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ORIGINAL PAPER

Validation of an accelerated solvent extraction liquid chromatography–tandem mass spectrometry method for Pacific ciguatoxin-1 in fish flesh and comparison with the mouse neuroblastoma assay

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Abstract Ciguatera fish poisoning (CFP) is a global foodborne illness caused by consumption of seafood containing ciguatoxins (CTXs) originating from dinoflagellates such as *Gambierdiscus toxicus*. P-CTX-1 has been suggested to be the most toxic CTX, causing ciguatera at 0.1 μg/kg in the flesh of carnivorous fish. CTXs are structurally complex and difficult to quantify, but there is a need for analytical methods for CFP toxins in coral reef fishes to protect human health. In this paper, we describe a sensitive and rapid extraction method using accelerated solvent extraction combined with high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) for the detection and quantification of P-CTX-1 in fish flesh. By the use of a more sensitive MS system (5500 QTRAP), the validated method has a limit of quantification (LOQ) of 0.01 μg/kg, linearity correlation coefficients above 0.99 for both solvent- and matrix-based standard solutions as well as matrix spike recoveries ranging from 49% to 85% in 17 coral reef fish species. Compared with previous methods, this method has better overall recovery, extraction efficiency and LOQ. Fish flesh from 12 blue-spotted groupers (*Cephalopholis argus*) was assessed for the presence of CTXs using HPLC-MS/MS analysis and the commonly used mouse neuroblastoma assay, and the results of the two methods were strongly correlated. This method is capable of detecting low concentrations of P-CTX-1 in fish at levels that are relevant to human health, making it suitable for monitoring of suspected ciguateric fish both in the environment and in the marketplace.

Keywords Ciguatera fish poisoning · Ciguatoxin · HPLC-MS/MS · Accelerated solvent extraction · Mouse neuroblastoma assay

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Introduction

Ciguatera fish poisoning (CFP) is a foodborne illness caused by consumption of tropical and subtropical fishes containing ciguatoxins (CTXs). CTXs are a family of heat-stable, lipid-soluble, colourless and odourless cyclic polyethers which cannot be degraded through cooking, drying or freezing [1]. Symptoms of CFP include gastrointestinal, neurological and cardiovascular disturbances, such as vomiting, diarrhoea, hypotension and paralysis [2]. Dinoflagellates in the genus *Gambierdiscus* such as *Gambierdiscus toxicus*, which is found on many kinds of macroalgae associated with coral reefs, are potential sources of CTXs [3]. They produce the less oxidised precursors, gambiertoxins (GTXs), which are biotransformed along the marine food chain by herbivorous

and carnivorous fishes, leading to the production of the more oxidised and toxic CTXs [4]. Various species of fishes at all trophic levels have been identified as ciguatoxic, such as moray eels, groupers, snappers and barracudas. Among these, moray eels are regarded as the most toxic ciguateric fishes [5–7]. More than 20 kinds of GTXs and CTXs have been identified in *G. toxicus* and in fishes [8].

Ciguatera is endemic to particular regions of the Pacific Ocean, western Indian Ocean and the Caribbean Sea. Among the three currently described groups of CTXs, the Pacific ciguatoxins (P-CTXs), Indian ciguatoxins (I-CTXs) and Caribbean ciguatoxins (C-CTXs), P-CTX-1 has been suggested to be the most toxic CTX, causing CFP at 0.1 μg/kg in the flesh of carnivorous fishes [8]. Some areas such as the western reef of Marakei and southern reef of Tarawa in the Republic of Kiribati have been identified as high-risk regions with respect to ciguatera [1]. The global incidence of CFP has increased in recent years due to the expansion and development of the international seafood trade; globally, it is estimated that more than 25,000 people are affected annually [1].

