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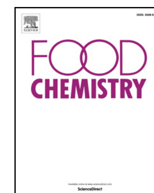

Implementation of liquid chromatography tandem mass spectrometry for the analysis of ciguatera fish poisoning in contaminated fish samples from Atlantic coasts

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ABSTRACT

The increased emergence of Ciguatera Fish Poisoning in the Canary Islands and Madeira demanded the development of confirmatory methods by liquid chromatography coupled to tandem mass spectrometry to characterize the risk. Efforts were particularly focused on the optimization of sample pretreatment, especially in the sample cleanup step, to efficiently remove matrix interferences as a critical factor to consider in mass spectrometry detection. Two different LC-MS/MS approaches have been used for confirmation purposes, the first one using the sodium adduct as precursor and product ion to allow an increased sensitivity in the detection, whereas additional fragments were also monitored for further confirmation. The optimized conditions above mentioned allowed the confirmation of Caribbean Ciguatoxin-1 as the main responsible for the samples analyzed from these geographical areas, while the presence of a new hydroxyl metabolite of C-CTX1 was also confirmed in one sample analyzed in this study.

1. Introduction

Ciguatera Fish Poisoning is an endemic illness in tropical and subtropical regions of the world (Lipp & Rose, 1997) in which lipophilic polyether ciguatoxins (CTXs) are involved. The risks to human health related to the consumption of CTXs in fish has been assessed by the EU Food Safety Authority (EFSA) Panel on Contaminants (EFSA Panel on Contaminants in the Food Chain, 2010). CTXs occur in fish as a result of bioaccumulation and metabolic transformation of precursor gambiertoxins produced by the benthic dinoflagellate Gambierdiscus toxicus. CTX toxins are mainly found in Pacific, Caribbean and Indian Ocean regions and are classified as Pacific (P), Caribbean (C) and Indian Ocean (I) CTXs (Lewis, 2000). CTXs are lipophilic, ca. 1100 Da, polyether compounds that are heat and pH stable. These toxins are selective activators of voltage-dependent Na channels in cells (Murata, Legrand, Ishibashi, & Yasumoto, 1989; Scheuer, Takahashi, Tsutsumi, & Yoshida, 1967; Strachan, Lewis, & Nicholson, 1999; Vetter et al., 2012). The chemical structure of the Indian CTXs, have not yet been resolved (Hamilton, Hurbungs, Vernoux, Jones, & Lewis, 2002). CTX1B is considered the most toxic congener, with an action level of 0.01 $ng{\cdot}g^{-1}$ while C-CTX1 is estimated to be 10 times less toxic (Dickey & Plakas, 2010).

CTXs are formed by the oxidation of gambiertoxins produced by dinoflagellates of the genus Gambierdiscus spp. and Fukuyoa spp., which are epiphytes of macroalgae growing in coral reef areas. Macroalgae serve as transmission vectors to fauna in the food web and giving rise to trophic bioaccumulation and metabolic transformation (Lewis & Holmes, 1993). Although Yasumoto and collaborators have elucidated 19 novel CTXs analogues by fast-atom bombardment tandem mass spectroscopy (Yasumoto et al., 2000), the identification of these toxins in the food web is hampered by the process of biotransformation which gives rise to an even larger number of CTX analogues, and the lack of certified reference materials (Amade, Mehiri, & Lewis, 2014).

Different strategies have been used to detect CFP toxins. Most of them involve initial toxicity screening using, among others, mouse bioassay (MBA) (Yasumoto, Raj, & Bagnis, 1984), neuroblastoma cell

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assays (Manger et al., 1995) or immunochemical approaches (ELISA) (Tsumuraya, Fujii, & Hirama, 2014), followed by a confirmation step which includes a chromatographic separation coupled to mass spectrometric detection. High Resolution Mass Spectrometry (HRMS) is also used as a powerful tool for CTXs confirmation based on their exact mass and isotopic pattern, which is very useful for identification when pure standards are not available (Suzuki, Ha, Uesugi, & Uchida, 2017; Silva et al., 2015). The main disadvantage of HRMS is the lack of sensitivity compared to low-resolution mass spectrometry (MS/MS), which is needed to meet the guidance level of 0.01 $ng \cdot g^{-1}$ CTX1B proposed by the Food and Drug Administration (FDA) (Dickey & Plakas, 2010). Several LC-MS/MS methods are described in the literature for CTXs determination in fishery products (Abraham, Jester, Granade, Plakas, & Dickey, 2012; Lewis, Yang, & Jones, 2009; Yogi, Oshiro, Inafuku, Hirama, & Yasumoto, 2011). The implementation of these methods and in particular assessing the efficiency of sample pretreatment protocols, is hampered by the lack of reference materials, this evaluation is considered a critical factor for a thorough characterization of the risk of CTXs in fish and consequently to the adequate evaluation of the risk caused by the consumption of fish contaminated with CTXs.

