-CTX1 isolated from several fractions and (ii) homogenates of fish tissue (FTRM).

A common initial pre-treatment using autoclave (121ºC, 45 min) was applied in the preparation of both purified C-CTX1 solutions and FTRMs with the aim of removing proteins and facilitating the homogenization also contributing to the preservation of the stability, avoiding possible degradation. The materials were kept at -20ºC for further distribution.

2.3.1. Preparation of C-CTX1 solutions

Toxin solutions containing C-CTX1 were prepared by the isolation of C-CTX1 after final HPLC fractionation. The toxicity of the HPLC fractions was confirmed by N2a before their characterization by LC-MS/MS.

A continuous Soxhlet extraction with acetone was used in to maximize the extraction efficiency (recovery estimated 99%). The low concentration of C-CTX1 in the fish samples selected was compensated by increasing the amount of sample taken for further preparation. With this aim, 1 Kg of fish homogenate was taken for Soxhlet extraction and the acetone extract was evaporated to an aqueous residue, under reduced pressure being further partitioned twice with diethyl ether. The organic layer was evaporated and the residue was dissolved in 90% MeOH and defatted with hexane. The methanol extract was evaporated to a solid residue and further purified. The protocol is described in Figure 4.



In brackets: mass/volume ratio.

Figure 4: Extraction protocol used for the preparation of the C-CTX1 solutions

- **PURIFICATION**

The purification of the extract was carried out using different SPE mechanisms (Florisil, C8), a further step of purification and fractionation by GPC was included followed by an HPLC fractionation to obtain purified fractions of isolated C-CTX1. The protocol used for this purification is described in Figure 5.

The solid residue obtained after extraction was dissolved in ethyl acetate and loaded into the Florisil SPE column in which the CTXs were eluted with ethyl acetate/methanol (9:1). The ethyl acetate eluate was evaporated to a solid residue and dissolved in MeOH. The MeOH extract was loaded into a glass column packed with Sephadex LH-20 (GPC) using MeOH as mobile phase. Fractions from GPC were collected and the CTX-like toxicity of these fractions was evaluated by N2a. The presence of C-CTX1 was subsequently confirmed by LC-MS/MS analysis. The toxic fractions containing C-CTX1 were combined and evaporated to dryness, being reconstituted in 50% MeOH to be further purified through C8 SPE and eluted with 80% MeOH. The C-CTX1 eluates were evaporated to dryness and reconstituted in 100 µL of MeOH. An additional fractionation was carried out by HPLC/UV using a C18 column following the optimised protocol developed in this SG4 and published in [8]. Isolated fractions of purified C-CTX1 were collected and evaporated to dryness and reconstituted in 1 mL of MeOH and then filtered through

0.22 µm before injection into the LC-MS/MS for final characterization. The full protocol used for purification is described in Figure 5.

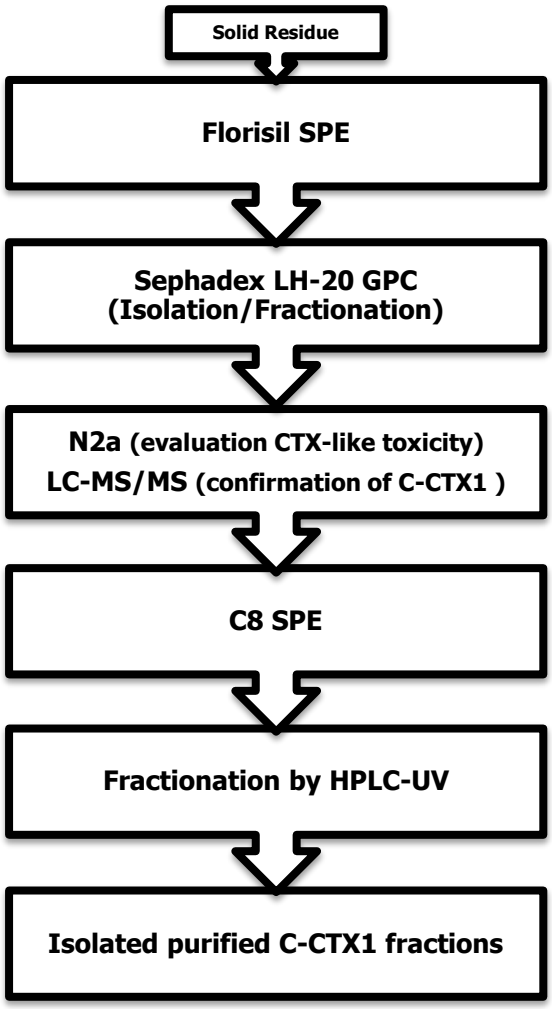


Figure 5: Scheme of C-CTX1 isolation and purification

2.3.2. Characterization of the FTRM

The selected fish materials consisted on two species prone to contain CTXs in the Canary Islands (Spain), Amberjack (*Seriola sp.*) and Dusky grouper (*Epinephelus marginatus*). FTRM was prepared by selecting fish samples containing C-CTX1. These materials were autoclaved, and after homogenization using a blender, they were kept at -20C in sealed plastic bags containing 1 Kg of FTRM. A portion of FTRM was initially taken for characterization following the SOP for the LC_MS/MS analysis developed in this SG4 reported in the Deliverable number 2 and published in [4]. The quantitative analysis of C-CTX1 was carried out using the calibration curves obtained with the C-CTX1 standard kindly provided by Dr. Robert Dickey (previously, U.S. Food and Drug Administration) via Dr. Ronald Manger (Fred Hutchinson Cancer Research Center, Seattle, USA). This calibration curve was compared with the one obtained using the Pacific CTX1B as calibration standard.

3. Assessment/Results

3.1. Development optimization and validation of LC-MS/MS and LC-HRMS

The validation of the optimised LC-MS/MS method has been carried out and the performance criteria obtained were as follows:

- *Limit of detection and Limit of quantitation*

Limits of detection (S/N>3) and quantification (S/N>10) of CTXs were 0.0045 ng g^{-1} and 0.0150 ng g^{-1}, respectively, calculated on matrix matched samples by spiking CTX1B standard. These limits are clearly below the safe level proposed for Caribbean CTX-1 in the United States, i.e. 0.1 ng g^{-1} [11]

The limited amount of C-CTX1 standard, did not allow to carry out the matrix match experiments to be able to analyse the LODs and LOQs for this particular CTX compound. As mentioned above, the good sensitivity obtained for CTX1B, which is considered 10 times more toxic than C-CTX1, would allow us to ensure that the levels for C-CTX1 are good enough to obtain the required sensitivity for the analysis of Caribbean CTX analogues at the recommended levels.

- *Linearity (Calibration curve)*

The calibration curve was carried out using standard solutions prepared by dilution from a stock solution of a CTX1B standard quantified by qNMR and kindly provided by Professor Takeshi Yasumoto. The concentration of the stock solution was 4466 ng mL^{-1} for CTX1B. Five calibration solutions were prepared by dilutions of the stock solution in MeOH in the range 0.28-27.88 ng/mL (Figure 6).

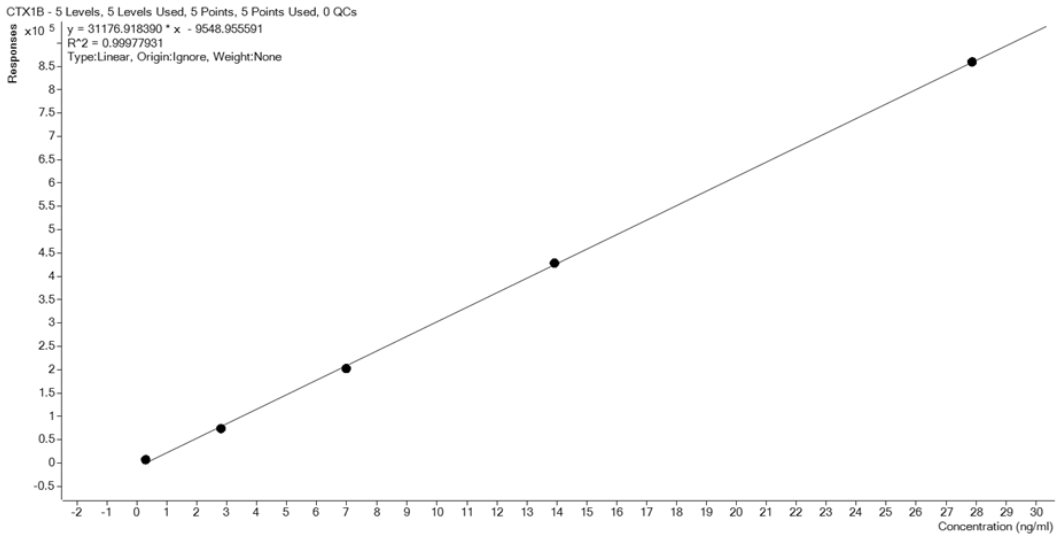


Figure 6: Example of the calibration curve using CTX1B standard kindly provided by Professor Yasumoto

- *Linearity plot*

To assure that the slope of the calibration curve showed a linear regression, the correlation coefficient, R^2, had to be ≥0.98. Linearity was evaluated in nine different days and correlation coefficient values above 0.99 were always obtained (Figure 7).