Ciguatoxic fishes may have normal appearance, taste and smell, and therefore, there is a need for methods that can be used to identify toxic fishes to protect human health. Several approaches have been developed in the past few decades to address this issue, such as feeding tests [7], immunoassays [9–11] and cell-based cytotoxicity test [12], but they all have some drawbacks. Feeding tests require large amounts of toxic extract, are time-consuming, raise ethical questions about animal use and lack reproducibility and specificity. Immunoassays and cytotoxicity tests may give false negative or positive responses due to structural differences among CTX congeners or other sodium ion channel activators, compromising the accuracy of these methods [8, 12]. To confirm the presence of CTXs in fish tissues, chemical methods such as high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) have been developed for analytical detection of CTXs in crude extracts [13, 14].

HPLC-MS/MS provides a sensitive and chemically specific analytical approach for the determination of CTXs at sub-parts per billion levels in fish flesh [13]. P-CTX-1 is the major toxin in flesh of carnivorous fishes of the Pacific Ocean, contributing to around 90% of the total lethality [15]. To date, two rapid chemical methods for detecting and quantifying P-CTX-1 have been established [14, 16]. However, matrix spike recovery of P-CTX-1 varies depending on matrix complexity (27% to 75%) [16], and further development of chemical methods has been hindered by a lack of analytical toxin standards [1].

By isolating and purifying authentic P-CTX-1 standard from moray eels collected from the Republic of Kiribati, a rapid, reliable and sensitive quantification method can be optimized using HPLC-MS/MS coupled with accelerated solvent extraction (ASE) for monitoring P-CTX-1 in fish flesh collected both in the environment and in the marketplace. ASE is a fully automated sample preparation method that provides elevated temperature and pressure to keep solvent in the liquid phase, allowing for rapid and efficient extraction of analytes from various matrices [17]. In this paper, we describe an optimization of a fast, effective and automatic extraction method using ASE combined with HPLC-MS/MS for the detection and quantification of P-CTX-1 in fish flesh.

Experimental

Chemicals and reagents

Methanol (HPLC-grade), ethanol (AR), sodium chloride and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chloroform (AR), hexane (AR), diethyl ether (AR) and acetonitrile (HPLC-grade) were purchased from RCI Labscan (Bangkok, Thailand). Acetone and dichloromethane were purchased from TEDIA (Fairfield, OH, USA). Formic acid (98–100%) was purchased from BDH (Poole, UK). ASE Prep diatomaceous earth was purchased from Dionex (Sunnyvale, CA, USA). Florisil (PR-grade, 60–100 mesh) was purchased from Supelco (Bellefonte, PA, USA). Sephadex LH-20 was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Toyopearl HW 40S was purchased from Tosoh Corporation (Tokyo, Japan).

Isolation and purification of P-CTX-1

Based on the P-CTX-1 standard provided by Prof R. Lewis, University of Queensland, 40 μg of P-CTX-1 was isolated and purified from the viscera of moray eels (*Gymnothorax flavimarginatus*, $n=11$, and *Gymnothorax undulatus*, $n=20$; 5.6 kg) collected from the Republic of Kiribati according to the method described previously [18, 19]. Briefly, the viscera was cooked at 70 °C for approximately 30 min and then homogenized with acetone (3 mL/g fish viscera). The acetone extract was filtered and dried using a rotary evaporator under reduced pressure. The resulting acetone extract was partitioned between hexane and 90% aqueous methanol, and the residue in the methanolic phase was further partitioned between 25% ethanol and diethyl ether. The residue in the organic layer was subjected to a Florisil column (14 g/g extract) prewashed with acetone:hexane (*v*:*v*, 1:3) and separated with gradients of acetone:hexane: methanol (*v*:*v*:*v*, 1:3:0, 1:0:0, 9:0:1, 7:0:3 and 0:0:1) to yield 12 fractions. Fractions containing P-CTX-1 were determined by comparing the retention times with those of the standard using HPLC-MS/MS. Combined toxic fractions were