The objective of this work is therefore to implement an LC-MS/MS method for the analysis of CFP toxins in contaminated fish samples from Atlantic coasts. Sample pretreatment was considered a critical step needing a suitable evaluation and optimization based on previously described methods with the aim not only to efficiently extract the analyte, but also to minimize the matrix effects without compromising limits of detection and quantitation. To accomplish this aim a sensitive LC-MS/MS method based on the use of Na adduct has been implemented for quantitation while a second method was used for confirmation by selecting specific fragments of the analogues suspected to be involved in the CFP contamination.

2. Materials and methods

2.1. Standards

CTX1B pure standard solution (4460 $ng\,mL^{-1}$) and a Pacific Ciguatoxins mixed qualitative standard that contain: CTX1B, M-seco-CTX3C, 2,3-dihydroxy-CTX3C, 51-hydroxy-CTX3C, 52-*epi*-54-deoxy-CTX1B/54-deoxy-CTX1B, M-seco-CTX4A/B, 2-hydroxy-CTX3C, M-seco-CTX3C − methylacetal, 49-*epi*-CTX3C, CTX4A/CTX4B were kindly supplied by Prof. Takeshi Yasumoto (Japan Food Research Laboratories). C-CTX1 pure standard solution (5 $ng\,mL^{-1}$) was kindly supplied by Dr. Robert Dickey (previously, U.S. Food and Drug Administration) via Dr. Ronald Manger (Fred Hutchinson Cancer Research Center, Seattle, USA).

2.2. Chemicals

Acetone, diethyl ether (Et_2O), methanol (MeOH), water (H_2O), hexane and ethyl acetate (AcOEt) used for sample preparation were HPLC grade (Merck KGaA, Darmstadt, Germany). MeOH, acetonitrile (ACN), formic acid and ammonium formate used for LC-MS/MS analysis were LC-MS grade (Merck KGaA, Darmstadt, Germany). H_2O for LC-MS/MS analysis was LC-MS grade (J. T. Baker, Batch No: 0000148893)

2.3. Samples

Different species of fish from Canary Islands (Spain), Madeira and Selvagens Islands (Portugal) were collected. Fish samples screened as positive for CTX-like toxicity by N2a cell assay at the Instituto Universitario de Sanidad Animal Seguridad Alimentaria (IUSA) (Manger et al., 1995), University of Las Palmas de Gran Canarias (SG3 of EuroCigua project) were submitted for confirmation by LC-MS/MS analysis. The fish tissue was stored at −20 °C prior to LC-MS/MS analysis.

Uncontaminated samples of Red Snapper (*Lutjanus malabaricus*) from German market and imported from Indonesia were used for spiking experiments.

2.4. Sample preparation

Sample pretreatment including extraction and purification steps was carried out following the conditions initially proposed by Yogi et al., 2011 with optimization described in this work, 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 of Et_2O and evaporated to dryness. The solid residue was dissolved in 4.5 mL 90% MeOH. The aqueous MeOH solution was defatted with hexane (9 mL) and evaporated under light nitrogen gas stream. The solid residue was dissolved in 2 mL of 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: 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), was dried under nitrogen stream at 50 °C (Murata, Legrand, Ishibashi, Fukui, & Yasumoto, 1990). 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. 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.

2.5. LC-MS/MS analysis

LC-MS/MS analysis was carried out following two different methods, the first based on the conditions proposed by Yogi et al., 2011, was used for a sensitive determination of CTX isomers and analogues while the second method, based on the conditions described by Abraham et al., 2012, was used for the confirmation of CTXs. Both methods were modified in this work to optimize LC-MS instrument conditions to improve sensitivity. LC-MS/MS analyses was performed using an 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 LC-MS/MS optimized conditions used in this work are briefly described:

Method A: Analytes were separated on a Poroshell 120 EC-C18 column (3.0 X 50 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 MeOH (B). The gradient used was: 78% to 88% B in 10 min and holding for 5 min. The flow rate was 0.4 mL/min, and the injection volume was 1 µL. The mass spectrometer was operated in positive mode in order to monitor sodium adduct ions ($[M+Na]^+$). The collision energy was 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 best sensitivity for all compounds.