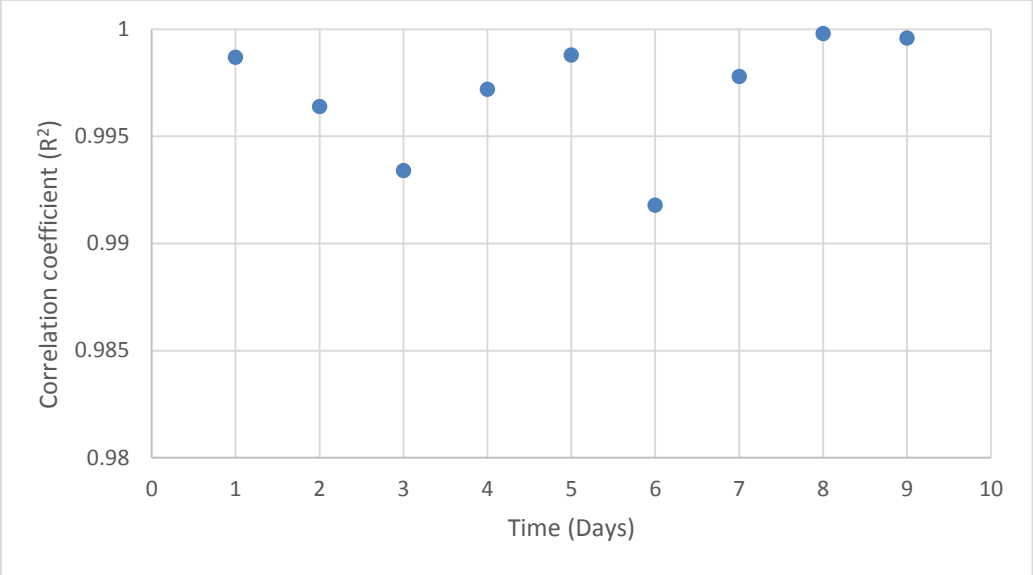


Figure 7: Variation of the correlation coefficient over the time

- *Robustness*

The variation of the slope was evaluated by injecting the calibration standards on nine different days and increased sensitivity was observed over the time (Figure 8). In contrast to other analytical methodologies, LC-MS does not detect directly the neutral compound, as it occurs in other techniques such as LC-UV or LC-FLD. Ions of the compound are formed in the ion source, and this process may decrease robustness because the ionization/evaporation process might be affected by many factors, including the preparation of the mobile phase, the state of cleanliness of the chromatographic column or the ionization source.

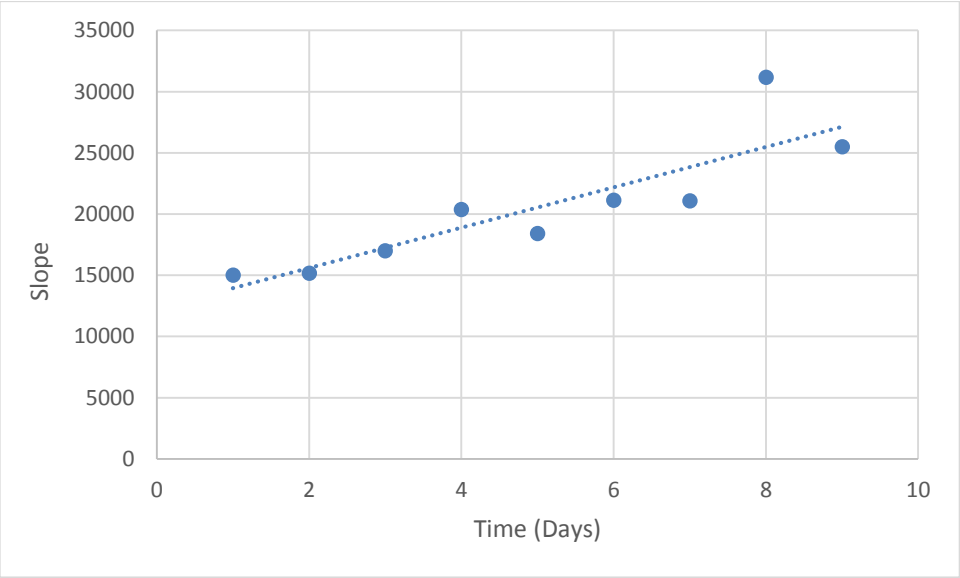


Figure 8: Variation of the slope of the calibration curve of CTX1B over the time

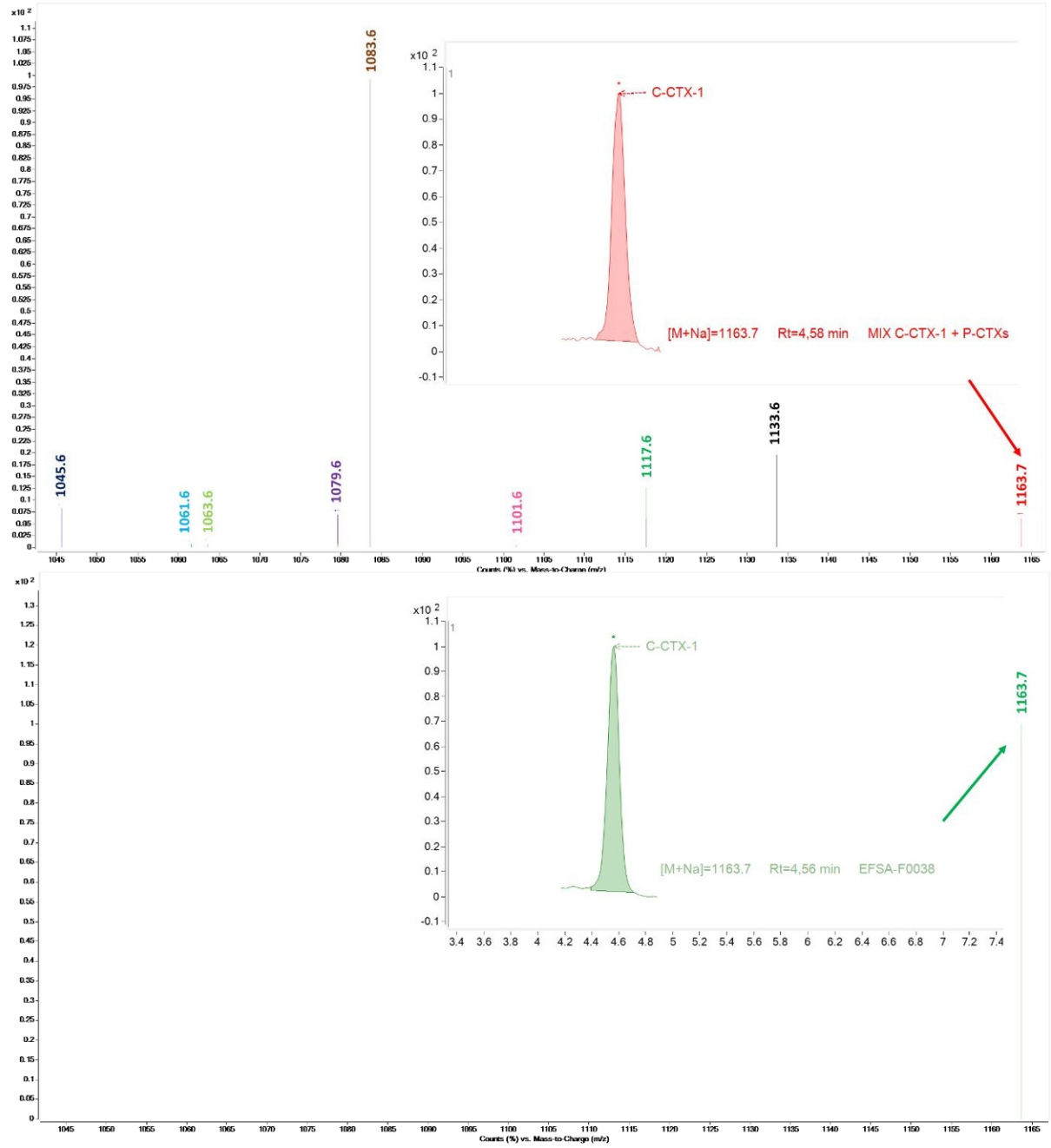
- *Identification and confirmation*

The identification of the presence of each CTX was performed with the reference standards available at this stage of the project which were kindly provided by Prof Yasumoto (Pacific CTX) and Dr. Dickey and Dr. Manger (Caribbean CTX) (Table 6).

Table 6: Retention time of the different CTXs analysed by dynamic Multiple Reaction Monitoring (dMRM) by LC-MS

Toxin	Start time (min)	Retention time (min)	End time (min)
CTX1B	3.55	3.68	3.83
C-CTX1	4.41	4.58	4.74
2,3-dihydroxiCTX3C	6.69	7.02	7.38
51-hydroxiCTX3C	7.01	7.12	7.28
52-*epi*-54-deoxyCTX1B/54-deoxyCTX1B	7.16	7.34	7.53
49-epi-CTX3C/CTX3C	12.23	12.42	12.62
CTX4A/CTX4B	13.60	13.83	14.17

The results obtained after the LC-MS/MS analysis of fish flesh samples from Canary Islands sent by the University of Las Palmas de Gran Canarias (ULPGC) (SG3) show C-CTX1 as main responsible for the toxicity. The identification of this toxin was achieved by comparison of retention times with the C-CTX1 standard, and also by comparison of the precursor/product ion transitions *m/z* 1163.7 $[M+Na]^+$→ *m/z* 1163.7 $[M+Na]^+$ by MRM of C-CTX1 with those of C-CTX1 standard. (Figure 9).