further purified on a Sephadex LH-20 column (2.8×110 cm) eluted with dichloromethane:methanol (*v*:*v*, 1:1), and the resulting toxic fraction was further applied to another Sephadex LH-20 column (3.0×95 cm) eluted with methanol and a TSK HW-40S column (3.0×29 cm) eluted with methanol. Further purification was carried out using a Hamilton PRP-1 column (150×4.1 mm i.d., 5 μm) eluted at 600 μL/min with 0.2% formic acid in 50% aqueous acetonitrile and finally purified by a Phenomenex Luna C18 (2) (250×2.0 mm i.d., 5 μm) column eluted at 250 μL/min with 0.2% formic acid in 43% aqueous acetonitrile. Purified P-CTX-1 was reapplied to HPLC columns and eluted with solvents of different polarities to confirm purity. A single and homogeneous peak was observed using different solvent ratios by a DAD detector. Purity of the authentic P-CTX-1 standard was over 95% by normalization of peak areas detected by HPLC-DAD.

Sample collection

Coral reef fishes were collected from the western reef of Marakei and southern reef of Tarawa in the Republic of Kiribati during May and June 2009. Fish species were identified based on their external characteristics such as body shape and coloration. Twelve species of coral reef fishes usually associated with CFP were selected for this study, including carnivores, herbivores and omnivores. They are *G. undulatus*, *Cephalopholis argus*, *Epinephelus corallicola*, *Epinephelus fuscoguttatus*, *Epinephelus spilotoceps*, *Variola louti*, *Lutjanus gibbus*, *Lutjanus bohar*, *Lutjanus fulvus*, *Monotaxis grandoculis*, *Acanthurus gahhm*, *Acanthurus lineatus*, *Acanthurus maculiceps*, *Siganus argenteus*, *Naso lituratus*, *Scarus ghobban* and *Ctenochaetus binotatus*. Prior to method optimization, all fish samples (ca. 20 g, fresh weight) were extracted using a method previously described by Lewis et al. [18] and Wong et al. [20] with acetone as the extraction solvent and doing liquid–liquid partition to facilitate the extraction of lipophilic CTXs from fish flesh to give relative clean extracts for use in the mouse neuroblastoma assay (MNA) for initial screening.

Extraction of fish and sample preparation

Fish flesh of non-toxic *G. undulatus* as determined by MNA was used for the optimization of ASE conditions. Homogenized fish flesh samples (ca. 5 g, fresh weight) were frozen at −80 °C and then freeze-dried overnight depending on the moisture content of fish flesh (Labconco FreeZone 12^{plus}, Kansas City, MO, USA) before analysis. During freeze-drying, the vacuum was set to 0.05 bar, and the temperature reached −60 °C. Each sample was mixed with 5 g diatomaceous earth (DE) in a porcelain mortar and transferred into a 22-mL stainless steel extraction cell (Dionex, Sunnyvale, CA, USA) with two Whatman glass fibre filters placed at the bottom of the cell. Samples were extracted using the ASE 200 system (Dionex, Sunnyvale, CA, USA) at 75 °C and 1,500 psi with 5 min of heating followed by a 5-min static extraction twice using methanol. The extraction cell was flushed with 60% methanol with a purge time of 100 s. The final extract was concentrated to 5 mL.

To compare the extraction efficiency of the present method with that of previously published methods, fish flesh samples were extracted based on the rapid extraction method described by Lewis et al. [14] and Stewart et al. [16] with some modifications. Briefly, 5 g homogenized fish flesh samples were cooked at 70 °C for 30 min in capped 50 mL Falcon tubes. Samples were cooled and homogenized with 20 mL methanol:hexane (3:1, *v*:*v*), followed by sonication for 15 min. After centrifuging at 3,500 rpm for 10 min, the upper hexane layer was discarded, and the methanol layer was decanted. This operation was repeated twice.