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, N_2 at 50 psi; capillary voltage, 5000 V; nozzle voltage: 300 V; fragmentor potential 380 V. Toxins were monitored as follows: CTX1B (*m/z* 1133.6), C-CTX1 (*m/z* 1163.7), M − seco − CTX3C (*m/z* 1063.6), 2,3-dihydroxy-CTX3C (*m/z* 1079.6), 51-Hydroxy-CTX3C (*m/z*

1061.6), 52epi-54deoxy-CTX1B/54deoxy-CTX1B (*m/z* 1117.6), M-seco-CTX4A/B (*m/z* 1101.6), 2-hydroxy-CTX3C (*m/z* 1063.6), M-seco-CTX3C – methylacetal (*m/z* 1077.6), 49-*epi*-CTX3C/CTX3C (*m/z* 1045.6), CTX4A/CTX4B (*m/z* 1083.6)

Method B: LC separation was achieved using a Poroshell 120 EC-C18 (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 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. The flow rate was 0.4 mL/min, and the injection volume was 5 µL. The mass spectrometer was operated in positive 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.

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.

3. Results and discussion

3.1. Sample pretreatment

In the face of the extremely limited availability of reference toxins, recovery tests on spiked sample had to be restricted to the minimum. The Pacific-type toxins were tested for the first time using fish from Canary Island for the applicability of the LC-MS method in the literature. The conditions used for sample pretreatment were evaluated and optimized in order to achieve and adequate recovery of the analytes. The complexity of the fish tissue matrix is a critical factor affecting efficiency of analyte recovery and thus the reliability of the LC-MS/MS analysis. To reduce matrix interference different sample amounts, 15 g and 25 g, were selected for initial extraction. The efficiency of the extraction was evaluated through recovery experiments using matrix match standard addition with CTX1B. The amount of fish sample (15 g) was selected as the optimal sample size to minimize the matrix effect without compromising analyte recovery and the sensitivity of LC-MS/MS analysis. The small amount of CTX standard available limited the number of standard addition experiments carried out through the extraction evaluation process. Accordingly, and in lieu of standard addition experiments, fish samples naturally contaminated with CTXs that had been previously analyzed were used as “laboratory reference materials” to assess possible analyte losses. Slight modifications, with an additional centrifugation step to reduce emulsions, were included in the extraction process to increase its efficiency by increasing the analyte recovery.

The lipophilic character of CTXs makes them difficult to separate from the high levels of lipids and fatty acids in fish tissues, thus compromising the efficiency of CTX analyte recovery and chromatographic resolution during LC-MS/MS analysis. Accordingly, additional prepurification steps are required to remove these interferences.

In the present study the purification of the extract was carried out using normal and reversed-phase SPE mechanisms with the aim of selectively removing interferences based on polarity. Initial experiments showed that isocratic elution of Florisil SPE resulted in poor analyte recovery and separation from matrix lipids (Yogi et al., 2011). Stepwise gradient elution with increasing solvent polarity improved analyte recovery from 58.5% to 77.2% (Table 1) and separation from matrix lipids. Better recoveries were found for CTX1B in standard addition experiments. The evaluation of C18 SPE was also carried out using CTX1B standard addition, and a recovery of 69.3% was obtained. After evaluating separately both clean-up cartridges, the whole clean-up step, Florisil SPE followed by C18 SPE, was evaluated obtaining a recovery of 56.7% in matrix matched samples by comparing with neat solution standard (see Table 2).

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 Wash: 3 mL AcOEt	Load: 2 mL sample extract in MeOH/H_2O (60%)
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%)

Matrix effects on mass spectrometry as signal suppression or enhancement in the ionization-evaporation process was evaluated in this study using CTX1B standard addition to SPE pre-treated fish extracts from uncontaminated fish tissue. The final eluate from C18 SPE pretreatment was spiked with a known amount of CTX1B and analyzed by LC-MS/MS. Signal intensity from standard addition was compared to pure standards at the same concentration. A 22% signal suppression was observed. The recovery of the sample pretreatment protocol that is not affected by the matrix would then correspond to 72.7%. Whereas recovery on the whole LC-MS/MS protocol including signal suppression would correspond to 57.6%. A correction factor of 1.74 for final calculation of the CTX content in fish matrices would also assume that all CTXs, isomers and analogues, would behave similarly to CTX1B in the protocol presented here as well as a similar matrix effect in the different fish species.

3.2. LC-MS/MS analysis

As described above, two LC-MS/MS methods were used in this work. Method A is based on Yogi et al., 2011, while Method B follows Abraham et al., 2012. Both methods have been slightly modified and optimized according to the performance characteristics of the mass spectrometer used in this work. The lack of pure standards and reference materials has been a critical limitation to perform the full validation of these methods. For this reason the most relevant performance criteria have been selected to perform the quantitation, including linear range, LODs, LOQs and a minimal evaluation of performance criteria as robustness was also evaluated. These criteria have been evaluated for Method A since this method has been used for quantitation purposes due to its ability to monitor the sodium adduct of a particular ion with a high sensitivity. Since method B has been only used to confirm the presence of C-CTX1 found as the main responsible

Table 2
Results of the recoveries for the optimized clean-up conditions.