Comparison among mass spectra and retention time of Mix C-CTX1 + P-CTXs standard (top) and sample EFSA-F0038 (bottom).

Figure 9: Example of identification of CTXs in naturally contaminated samples

- *Quantification*

The limited amount of standard available for C-CTX1, the main responsible of the toxicity in the Canary Island fish flesh samples, did not allow the full calibration for this particular CTX analogue. Nevertheless, the calibration carried out with other CTXs analogues was taken into account for the quantitative estimation of the C-CTX1 present in the fish samples analysed.

For this purpose, C-CTX1 reference material with a concentration of 5 ng mL^{-1} was expressed in CTX1B equivalents by interpolation in the calibration curve. Samples where C-CTX1 was detected were quantified as CTX1B and converted to equivalents of C-CTX1 with the value obtained with the reference material previously (See below an example of quantification).

Example of quantification:

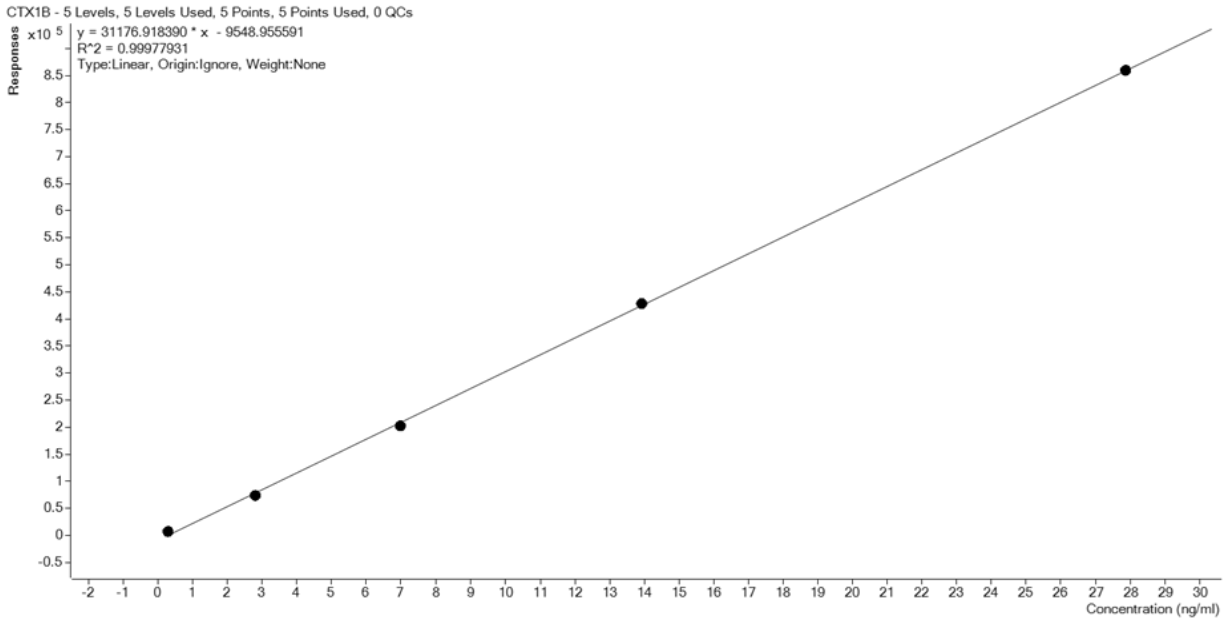


Figure 10: Calibration curve obtained for CTX1B

Table 7: Data obtained from the calibration curve, C-CTX1 standard concentration and CTX1B equivalent

Equation of the calibration curve	Correlation coefficient (R^2)	C-CTX1 standard (ng mL^{-1})	CTX1B equivalent (ng mL^{-1})
y=31176.92x-9548.96	0.9998	5	1.47

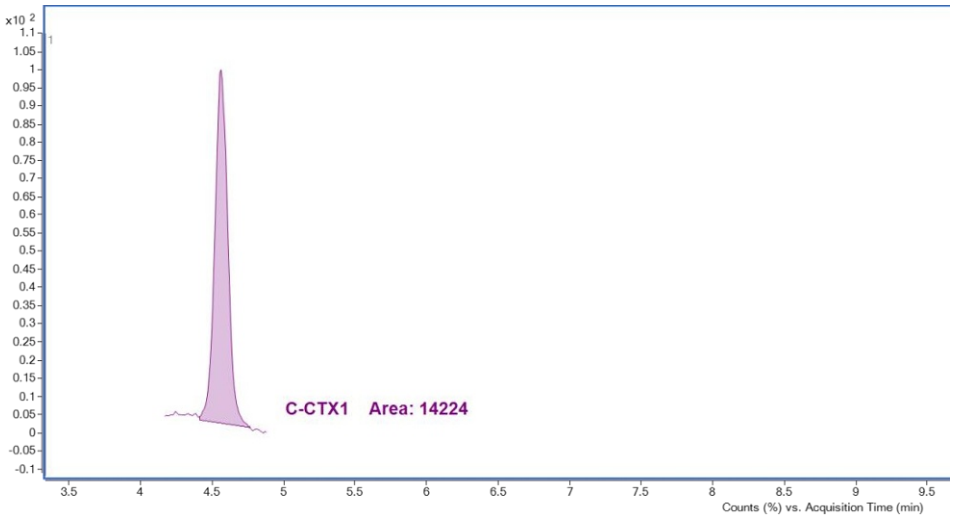


Figure 11: Example of sample where was detected C-CTX1

It is transformed to area from concentration of CTX1B, in ng mL^{-1}, with the equation of the calibration curve:

$$y = 31176.92x - 9548.96; \qquad Area_{sample} = 14224$$

$$14224 = 31176.92x - 9548.96\ ; \qquad x = \frac{14224 + 9548.96}{31176.92}; \qquad x = 0.76\ ng/mL\ (CTX1B)$$

Using CTX1B equivalent (Table 7), it is transformed to C-CTX1 concentration in ng mL^{-1}:

$$[CTX1B]_{sample}\left(\frac{ng}{mL}\right) \cdot \frac{[C-CTX1]\ (ng/mL)}{[CTX1B]_{equivalent}\ (ng/mL)} = [C-CTX1]\ \left(\frac{ng}{mL}\right)$$

$$0.76\frac{ng}{mL} \cdot \frac{5\ ng/mL}{1.47\ ng/mL} = 2.59\ ng/mL$$

With the mass of the fish flesh sample, the final volume and applying the recovery of the purification step (70.3%) it is calculated the concentration of C-CTX1 in terms of ng/g (ppb):

$$[C-CTX1]\ \left(\frac{ng}{mL}\right) \cdot \frac{V_{final}\ (mL)}{m_{sample}\ (g)} \cdot Recovery\ Factor_{purification} = [C-CTX1]_{sample}\left(\frac{ng}{g}\right)$$

$$2.59\frac{ng}{mL} \cdot \frac{0.5\ mL}{15\ g} \cdot \frac{100}{70.3} = \mathbf{0.12}\ \boldsymbol{\frac{ng}{g}}$$

3.2. Analysis of fish samples by LC-MS/MS and LC-HRMS

Both LC-MS/MS approaches implemented in this SG4 were applied to the analysis of fish samples from the Canary Islands (Spain) (n = 212) and Madeira archipelago (Portugal) (n = 66). The samples from the Canary Islands and Madeira archipelago were positive in the N2a for CTX-like compounds. All the samples were extracted, purified and analysed by following the conditions described in section 2.1.

Among the monitored CTXs analogues, including P-CTXs and C-CTX1, only C-CTX1 was detected as main compound responsible for the CTX-like toxicity (Figure 12).

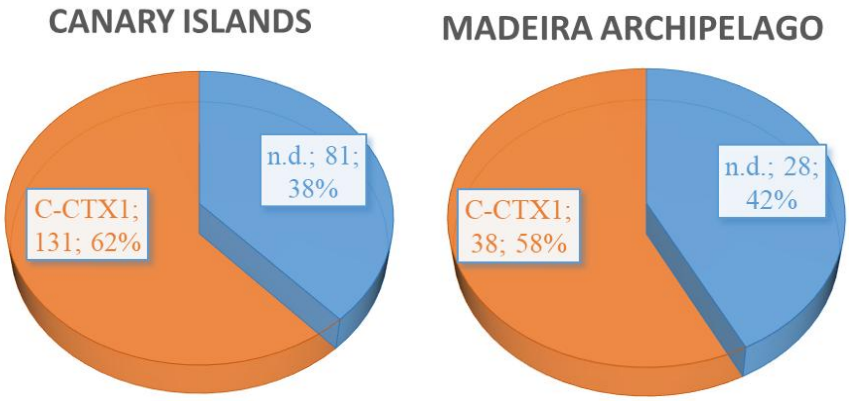


Figure 12. Samples from the Canary Islands (Spain) and Madeira archipelago (Portugal) in which C-CTX1 was detected as responsible for the CTX-like toxicity

3.2.1. Canary Islands samples

In 131 out of 212 samples, C-CTX1 was detected as main compound responsible for the CTX-like toxicity. In the remaining 81 samples, neither C-CTX1 nor any other P-CTX with standard available were detected. This discrepancy can be explained due to the low toxicity detected in the N2a in most of the samples where no CTXs were detected. In a sample with a high toxicity in the N2a, C-CTX1 was not detected but a different compound was detected at a retention time of 5.6 min with *m/z* 1139 (Putative C-CTX-1157 described by [12]). The analysis in MRM mode revealed the detection of up to three water losses as well as a C-CTX1 specific fragment *m/z* 191.1 (Figure 13). Therefore, it can be assumed that beside the presence of C-CTX1 as main compound responsible of the CTX-like toxicity, other C-CTXs may be present in the samples from this geographical region. Additionally, a possible matrix effect of signal suppression in the LC-MS/MS analysis should be considered. However, the purification through the use of two consecutive SPEs reduce the probability of having the above mentioned matrix effect.