The crude extract was adjusted to 50% aqueous methanol before being subjected to a cleanup step according to the procedures reported by Lewis et al. [14]. Different brands of reverse-phase C18 cartridges were tested, and the C18 Bond Elute cartridge (Varian, 500 mg, 6 mL) was finally used for the first cleanup procedure. The cartridges were preconditioned with 15 mL methanol and 10 mL MilliQ water successively. The crude extract was passed through the pre-conditioned cartridges at a rate of 1 drop/s. The cartridges were then washed with 6.5 mL 65% aqueous methanol, and the target analyte was eluted with 12 mL 80% aqueous methanol. A total of 10 mL chloroform and 6.3 mL 1 M NaCl was added and mixed well with the eluate. The upper aqueous layer was removed, and the lower chloroform layer was dried under a stream of N_2. The residue was dissolved in 4 mL of chloroform and loaded on a Sep-Pak silica cartridge (Waters, 500 mg, 6 mL) that was preconditioned with 30 mL chloroform. The cartridge was then washed with 4.5 mL chloroform, and the target analyte was eluted with 8 mL chloroform:methanol (9:1, *v*:*v*). The eluent was dried under a stream of N_2 and reconstituted with 100 μL methanol before injection. A flow diagram of the overall method is shown in Fig. 1.

Liquid chromatography-quadrupole-linear ion-trap mass spectrometry

Two models of QTRAP mass spectrometers (AB Sciex, Foster City, CA, USA) equipped with a turbo ionspray interface were used for the detection of P-CTX-1 in this study. A 3200 QTRAP mass spectrometer coupled to an Agilent 1200 HPLC system (Agilent, Palo Alto, CA, USA) running Analyst 1.4.2 software was employed for the optimization of ASE conditions and sample analysis. A Phenomenex Luna C18 (2) column