Adsorbent	Recoveries, mean ± SD, %; (%RSD) Intra-day (n = 3)
	CTX1B
500 mg Florisil	*77.2* ± 3.3 (4.3)
500 mg C18	69.3 ± 3.3 (4.8)
500 mg Florsil + 500 mg C18	57.6 ± 2.4 (4.2)

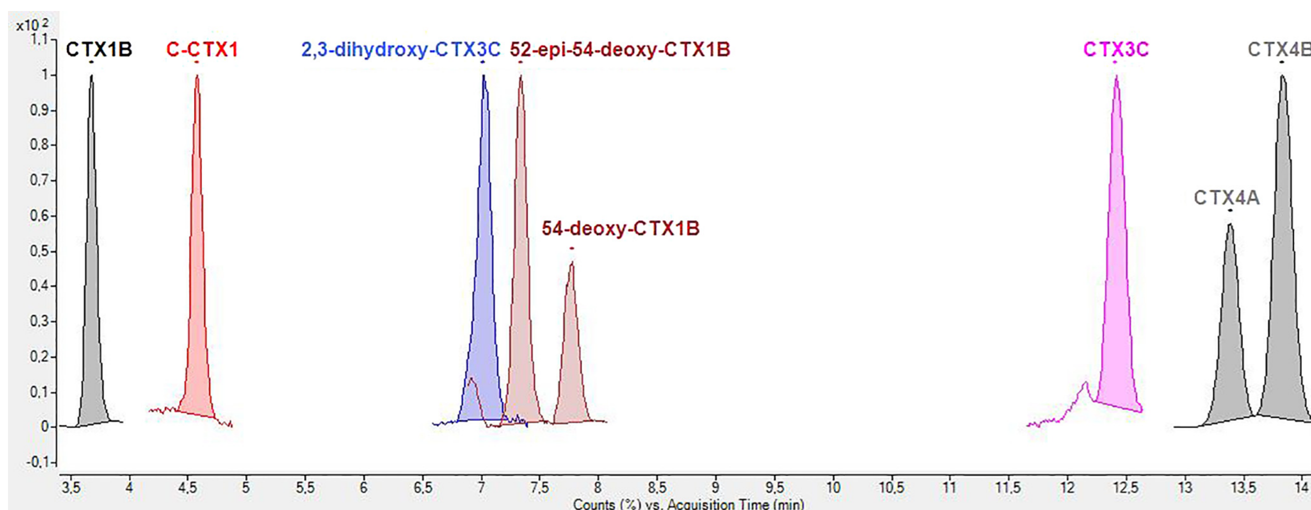

Fig. 1. Example of LC-MS/MS analysis with Method 1 of a mixture of standards of Pacific Ciguatoxins and Caribbean Ciguatoxin-1 kindly provided by Professor Yasumoto and Dr. Dickey and Dr. Manger.

of the CFP in the contaminated samples, the evaluation of these performance criteria was not considered strictly necessary thus allowing to minimize the use of pure standards.

Method A: Aqueous MeOH mobile phase with ammonium formate and formic acid modifiers resulted in the production of high intensity $[M+Na]^+$ ions. The selection of highly stable $[M+Na]^+$ CTXs ions as precursor and product ions by MRM simplified the identification and quantitation of CTXs analogues. The high sensitivity achieved with this method allowed for the reduction in the injection volume (1 µL) and associated matrix effects. Limits of detection (S/N > 3) and quantitation (S/N > 10) of CTXs were 0.0045 ng g^{-1} and 0.0150 ng g^{-1}, in matrix matched samples spiked with CTX1B standard. These limits are clearly below the guidance level proposed by de FDA, USA for C-CTX1, 0.1 ng g^{-1} (Dickey & Plakas, 2010).

The identification of CTX analogues was made using reference standards by comparing retention time (Fig. 1). Linearity was assessed for CTX1B over a concentration range of 0.28–27.88 ng mL^{-1} (R^2 = 0.999) (Fig. 2). To ensure that the slope of the calibration curve showed a linear regression, the correlation coefficient, R^2, should be ≥0.98. Consequently, linearity was evaluated in nine different days and correlation coefficient values above 0.99 were always obtained. Robustness was also minimally evaluated trough the variation of the slope by injection on nine different days and an increased sensitivity was observed over the time. For quantitation purpose and due to the limited amount of C-CTX1 pure standard, C-CTX1 content was expressed in CTX1B equivalents. Samples where was detected C-CTX1 were quantified as CTX1B and converted to equivalents of C-CTX1 with the value obtained with the reference material previously.