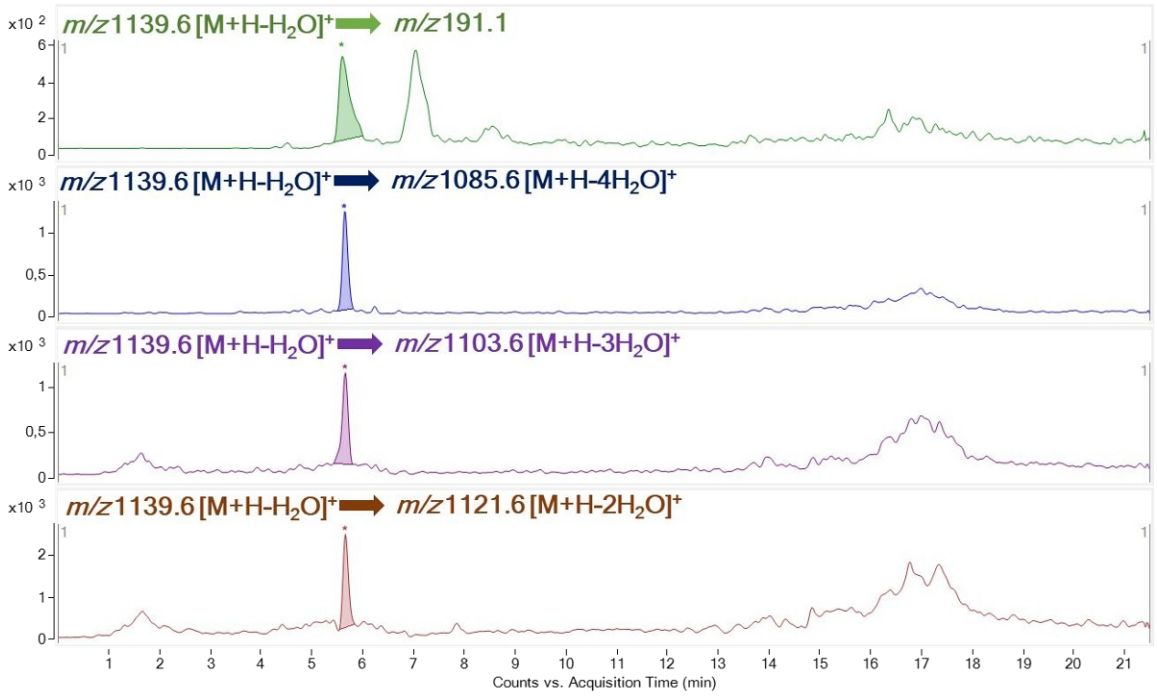


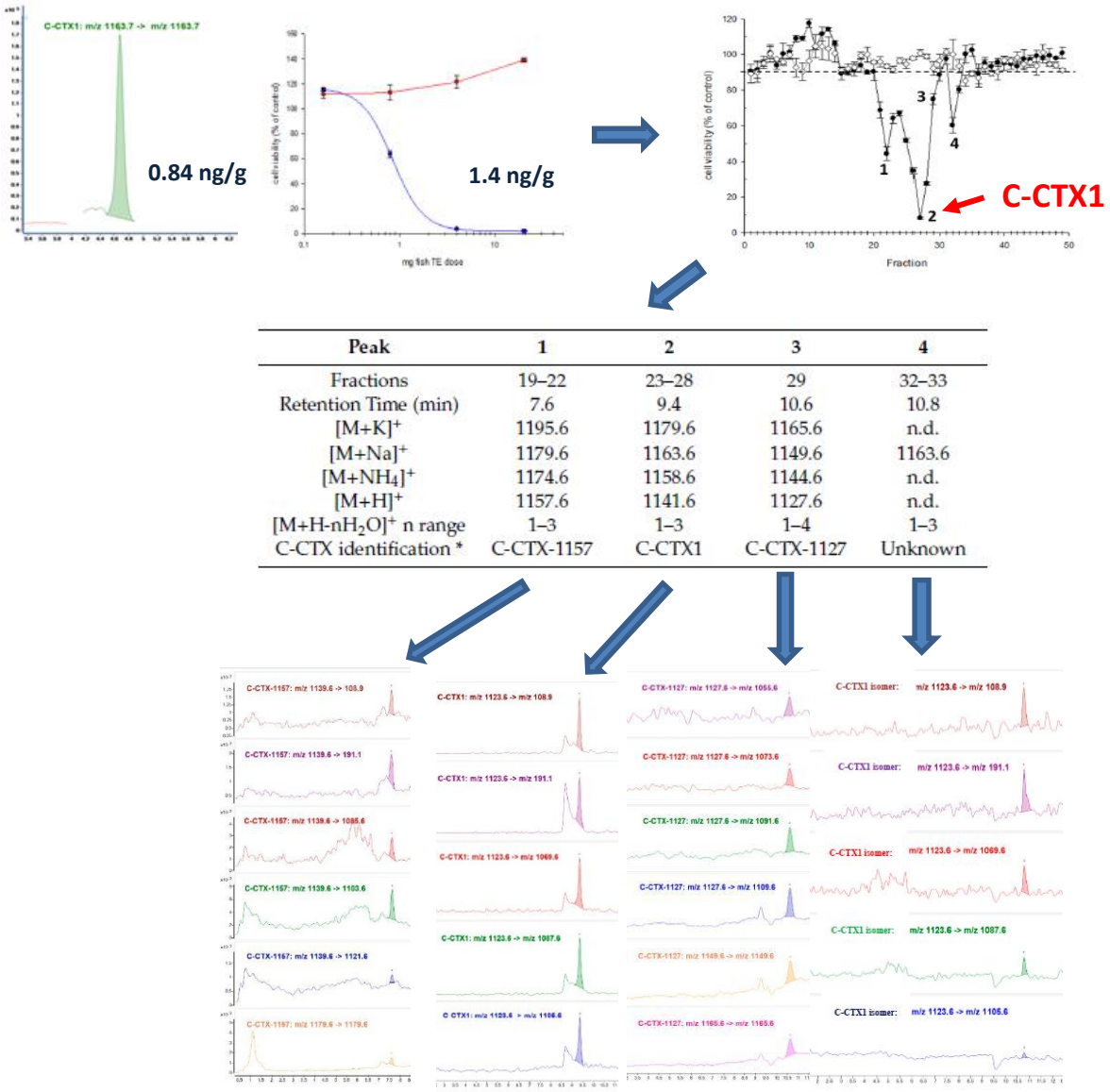
Figure 13: Putative C-CTX-1157 analogue at 5.6 min detected in a black moray (*Muraena augusti*) from El Hierro (Canary Islands, Spain)

3.2.2. Madeira archipelago samples

In 38 out of 66 samples, C-CTX1 was detected as main compound responsible of the CTX-like toxicity. In the remaining 28 samples neither C-CTX1 nor any other P-CTX with standard available were detected. The lack of CTXs in these samples can be justified by considering the same arguments indicated in Section 3.2.1. It is important to highlight that the samples from this region showed a higher concentration of C-CTX1, specifically the samples from the Selvagens Islands (Portugal).

3.3. CTXs profile characterization

The approach used to characterize the CTX profile was to couple the sample fractionation through HPLC developed and already reported in previous deliverable, with the toxicity evaluation of each fraction with N2a to be further analysed by LC-MS/MS to identify the responsible toxins [8]. The LC-MS/MS confirmatory method developed was used to characterize these compounds due to the ability to monitor different ion transition and specific fragments that allow the confirmation in contrast with the selection of the single sodium adduct. Following this approach, it was concluded that C-CTX1 is the main responsible of the toxicity in the samples fractionated, and that several analogues of C-CTXs (C-CTX-1157, C-CTX-1127 and C-CTX1 isomers) seem to be present in these samples, confirming that the profile of the contamination is similar to that detected in the Caribbean Sea [12] (Scheme 1).

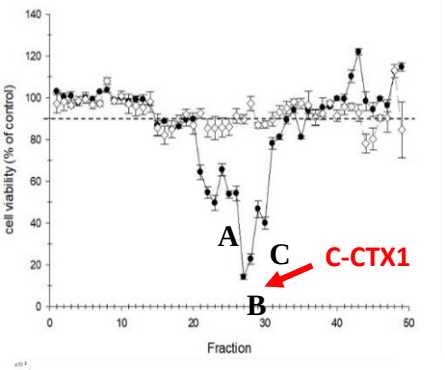


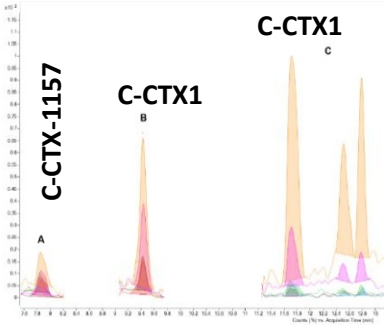
Peak	1	2	3	4
Fractions	19–22	23–28	29	32–33
Retention Time (min)	7.6	9.4	10.6	10.8
$[M+K]^+$	1195.6	1179.6	1165.6	n.d.
$[M+Na]^+$	1179.6	1163.6	1149.6	1163.6
$[M+NH_4]^+$	1174.6	1158.6	1144.6	n.d.
$[M+H]^+$	1157.6	1141.6	1127.6	n.d.
$[M+H-nH_2O]^+$ n range	1–3	1–3	1–4	1–3
C-CTX identification *	C-CTX-1157	C-CTX1	C-CTX-1127	Unknown

Scheme 1. Example of the approach used to characterize the CTX profile of a *Seriola Fasiata* involved in a Ciguatera Fish Poisoning outbreak in Tenerife in 2008.