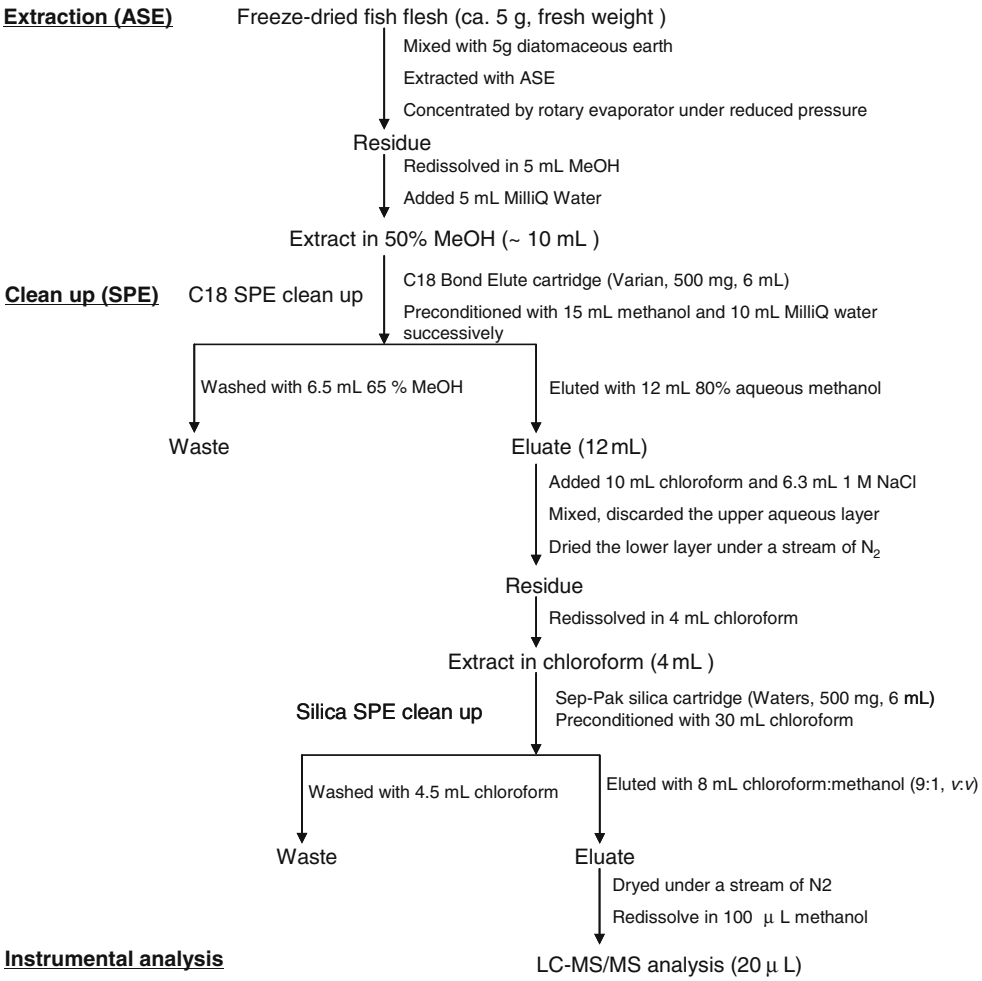

Fig. 1 Flow diagram of the extraction and cleanup method of P-CTX-1 in fish flesh

(250×2.0 mm i.d., 5 μm) preceded by a Phenomenex Luna C18 guard column (4×2.0 mm i.d., 5 μm) were selected for analysis. The mobile phase comprised 0.1% formic acid in MilliQ water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and was delivered at a flow rate of 200 μL/min. A linear gradient program starting at 55% B to 95% B in 15 min was used to elute P-CTX-1. Ninety-five percent B was then held for 10 min before returning to 55% B in 2 min. The column was equilibrated for 10 min with 55% B prior to the next run. The injection volume was 20 μL. The mass spectrometer was operated in positive ion, multiple reaction monitoring (MRM) mode with two transitions with resolution for both Q1 and Q3 set at low. MS/MS conditions were established using P-CTX-1 standard in 50% aqueous acetonitrile and 0.1% formic acid injected directly into the MS with a flow rate of 10 μL/min. MS/MS conditions of the two dominant product ions generated from the $[M + H]^+$ ion of P-CTX-1 (*m/z* 1,117.7>1,093.3, 1,117.7>1,075.7) were further optimized by flow injection analysis.

A 5500 QTRAP mass spectrometer coupled to a Shimadzu Prominence UFLC LC-20 system (Shimadzu, Tokyo, Japan) running Analyst 1.5.2 software was employed for the determination of matrix effects, and the limits of quantification (LOQ) and detection (LOD). A Phenomenex Luna C18 (2) column (150×3.0 mm i.d., 3 μm) was selected for analysis. The mobile phase comprised 5 mM ammonium formate with 0.1% formic acid in MilliQ water (Solvent A) and acetonitrile (Solvent B) and was delivered at a flow rate of 350 μL/min. The gradient program started at 60% B for 3 min, and then was ramped to 90% B in 1 min. Ninety percent B was held for 6 min before returning to 60% B in 0.1 min. The column was then equilibrated for 4.9 min with 60% B prior to the next run. The injection volume was 10 μL. The mass spectrometer was operated in positive ion. Other mass spectrometer operating parameters are shown in Table 1.

Method validation

P-CTX-1 standard solutions of eight concentrations ranging from 0.5 to 500 ppb were obtained by serial dilution with methanol for constructing an external calibration curve. In addition, standards ranging from 2.5 to 500 ppb were also spiked in extracts of non-toxic *G. undulatus* and *C. argus* for matrix-matched calibration. The calibration curve was