Method B: Source and interface conditions were optimized using CTX3C (Wako), whereas MS/MS conditions for C-CTX1 were optimized using C-CTX1 "laboratory reference materials". In contrast with MeOH-H_2O mobile phases, which produced prominent $[M+Na]^+$ ions for the three principal CTXs (CTX1B, CTX3C and C-CTX1), the ACN-H_2O mobile phase gave rise to prominent $[M+H]^+$, $[M+NH_4]^+$ and serial

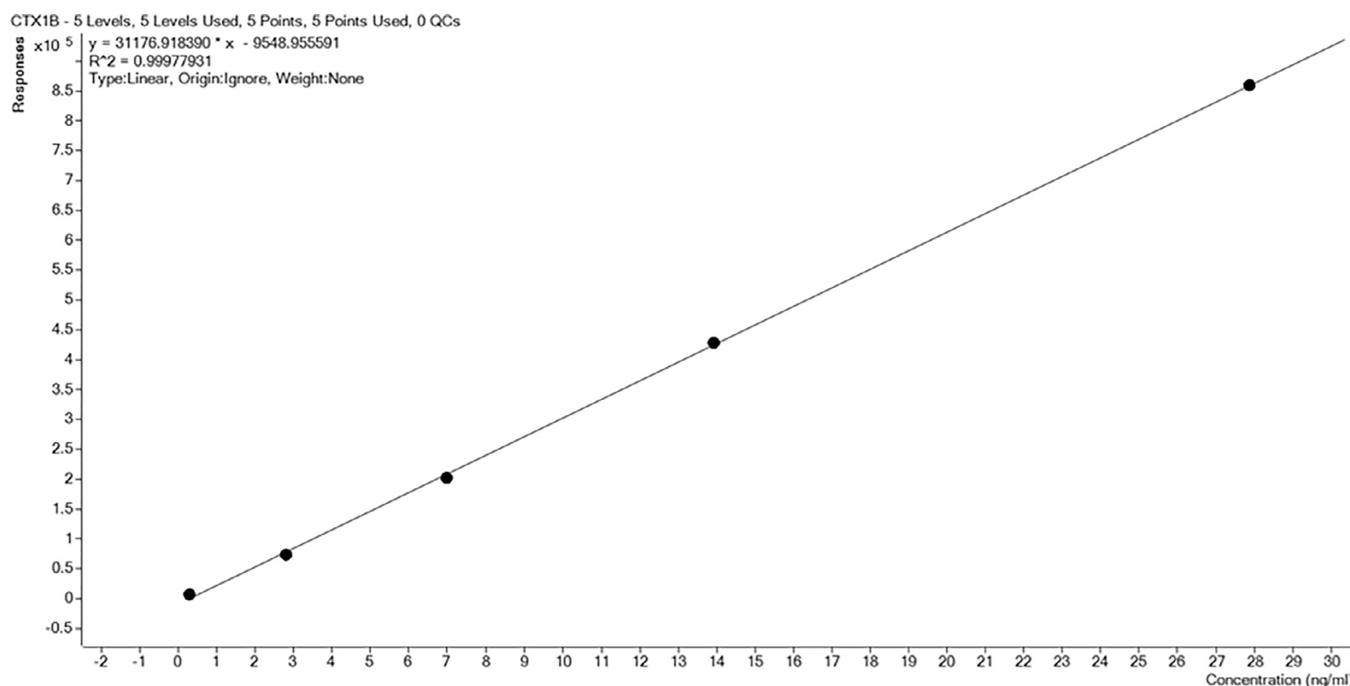

Fig. 2. Example of CTX1B calibration curve.

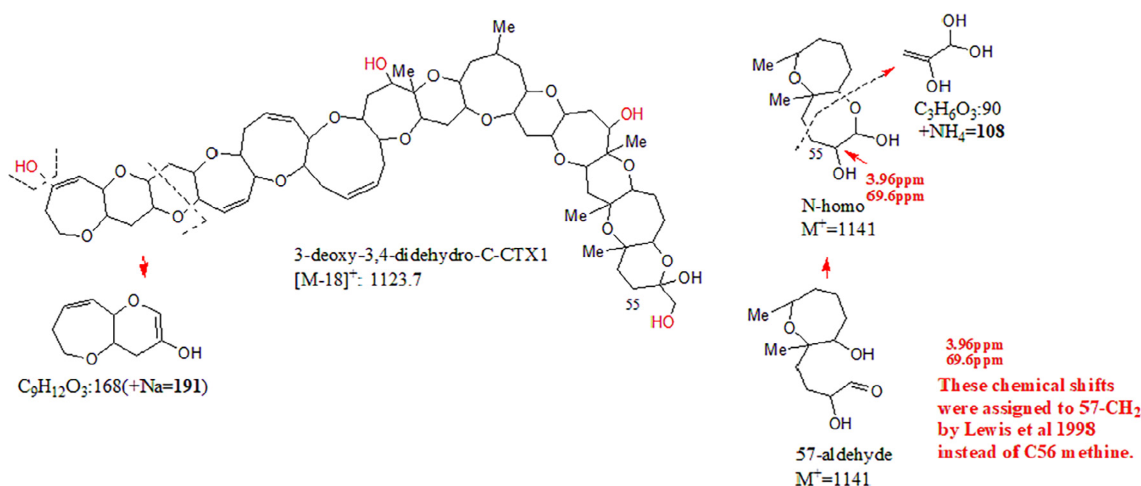

Fig. 3. Proposal for the revision of the N-ring in C-CTX1 mainly based on the MS/MS data selection a dehydrated ion at m/z 1123 as target ion and fragments assignments.