This approach was extended to other samples from different regions such as Selvagens Islands or Canary Islands, being always C-CTX1 the main toxic analogue (Scheme 2).

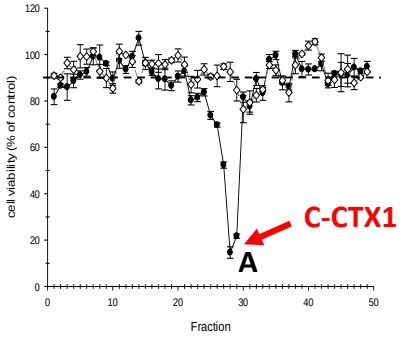
Canary Island sample with 0.49 ng/g (Lutjanus Cyanopterus)

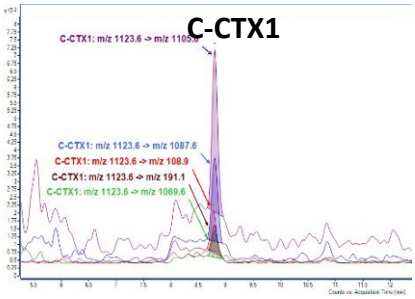




Peak	A	B	C
Fraction	23	25-28	30
Time (min)	7.85	9.4	**11.7**, 12.5, 12.8
$[M+K]^+$	1195.6	1179.6	n.d
$[M+Na]^+$	1179.6	1163.6	1163.7
$[M+NH_4]^+$	1174.6	1158.6	1158.6
$[M+H]^+$	1157.6	1141.6	1141.6
$[M+H-nH_2O]^+$ n range	1-3	1-3	1-3
C-CTX identification*	C-CTX-1157 isomer	C-CTX1	C-CTX1 isomer

Selvagens Islands sample with 0.35 ng/g (Bodianus scrofa)





Peak	A
Fraction	25-28
Time (min)	9.4
$[M+K]^+$	1179.6
$[M+Na]^+$	1163.6
$[M+NH_4]^+$	1158.6
$[M+H]^+$	1141.6
$[M+H-nH_2O]^+$ n range	1-3
C-CTX identification*	C-CTX1

Scheme 2. Results of other examples using the selected approach to characterize the CTX profile of the contaminated samples.

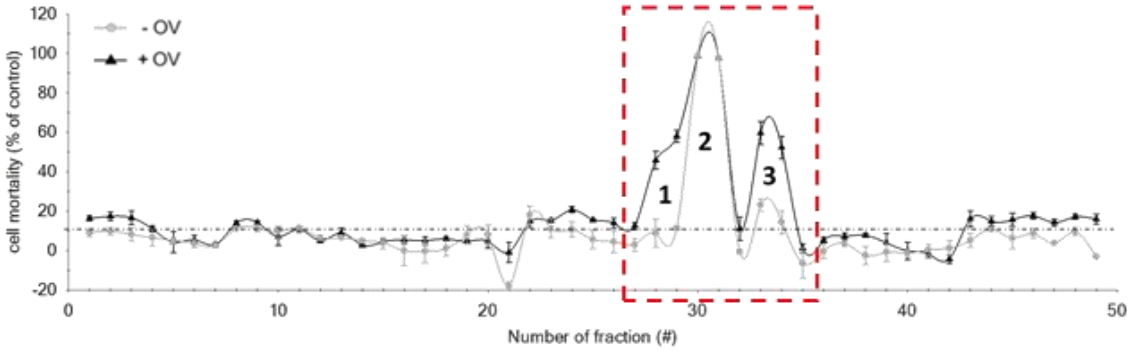
Therefore, the CTXs profile of the samples from this geographical region, Canary Islands (Spain) and Madeira archipelago (Portugal), was confirmed, with C-CTX1 as the main compound responsible of the contamination.

3.4. Analysis of *Gambierdiscus* and *Fukuyoa* extracts by LC-MS/MS

The LC-MS/MS analysis of the dinoflagellate samples supplied by the SG3 did not reveal a CTX profile since neither P-CTXs nor C-CTX1 were detected (Table A1). As a consequence of that, the approach developed by the research group in this SG4 using HPLC/N2a/LC-MS/MS was used to characterize the toxic profile of these *Gambierdiscus sp* and to determine the presence of other toxic analogues also reported in the literature for these particular strains.

3.4.1. Characterization of *Gambierdiscus* extract by HPLC/N2a/LC-MS/MS

One of the *Gambierdiscus* extracts with the higher CTX-like toxicity in the N2a (*G. australes*, IRTA-SMN-17-271, from Balearic Islands (Spain), see Table A1) was characterized by using the combination of sample fractionation by HPLC with toxicity evaluation by N2a and final confirmation of the toxic fractions by LC-MS/MS (*Manuscript in preparation*). The sample showed three toxic peaks after the sample fractionation and toxicity evaluation (Figure 14).



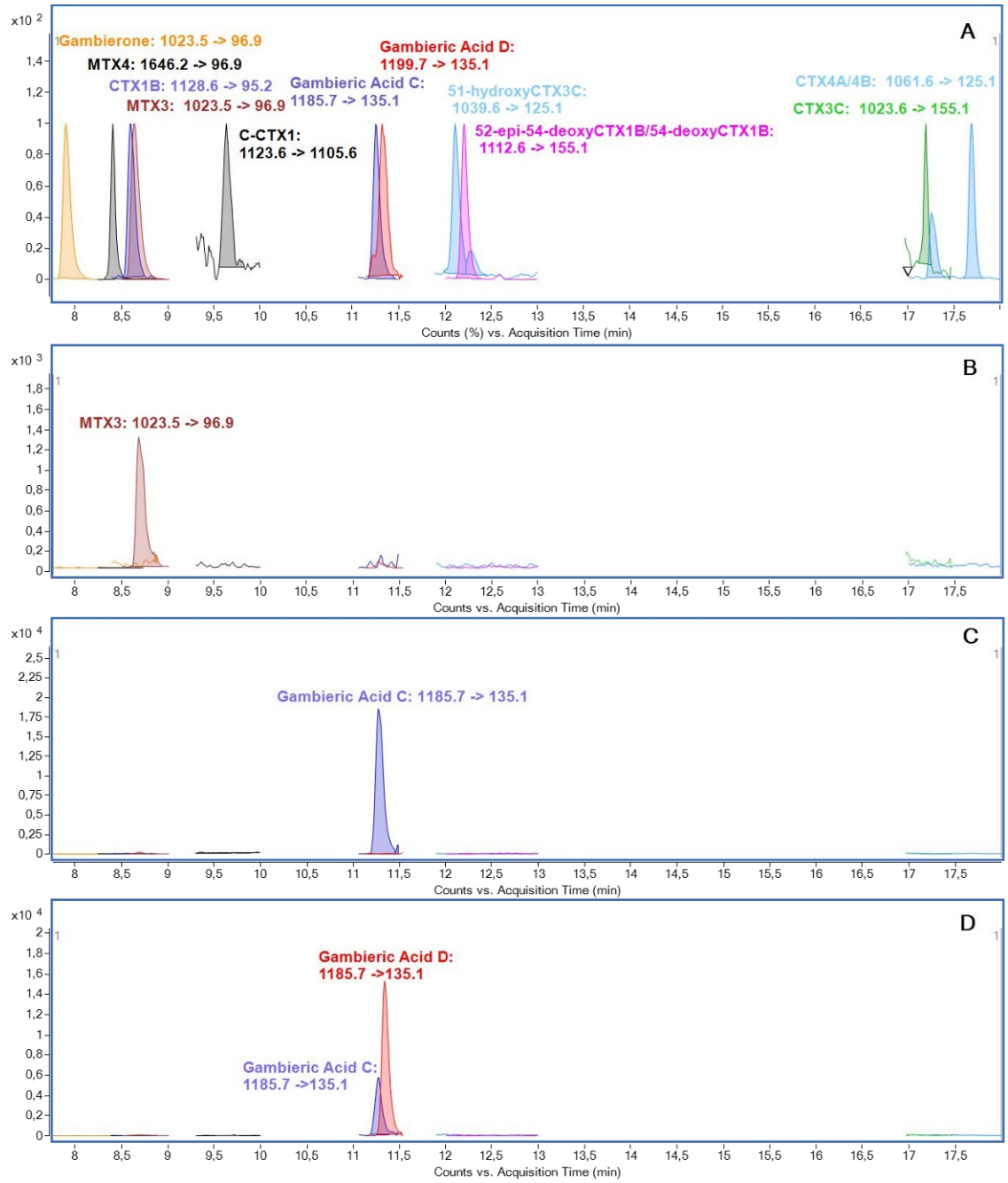
Cytotoxicity effect of fractions in presence (—▲—) and absence (—●—) of OV. The red box highlights the main toxic region including the fractions ranging from 28 to 34, where 3 peaks with a cytotoxic activity can be distinguished.