Table 1 Mass spectrometer operating parameters used for analysis of P-CTX-1

Mass spectrometer	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)	CUR	IS	TEM (°C)	GS1	GS2	Dwell time (ms)
3200 QTRAP	1,111.7	1,093.3	51	8	23	8	10	5,500	400	60	10	50
		1,075.7	51	8	23	8						
5500 QTRAP	1,128.6	1,093.7	76	8.6	36	17.6	10	5,500	450	40	50	50
		1,075.7	76	8.6	25	17.6						
		1,057.7	76	8.6	28	17.6						
		1,039.7	76	8.6	31	17.6						

constructed by plotting peak areas of $[M + H - H_2O]^+$ against concentrations of the corresponding standard solutions (parts per billion) using "1/*x*" weighed linear regression analysis with a correlation coefficient better than 0.990, and the deviation of each point from the regression line was less than 20% of its theoretical value. LOD and LOQ values were estimated based on the lower limit of the calibration curve with signal-to-noise ratio larger or equal to 3 and 10, respectively, with concentration factors of 50. The overall method recovery was performed by spiking 0.5 mL 40 ppb P-CTX-1 standard solution in pure methanol in 5 g fish flesh of non-ciguatoxic coral fish and waiting for 30 min for toxin absorption before mixing the sample with DE or cooking in duplicate. The cleanup procedural recovery was performed by spiking 20 ng of P-CTX-1 directly after extraction and before loading the extract onto cartridges in duplicate. Intermediate precision and repeatability were calculated by spiking standard on the same sample set used for the recovery test. Two working solutions prepared from the same sample during the same day and a total of six different working solutions were analyzed to determine the repeatability and intermediate precision, respectively. Precision tests were carried out on the basis of intra- and inter-day variation (n=3) of the same standard solution.

Mouse neuroblastoma assay (MNA)

Neuroblastoma cells, Neuro-2a (ATCC CCL131; ATCC, Manassas, VA, USA), were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) at 37 °C in 5% CO_2. The RPMI-1640 was supplemented with 10% foetal bovine serum (BD Biosciences, San Jose, CA, USA), 2 g/L Na_2CO_3 and antibiotic solution (50 units/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml Fungizone® (Gibco, Life Technologies, Carlsbad, CA, USA)). Cell proliferation was measured by MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were seeded at a density of 2.5×10^5 cells per well in 96-well plates. After 24 h, medium was renewed with complete RPMI-1640 containing 0.1 mM ouabain and 0.01 mM veratridine. Cells were dosed with 10 μL/well P-CTX-1 standards at seven concentrations ranging from 0.97 to 62.5 pg/mL in four replicates. *C. argus* flesh extracts were also tested in four replicates. Absorbance was measured using a microplate reader (Molecular Devices Spectra Max 340 PC) at 595 nm with a reference wavelength of 655 nm. The optical density acquired for each well was then normalized by the MTT blank. IC_{50} values were determined from the dose–response curve. All the toxicity of fish samples was determined from the standard curve. The assays were conducted twice, and the toxicity results are reported as mean P-CTX-1 equivalents between the two assays.

Data analysis

Normality of data was evaluated using Kolmogorov–Smirnov tests. Correlation analysis was carried out using Pearson product moment correlations if data passed the normality tests; otherwise, Spearman rank order correlations were used to analyze the relationship between the toxin concentration obtained by MNA and HPLC-MS/MS. Statistical significance was accepted at $p<0.05$ (SigmaStat 3.5, Aspire Software International, Washburn, VA, USA).

Results and discussion

Optimization of HPLC-5500 QTRAP-MS/MS conditions

In order to achieve higher sensitivity, a 5500 QTRAP mass spectrometer was also employed for the determination of matrix effects, LOQ and LOD. Because of the high abundance of $[M + Na]^+$, P-CTX-1 was dissolved in 50% aqueous acetonitrile which contained 1% formic acid and 30 mM ammonium formate to promote the formation of precursor ions of interest such as $[M + NH_4]^+$ and $[M + H]^+$. P-CTX-1 standard was injected directly into the 5500 QTRAP mass spectrometer with a flow rate of 7 μL/min. The intensity of $[M + NH_4]^+$ improved, and it was selected as the precursor ion of P-CTX-1. With the higher sensitivity of