dehydrated ions. These three principal CTXs produced different ion profiles in the ACN-H_2O solvent system. $[M+NH_4]^+$ m/z 1128.6 was the major ion detected for CTX1B, $[M+H]^+$ m/z 1023.6 for CTX3C and $[M+H\text{-}H_2O]^+$ m/z 1123.6 for C-CTX1. Hence, $[M+H\text{-}H_2O]^+$ m/z 1123.6 was selected for monitoring C-CTX1 in the Multiple Reaction Monitoring (MRM) analysis. Prominent ions were also observed at m/z 108.9 and 191.1 in product ion analysis of $[M+H\text{-}H_2O]^+$ m/z 1123.6 with high collision energies whereas water loss ions $[M+H\text{-}2H_2O]^+$ m/z 1105.6, $[M+H-3H_2O]^+$ m/z 1087.6 and $[M+H\text{-}4H_2O]^+$ m/z 1069.6 were observed at low collision energies. The MS/MS data obtained for C-CTX1 at high collision energies show that the N-ring of C-CTX1 is more likely a seven-membered ring rather than the six-membered ring as proposed by Lewis, Vernoux, & Brereton, 1998. The NMR data in the original paper suggest the data on C57 and C56 are interconvertible and do not contradict the revised version. In Fig. 3 is shown the assignments of the fragments obtained from C-CTX1 structure. Another key advantage in this approach was the use of ammonium formate, which significantly reduced matrix effects, allowing for the larger injection volumes without compromising detection. Further studies are needed to improve sensitivity because results were not comparable to those obtained using $[M+Na]^+$ ions generated in MeOH-H_2O mobile phase. As mentioned above the evaluation of the linearity range, detection and quantitation limits was not fully performed for method B due to the limited amount of C-CTX1 pure standard available. Since the applicability of this method was based on its ability for the confirmation of the presence of C-CTX1 previously identified and quantified in method A, the evaluation of these criteria was not considered strictly necessary. Despite of this, method B allowed the unambiguously confirmation of C-CTX1 using successive water loss ions and specific fragmentation ions detected in the C-CTX1 pure standard solution at concentrations bracketing the guidance level for C-CTX1 of 0.1 ng C-CTX1/g tissue. This method could be also useful for the identification of potential CTX analogues since provides an increased information compared to the single use of $[M+Na]^+$ ions.

3.3. LC-MS/MS analysis of naturally contaminated samples

The implementation of the methods allowed identifying C-CTX1 as the main responsible for the CFP contamination in the samples evaluated from Canary Islands (Spain) and Madeira (Portugal). Some examples of the results obtained for the quantification of these samples are included in Table 3. Among the samples included in this table some of them (E1, S1 and P1), were related with CFP outbreaks whereas sample L1 was obtained through the official control implemented in the Canary Islands. As previously mentioned, identification and quantitation was carried out following method A. Retention time and precursor/product ion transitions m/z 1163.7 → m/z 1163.7 by MRM of C-CTX1 in samples were consistent with those obtained for the pure standard solution of C-CTX1 (Fig. 4) with concentrations ranging from 0.78 ng C-CTX1/g tissue to 0.12 ng C-CTX1/g tissue. The concentrations of C-CTX1 in the samples related to the CFP outbreaks were higher than the ones established in the FDA guidance levels 0.1 ng C-CTX1/g tissue while the concentration levels obtained in other samples from official control were lower or even around these guidance levels in some cases (data not shown). As it was also mentioned above, the confirmation of the CTX analogues involved in the contamination was carried out using method B by detecting C-CTX1 water losses m/z 1123.6 → m/z 1105.6, m/z 1123.6 → m/z 1087.6, m/z 1123.6 → m/z 1069.6 as well as C-CTX1 fragments m/z 1123.6 → m/z 108.9 and m/z 1123.6 → m/z 191.1 by MRM with retention time and peak area ratios for the precursor/product ion transitions consistent with those of C-CTX1 pure standard solution.