Figure 14: Number of fractions collected after HPLC-C18 fractionation *vs* cell mortality relative to control cells

The LC-MS/MS method was used to confirm the presence of compounds responsible for the toxicity found by N2a. As a result of this initial confirmation, MTX3 was detected in the fraction 28 (positive for toxicity by N2a), whilst gambieric Acid C was detected in fraction 33, and the coelution of gambieric Acid C and D was detected in fraction 34 (Figure 15).

On the other hand, the confirmation of the toxicity corresponding to the chromatographic peak 2 was achieved in both fractions treated and untreated with OV. This toxicity can be atributed to Maitotoxins (MTX), e.g MTX1 or MTX4. The confirmation carried out by LC-MS/MS allowed to identify the presence of a novel MTX compound in the fractions 30 and 31 (corresponding to the chromatographic peak 2). This novel MTX compound shows similar ion pattern and fragmentation as MTX1 or MTX4.

The toxic profile of the *Gambierdiscus sp* analysed in this SG includes MTXs and gambieric acids C and D. The presence of these compounds was also confirmed by LC-HRMS. No C-CTX1 or P-CTXs or known potential precursors of these compounds were detected in any of the toxic fractions analysed. This could indicate that these compounds might be present at very low levels, or that the correlation with the CTXs profile is still unknown for these particular *Gambierdiscus* and *Fukuyoa* strains.



A) Reference Material containing a mixture of P-CTXs, C-CTX1, MTXs, Gambierone and Gambieric Acids. B) MTX3 detected in the CTX-like toxic Fraction 28. C) Gambieric Acid C detected in the CTX-like toxic Fraction 33. D) Coelution of gambieric acid C and D in the CTX-like toxic Fraction 34.

Figure 15: LC-MS/MS analysis in MRM mode showing the most remarkable transition for each compound in order to simplify and having an overview of the compounds present in the *G. australes*

3.5. Preparation of Fish Tissue Reference Material (FTRM)

3.5.1. Preparation of C-CTX1 solutions

As described above, C-CTX1 was isolated from fish tissue and liver to maximize the concentration of C-CTX1. This is due to the generally low concentration of the toxin in the fish samples collected during this project. The efficiency of the isolation and purification of C-CTX1 was highly dependent on the matrix complexity, and this justifies the additional steps included on the sample preparation protocol. The full purification of this toxin was compromised by the limited concentration, which also required a complete purification procedure with multiple steps, affecting the recovery of the toxin. Despite of this, the optimization carried out on the protocols developed allowed to conclude that the C-CTX1 solutions obtained are suitable as laboratory reference materials with an adequate estimation of the concentration. The reference material cannot be considered certified, but appropriate to be used for quantitation purposes.

Different concentrations of C-CTX1 have been prepared depending on their source, being with higher levels those ones extracted from livers than the ones extracted from fish tissue. The C-CTX1 solutions extracted from liver are scarce and therefore their adequate use needs to be ensured. An important consideration to take into account is the possible presence of a methylated congener of C-CTX1 (C-CTX1-Me) in some of the C-CTX1 fractions. This C-CTX1-Me is a 56-methoxy congener resulting from the transformation of C-CTX1 during the isolation and purification process, as published in [13]. This transformation takes place during the HPLC fractionation, in which C-CTX1 is selectively collected in a single fraction. The presence of slight acidic conditions in the mobile phase triggers the transformation of C-CTX1 into C-CTX1-Me. This transformation has been also observed in the standard of C-CTX1 kindly provided by Dr. Dickey (Figure 16) and used in these studies. Therefore, the concentrations of this methylated congener are also included in the C-CTX1 toxin solutions prepared.

The preparation of these fractions from fish tissue is summarized as follows: 19 kg of fish tissue were obtained from the autoclave and homogenization of an initial amount of 50 kg of fish. Then, 1 kg fractions were extracted and purified following the conditions described in section 2.3.1. A total of 48 fractions of toxin solutions of C-CTX1 were finally obtained (Table 8). C-CTX1 concentrations ranged from 1.350 ng/g to 0.015 ng/g. 20 out of 48 fractions were above the C-CTX1 guidance level of 0.100 ng/g proposed by the USA-FDA. 31 out of 48 fractions contained traces of C-CTX1-Me with concentrations ranging from 0.153 ng/g to 0.015 ng/g.

The C-CTX1 solutions once characterized were evaporated to dryness and kept at -20ºC in vials.

Table 8: List of toxin solutions of C-CTX1 obtained from the extraction of the autoclaved fish tissue. The dotted line showed samples above the threshold of C-CTX1 guidance level proposed by the USA-FDA (0.1 ng/g). n.d. not detected

RM ID	C-CTX1 (ng)	C-CTX1 (ng/g)*	C-CTX1-Me (ng)	C-CTX1-Me (ng/g)*
F64-6-Fr2	40.490	1.350	1.353	0.045
F0071-BS-4-Fr2	36.527	1.218	1.047	0.035
F178-1-Fr3	27.748	0.925	3.409	0.114
F178-2-Fr3	27.366	0.912	1.881	0.063
F178-3-Fr3	23.894	0.796	1.098	0.037
F0071-BS-2-Fr2	23.308	0.777	1.320	0.044
F0071-BS-5-Fr2	18.365	0.612	0.623	0.021
F64-4-Fr2	12.982	0.433	0.920	0.031
F0071-BS-3-Fr2	9.644	0.321	1.227	0.041
F349-F350-1-Fr3	8.236	0.275	1.044	0.035
F0071-BS-6-Fr2	7.371	0.246	0.449	0.015
F64-5-Fr2	6.906	0.230	n.d.	n.d.
F350-1-Fr2	6.026	0.201	0.477	0.016

F178-2-Fr2	5.420	0.181	n.d.	n.d.
F0071-BS-1-Fr3	5.087	0.170	2.534	0.084
F64-1-Fr2	4.621	0.154	n.d.	n.d.
F178-3-Fr4	4.583	0.153	0.689	0.023
F64-3-Fr2	3.730	0.124	0.470	0.016
F0071-BS-1-Fr2	3.683	0.123	n.d.	n.d.
F64-2-Fr3	3.037	0.101	1.098	0.037
F0071-BS-6-Fr3	2.924	0.097	1.649	0.055
F0071-BS-4-Fr3	2.750	0.092	3.695	0.123
F350-1-Fr3	2.717	0.091	3.216	0.107
F0071-BS-5-Fr3	2.433	0.081	1.601	0.053
F64-5-Fr3	2.345	0.078	0.688	0.023
F0071-BS-2-Fr3	2.291	0.076	4.605	0.153
F64-3-Fr3	2.218	0.074	3.235	0.108
F64-1-Fr3	2.183	0.073	1.098	0.037
F710-1-Fr2	2.092	0.070	n.d.	n.d.
F349-F350-1-Fr2	2.076	0.069	n.d.	n.d.
F0071-BS-3-Fr3	1.926	0.064	2.225	0.074
F64-4-Fr3	1.671	0.056	1.359	0.045
F64-2-Fr2	1.626	0.054	n.d.	n.d.
F350-1-Fr4	1.098	0.037	n.d.	n.d.
F349-1-Fr3	0.993	0.033	0.436	0.015
F349-1-Fr2	0.715	0.024	n.d.	n.d.
F0071-BS-3-Fr4	0.667	0.022	n.d.	n.d.
F0071-BS-4-Fr4	0.653	0.022	0.905	0.030
F0071-BS-2-Fr4	0.648	0.022	n.d.	n.d.
F178-3-Fr2	0.616	0.021	n.d.	n.d.
F0071-BS-3-Fr1	0.592	0.020	n.d.	n.d.
F710-1-Fr4	0.561	0.019	n.d.	n.d.
F64-6-Fr1	0.536	0.018	n.d.	n.d.
F178-2-Fr4	0.519	0.017	0.622	0.021
F178-1-Fr4	0.481	0.016	1.160	0.039
F64-4-Fr1	0.475	0.016	n.d.	n.d.
F64-6-Fr4	0.454	0.015	n.d.	n.d.
F349-F350-1-Fr4	0.437	0.015	n.d.	n.d.

*If reconstituted in 1 mL.

A pool of 6 kg of fish liver from positive fish containing C-CTX1 was selected to also produce toxin solutions of C-CTX1. Then, 1 kg fractions were extracted and purified following the conditions described in section 2.3.1, resulting in 41 fractions of C-CTX1 toxin solutions (Table 9). C-CTX1 concentrations ranged from 1.896 ng/g to 0.015 ng/g. 20 out of 41 fractions were above the C-CTX1 guidance level of 0.100 ng/g proposed by the USA-FDA. 29 out of 41 fractions contained traces of C-CTX1-Me with concentrations ranging from 1.341 ng/g to 0.015 ng/g.