the 5500 QTRAP, product ions were detected using MRM with resolution for both Q1 and Q3 set at unit, ensuring the mass accuracy of the analytes of interest. MS/MS conditions of the four dominant product ions generated from the $[M + NH_4]^+$ ion of P-CTX-1 (*m/z* 1,128.6>1,093.7, 1,128.6>1,075.7, 1,128.6>1,057.7 and 1,128.6>1,039.7) were further optimized. Other mass spectrometer operating parameters are listed in Table 1. LC conditions including gradient program and flow rate were optimized for improving peak shape and preventing co-elution of matrices in the sample. As the presence of ammonium formate can promote the formation of the ammonium adduct of P-CTX-1, ammonium formate was added to the aqueous mobile phase when the 5500 QTRAP was used.

After optimization of the MS/MS conditions, the identification of other CTXs in the moray eel extract was also carried out. A toxic sample was analyzed in scan mass mode (*m/z* 1,000–1,150). It was found that a prominent molecule around *m/z* 1,112.7 could be identified as an ammonium adduct of CTXs, and a typical fragmentation pattern of a cyclic polyether, including proton adducts and ions of additional loss of one and two water molecules and their corresponding masses, was the same as those of P-CTX-2 or 3 reported in the literature [16]. The mass spectrum (Fig. 2, inset) showed an intense ion at $[M + NH_4]^+$ *m/z* 1,112.7, a protonated ion at $[M + H]^+$ *m/z* 1,095.8 and one and two losses of water at *m/z* 1,077.6 and 1,059.7. By setting the MRM transitions as *m/z* 1,128.6→1,075.7 for P-CTX-1 and *m/z* 1,112.7→1,077.6 for the analogs of P-CTX-2/3, a chromatogram was obtained in which the compounds of interest were eluted at 5.65, 8.04 and 8.94 min, respectively, in the moray eel extract (Fig. 2). Once the standards of P-CTX-2 and 3 are available, the optimized HPLC-MS/MS conditions may be suitable for detecting and quantifying a range of CTXs for toxic fish screening as shown by the additional peaks in the chromatogram (Fig. 2).

Optimization of the ASE

Fish flesh of non-ciguatoxic *G. undulatus* was ground and dispersed with DE to increase the interface with the extraction solvent and avoid the aggregation of sample particles. Samples were freeze-dried before extraction since lyophilization can remove moisture that may diminish extraction efficiency of P-CTX-1 [17]. The influence of extraction solvent, temperature, static time and number of extraction cycles of ASE on the extraction