The LC-MS/MS conditions applied in this work for the analysis of fish samples from Canary Islands allow the identification for the first time of a prominent ion at m/z 1181.7 in fractions found as CTX-like positive in the Neuro2a assay, but devoid of the ion corresponding to C-CTX1 m/z 1163.7. The mass for the ion at m/z 1181.7 corresponds to a sodium adduct to a hydrate product of C-CTX (C-CTX+H_2O, or 52,56,56-trihydroxy-N-seco-C-CTX1). The structure needs confirmation but could explain the generation of a unique ion at m/z 108 when a dehydration product ion (m/z 1123.6) was used as a target ion in an MS/MS experiment. The product ion at m/z 191 also could be explained for its generation. The MS/MS experiments by monitoring the ions shown in Fig. 5 verified that the compound giving the ion at m/z 1181 corresponds to a hydrate of C-CTX1. Further work is necessary to demonstrate the presence of C-CTX1 isomers and analogues in this fish sample and their toxicity (Pottier, Vernoux, Jones, & Lewis, 2002).

To our knowledge, most references about the detection of C-CTX1 in fish samples are on the Caribbean Sea (Abraham et al., 2012; Loeffler et al., 2018) and the estimation of the C-CTX1 concentration was based on cell assay (N2a) evaluating the total presence of Na channel activators, whereas LC-MS/MS was used in these references as a confirmatory tool, monitoring C-CTX1 water losses. The approach used in this work allows both quantitation and confirmation of C-CTX1 through the specificity provided by LC-MS/MS also using the sensitive identification and quantification monitoring $[M+Na]^+$ and the further confirmation by monitoring C-CTX1 water losses and specific fragments.

Further studies are being carried out to implement the knowledge about the incidence of CFPs in these areas even to relate their incidence

Table 3
Detailed list of naturally contaminated samples analyzed by LC-MS/MS.

Sample ID	Specie	Common name	Weight (g)	Island	Capture date (mm/dd/yy)	CFP Outbreak	C-CTX1 (ng g^{-1})
E1	*Epinephelus marginatus*	Dusky grouper	7000	Tenerife (Spain)	11/09/16	Yes	0.12
S1	*Seriola* spp.	Amberjack	Unknown	Tenerife (Spain)	12/27/16	Yes	0.37
L1	*Lutjanus cyanopterus*	Cubera Snapper	16,000	Fuerteventura (Spain)	07/11/16	No	0.49
P1	*Pagrus Pagrus*	Red Porgy	4000	Selvagem Islands (Portugal)	12/30/16	Yes	0.76

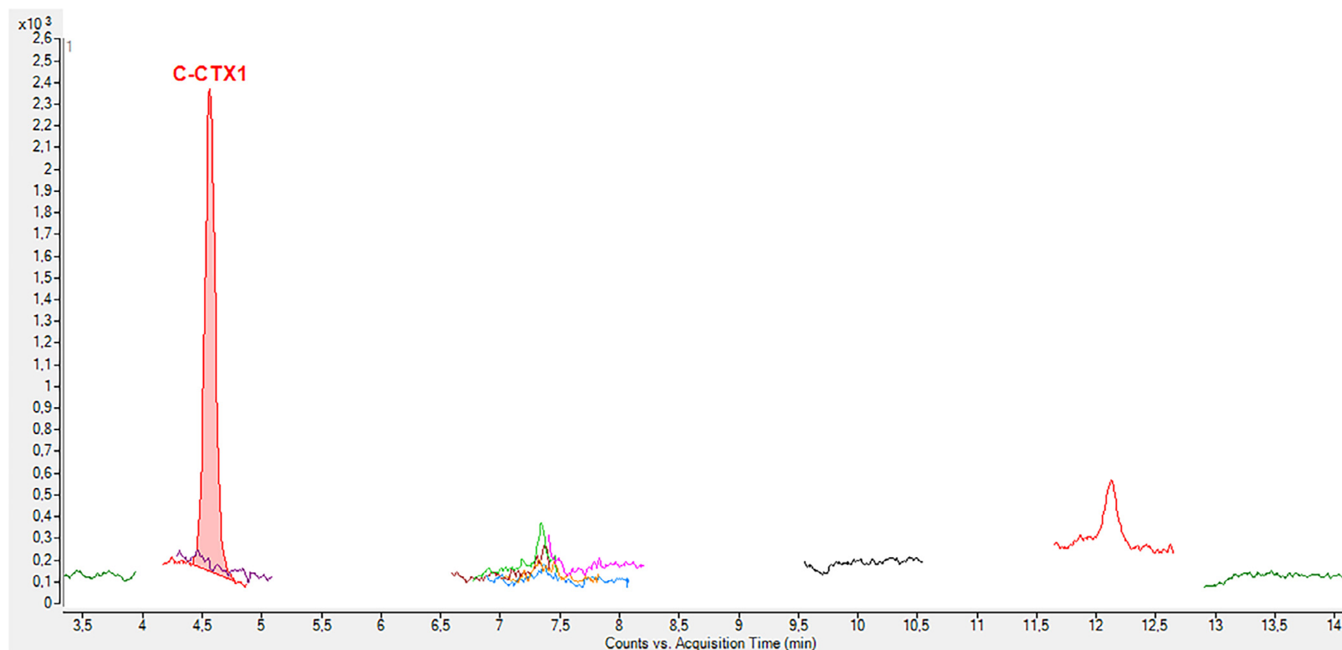

Fig. 4. Toxin profile of Grouper (*Epinephelinae*) from Canary Islands selecting sodium adduct with Method 1.