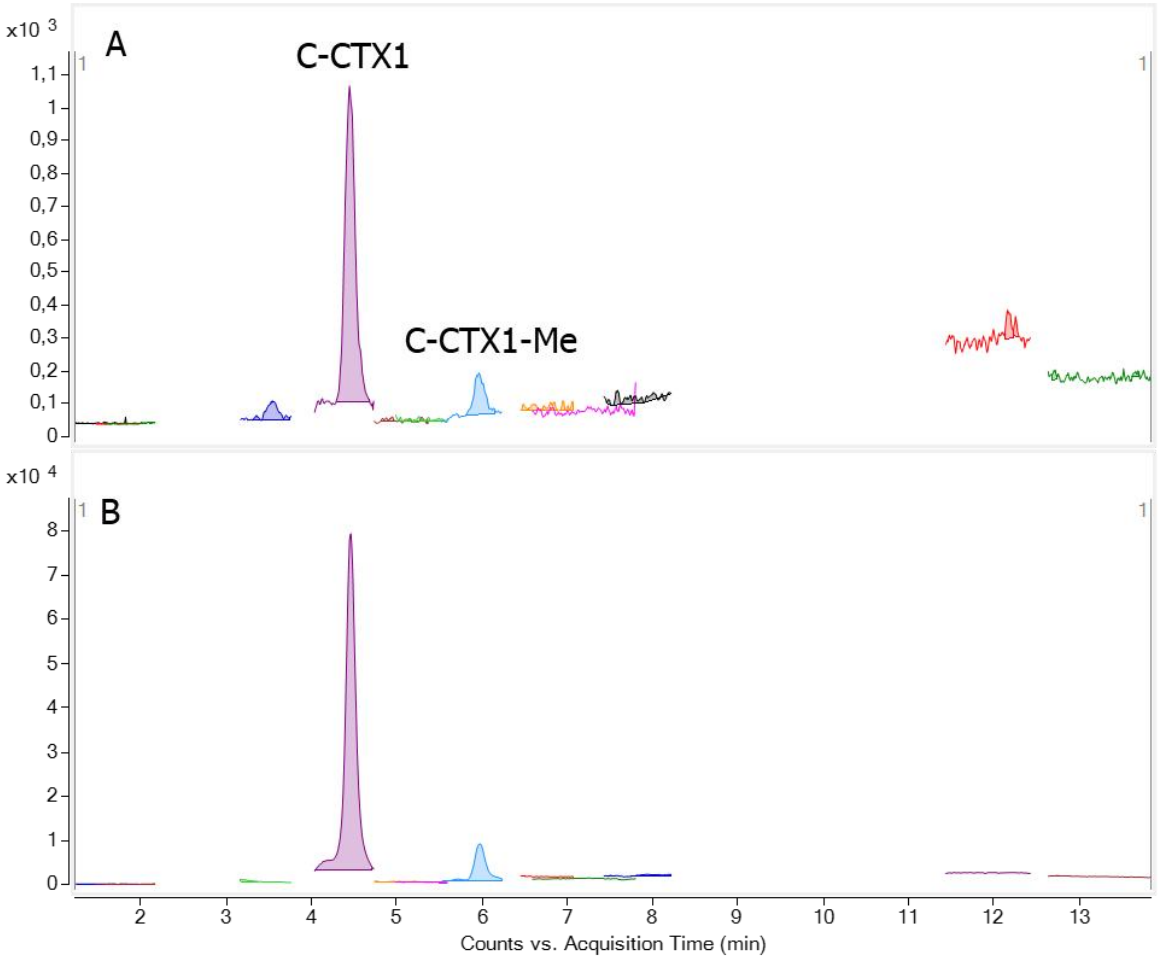
After the characterization of these toxin solutions of C-CTX1 they were evaporated to dryness and kept at -20ºC.

Table 9: List of the toxin solutions of C-CTX1 obtained from the extraction of autoclaved fish liver. The dotted line showed samples above the threshold of C-CTX1 guidance level proposed by the USA-FDA (0.1 ng/g).

RM ID	C-CTX1 (ng)	C-CTX1 (ng/g)*	C-CTX1-Me (ng)	C-CTX1-Me (ng/g)*
LOTE-2-LIV-R1-Fr2	56.888	1.896	9.110	0.304
LOTE-2-LIV-R2-Fr2	42.485	1.416	3.806	0.127
LOTE-2-LIV-R3-Fr2	38.856	1.295	3.895	0.130
LOTE-3-LIV-R2-Fr2	25.737	0.858	2.272	0.076
LOTE-4-LIV-R1-Fr2	14.787	0.493	n.d.	n.d.

LOTE-3-LIV-R1-Fr2	10.750	0.358	0.757	0.025
LOTE-4-LIV-R2-Fr2	10.696	0.357	1.889	0.063
RESIDUE FRACT-Fr2	10.518	0.351	0.814	0.027
LOTE-3-LIV-R3-Fr4	7.844	0.261	n.d.	n.d.
LOTE-3-LIV-R3-Fr2	7.462	0.249	0.749	0.025
LOTE-4-LIV-R5-Fr2	6.596	0.220	0.657	0.022
LOTE-3-LIV-R1-Fr4	5.455	0.182	n.d.	n.d.
LOTE-4-LIV-R3-Fr2	4.984	0.166	0.767	0.026
LOTE-3-LIV-R2-Fr4	4.864	0.162	n.d.	n.d.
LOTE-4-LIV-R4-Fr2	4.831	0.161	0.788	0.026
LOTE-2-LIV-R3-Fr4	4.816	0.161	n.d.	n.d.
LOTE-2-LIV-R2-Fr4	4.806	0.160	n.d.	n.d.
LOTE-2-LIV-R1-Fr4	4.310	0.144	n.d.	n.d.
LOTE-4-LIV-R3-Fr4	3.394	0.113	n.d.	n.d.
LOTE-4-LIV-R4-Fr4	3.298	0.110	n.d.	n.d.
LOTE-4-LIV-R5-Fr1	2.207	0.074	n.d.	n.d.
RESIDUE FRACT-Fr3	2.093	0.070	1.827	0.061
LOTE-4-LIV-R1-Fr1	2.084	0.069	2.573	0.086
LOTE-4-LIV-R2-Fr1	1.889	0.063	n.d.	n.d.
LOTE-2-LIV-R3-Fr3	1.640	0.055	18.253	0.608
LOTE-2-LIV-R2-Fr3	1.390	0.046	40.236	1.341
LOTE-2-LIV-R1-Fr3	1.349	0.045	26.234	0.874
LOTE-4-LIV-R3-Fr1	1.243	0.041	n.d.	n.d.
LOTE-3-LIV-R1-Fr3	1.240	0.041	4.455	0.148
LOTE-3-LIV-R2-Fr3	1.061	0.035	3.089	0.103
LOTE-3-LIV-R3-Fr3	1.040	0.035	1.729	0.058
LOTE-3-LIV-R2-Fr1	0.875	0.029	0.457	0.015
LOTE-2-LIV-R1-Fr5	0.633	0.021	3.110	0.104
LOTE-2-LIV-R3-Fr5	0.538	0.018	4.239	0.141
LOTE-3-LIV-R3-Fr1	0.445	0.015	0.749	0.025
LOTE-3-LIV-R1-Fr5	n.d.	n.d.	3.254	0.108
LOTE-4-LIV-R1-Fr3	n.d.	n.d.	3.552	0.118
LOTE-4-LIV-R2-Fr3	n.d.	n.d.	2.661	0.089
LOTE-4-LIV-R3-Fr3	n.d.	n.d.	1.283	0.043
LOTE-4-LIV-R4-Fr3	n.d.	n.d.	1.310	0.044
LOTE-4-LIV-R5-Fr3	n.d.	n.d.	1.528	0.051

n.d. not detected.
*If reconstituted in 1 mL.



A) C-CTX1 pure standard (1 ng/mL); B) C-CTX1 toxin solution (LOTE-2-LIV-R1-Fr2).

Figure 16: Example of a chromatogram after the LC-MS/MS analysis selecting the *m/z* $[M+Na]^+$ as precursor and product ion

3.5.2. Characterization of FTRM: Homogeneity and Stability studies

The homogeneity and stability studies of the FTRM developed in this project were limited by the restrictions imposed by the COVID situation concerning the lock down of the laboratory activities.

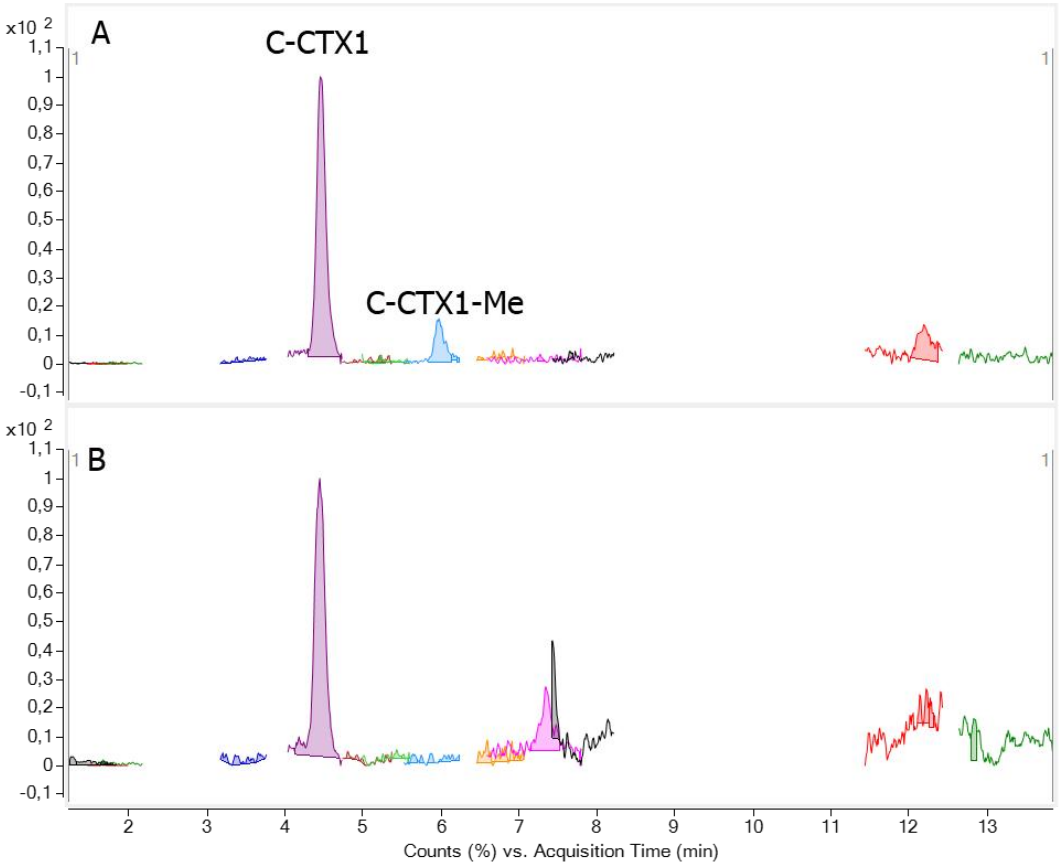
A contingency plan was established to minimise the effects of this situation.

The evaluation of the homogeneity of the FTRM was carried out selecting randomly one sample from each batch of the four different materials. Each sample was analysed by triplicate under repeatability conditions in the same day, following the LC-MS/MS SOP developed in this SG4 (Deliverable 2).

The stability of the materials was also evaluated by analysing triplicates of each batch of the four different FTRM selected randomly and this evaluation was carried out three times during one month. A mean concentration valued was assigned to C-CTX1 after this evaluation (Table A2, Figure 17).

Samples were selected to cover a wide range of C-CTX1 concentrations ranging from 0.039 to 0.284 ng/g. The detection of C-CTX1 in the FTRM with lower concentrations (e.g. FTRM-EFSA-ULPGC-F0349 or FTRM-EFSA-ULPGC-F0350) will be helpful to assess the sensitivity of the methods implemented in the laboratories. On the other hand, the FTRM with higher content of C-CTX1, above the C-CTX1

guidance level of 0.100 ng/g (e.g. FTRM-EFSA-ULPGC-F0710 and FTRM-EFSA-ULPGC-F0178) will guarantee that the laboratories can detect samples containing C-CTX1 which might cause CP.



A) C-CTX1 pure standard (1 ng/mL); B) C-CTX1 FTRM amberjack *(Seriola sp.)* (EFSA-ULPGC-F0349).

Figure 17. Chromatogram of the LC-MS/MS analysis selecting the *m/z* $[M+Na]^+$ as precursor and product ion

4. Conclusions

An optimised LC-MS/MS method was developed, in which the optimization of the conditions for the sample pre-treatment, in particular extraction and purification steps, has been critical. The optimized LC-MS/MS method developed allows the sensitive determination of the CTXs present in fish flesh according to the recommended safety limits described in the literature. In the absence of the adequate analytical standards, the monitoring of sodium adducts, water losses and characteristics fragments was used as a contingency for monitoring CTXs, but further confirmation by HRMS is still required. The use of sodium adduct allows a very good sensitivity.

Under the optimized conditions, LC-MS/MS allowed the identification of C-CTX1 as the main responsible for the CTX contamination of the fish samples from Canary Islands and Madeira archipelago, which had been screened as positive by cell assay (N2a).

An LC-HRMS method was evaluated and optimised selecting the best conditions for detection CTXs, including analogues from the Pacific and Atlantic Oceans. The HRMS methodology based on Quadrupole-

Time-of-flight technology remains less sensitive than the low resolution mass spectrometry approach using triple-stage quadrupole technology. HRMS detection is powerful for the confirmation of C-CTX1 and has been applied to confirm C-CTX1 in a reference material developed by University of Vigo and in a fish sample from the Selvagens Islands (Portugal). Limits of detection and quantification are significantly higher for HRMS, which currently results in the need for a significantly higher sample intake and additional clean-up steps.

The concentrations of C-CTX1 in the samples analysed was generally low, and only few samples were above the recommended limits (0.1 ng/g). This complicated the use of these samples for the preparation of reference materials and a contingency plan was established to accomplish this objective. The task of preparation of standards and reference materials has been adapted due to the unavailability of samples contaminated with adequate concentration of CTXs. The contingency plan in place was the preparation of FTRM contaminated with C-CTX1, found as the main responsible for the CTX toxicity in the evaluated samples, and C-CTX1 isolated from fish extracts after fractionation.
Vials containing C-CTX1 solutions evaporated to dryness have been kept in the freezer at -20C for further distribution. Additional reference materials have been also prepared including the fish matrix (FTRM), to help laboratories to evaluate, not only the sample preparation protocols, but also the matrix effect, as a critical factor to control when performing MS/MS analysis. These FTRM have been prepared as autoclaved homogenates and after characterization by LC-MS/MS were also kept in the freezer at -20ºC

Regarding the characterization of the toxic profiles of the *Gambierdiscus* and *Fukuyoa* extracts analysed in this SG4 from the Canary Islands and the Mediterranean Sea, it was found that the toxicity was mainly associated with MTXs, Gambierone and Gambieric acids. No precursors of CTXs, in particular C-CTX1, were found in any of the extracts analysed. For the first time, it was possible to study the full profiles of *Gambierdiscus* and *Fukuyoa* strains from the Mediterranean Sea (Balearic Islands and Crete). While no CTXs were detected in *Gambierdiscus australes*, *G. excentricus* and *Fukuyoa paulensis*, gambierone, 44-methyl-gambierone (= maitotoxin-3), gambieric acids C and –D and a putative gambierone analog were tentatively identified for the first time. Interestingly, none of the 6 *G. australes* strains from the Balearic Islands produced maitotoxin (MTX1), previously reported to be produced by *G. australes* strains from Japan.

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Abbreviations

CTX	Ciguatoxin
EC-C18	End Caped-C18
EFSA	European Food Safety Authority
EU	European Union
eV	Electronvolt
FDA	Food and Drug Administration
FLD	Fluorescence detection
FTRM	Fish Tissues Reference Materials
GPC	Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography
IFREMER	Institute Français de Recherche pour l`Explotation de la Mer
IRTA	Institut de recerca i tecnologia agroalimentàries

IUSA	Instituto universitario de sanidad animal y seguridad alimentaria
LC	Liquid Chromatography
LC-HRMS	Liquid Chromatography High Resolution Mass Spectrometry
LC-MS/MS	Liquid Chromatography- tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MTX	Maitotoxin
N2a	Neuroblastoma assay
OV	Ouabain/Veratridine
Ppb	Parts per billion
Ppm	Parts per million
S/N	Signal/Noise
SA	Specific Agreement
SA4	Specific Agreement 4
SG	Specific Grant
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
UV	Ultraviolet

Annex A

Table A1. Detailed information about the dinoflagellate extracts analyzed by LC-MS/MS

Species	Strain code	Location	Number of cells extracted	Volume of culture (L)	CTX-like (fg CTX1B equiv./cell)
G. australes	IRTA-SMM-17-189	Torret, Menorca, Balearic Islands, Spain	17 134 000	20	83 ± 12 [a]
G. australes	IRTA-SMM-17-162	St. Adeodat, Menorca, Balearic Islands, Spain	27 811 000	20	101 ± 7.5
G. australes	IRTA-SMM-17-164	St. Adeodat, Menorca, Balearic Islands, Spain	4 257 000	20	> 62.5
G. australes	IRTA-SMM-17-271	Macarella, Menorca, Balearic Islands, Spain	14 007 000	20	271 ± 29
F. paulensis	IRTA-SMM-17-209	Sacaleta, Menorca, Balearic Islands, Spain	6 964 000	20	16 ± 1.7 (a)
G. australes	IRTA-SMM-17-253	Anguila, Menorca, Balearic Islands, Spain	13 735 000	20	164 ± 16
G. australes	IRTA-SMM-17-244	Camp de Mar, Mallorca, Balearic Islands, Spain	4 121 000	5	155 ± 25
***Gambierdiscus* sp.2**	0010G-CR-CCAUTH	Kolimpari, Crete, Greece	2 300 000	5	NQ
G. excentricus	IRTA-SMM-17-407	Playa de vueltas, La Gomera, Canary Islands, Spain	6 084 000	5	>794 (NQ)

NQ: not quantifiable.

(a): CTX-like toxicity evaluated in [14].

Table A2. List of the autoclaved fish tissue reference materials

Sample ID	Island	Species	Autoclaved FTRM available (kg)	C-CTX1 (ng/g)
FTRM-EFSA-ULPGC-F0178	Tenerife	Amberjack *(Seriola sp.)*	6	0.284 ± 0.019
FTRM-EFSA-ULPGC-F0710	Lanzarote	Dusky grouper *(Epinephelus marginatus)*	1.5	0.102 ± 0.017
FTRM-EFSA-ULPGC-F0350	Lanzarote	Dusky grouper *(Epinephelus marginatus)*	15	0.081 ± 0.007
FTRM-EFSA-ULPGC-F0349	Lanzarote	Amberjack *(Seriola sp.)*	3	0.039 ± 0.006