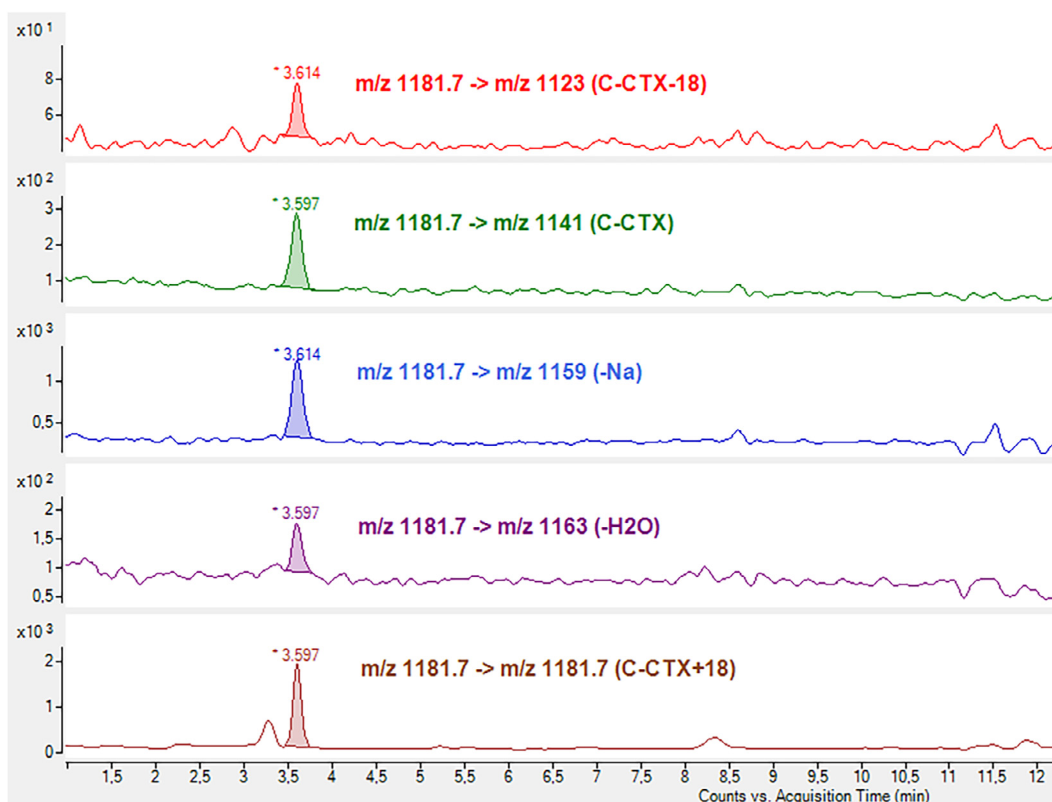

Fig. 5. MS/MS experiment for verifying that the compound giving the ion at *m/z* 1181 is a hydrate of C-CTX1.

with the environmental seasonal conditions, etc. The results obtained so far evidence the presence of Caribbean Ciguatoxin-1 as the main responsible for the CFP contamination in the Canary islands and Madeira in concentrations around the guidance level of 0.1 ng C-CTX1/g tissue established by FDA, USA.

4. Conclusions

This study suggests that sample pretreatment, in particular the extraction and SPE cleanup steps are critical, being necessary to evaluate their efficiency specially when the CTXs concentration levels are around the recommended guidance levels. The optimized conditions described in this work for the LC-MS/MS analysis of CTXs allows the confirmation of Caribbean CTXs, in particular C-CTX-1 and even some derivatives, in contaminated fish with a sensitivity clearly below the guidance levels. The lack of standards and reference materials represent a significant limitation to progress in applied research on ciguatera, nevertheless the use of purified extracts from naturally contaminated fish samples as laboratory reference materials has contributed to the identification of critical factors to be considered in the method development, not only to minimize matrix effects, but also to increase extraction efficiency. The mass spectrometry conditions used in this work, as well as the monitoring of adducts and fragments, contributed to an enhancement of the sensitivity which also facilitated the confirmation of C-CTX1 as the main CTX analogue present in the contaminated samples from Canary Islands (Spain) and Selvagens Islands (Portugal). The detection of the ion at m/z 1181.7 can be regarded as the most important finding in the MS/MS analysis of C-CTX demonstrating that it is a hydrate of C-CTX1.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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