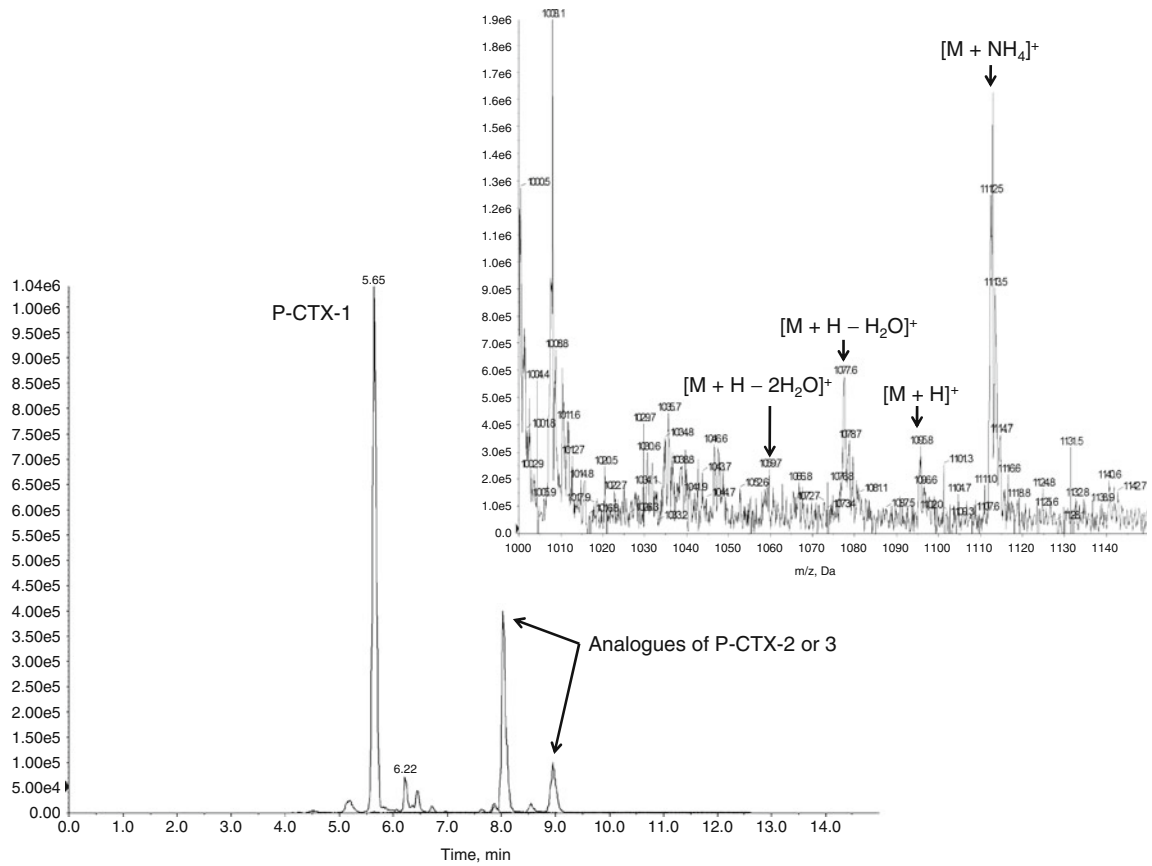

Fig. 2 Chromatogram of P-CTX-1 and analogues of P-CTX-2/3 in toxic *Gymnothorax undulatus* extracted by ASE. The *inset* shows the Q1 mass spectrum of the analogues of P-CTX-2 or 3 (*m/z* 1,000–1,150)

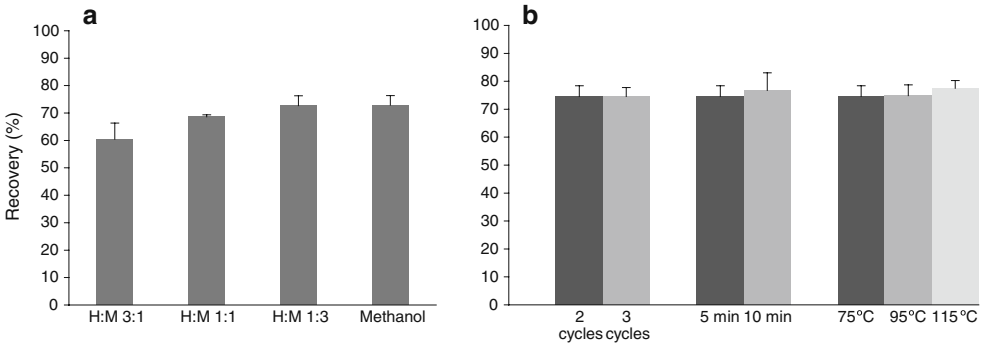

Fig. 3 Effect of **a** solvent, **b** number of cycles, static time and temperature on the extraction of P-CTX-1 in fish flesh (H:M = hexane:methanol; 20 ng of P-CTX-1 was spiked into 5 g non-toxic *Gymnothorax undulatus* for the optimization)

efficiency of P-CTX-1 in fish flesh was examined. Pressure is usually a minor variable for the ASE efficiency, as it is only required to maintain the extraction solvent in the liquid phase; it was set at 1,500 psi during the whole process.

The nature of the extraction solvent has a profound effect on the ASE process, and therefore, an appropriate choice of the extraction solvent is very important. Often, the same solvent used in conventional extractions will also work well in ASE. Methanol:hexane (M:H; 3:1, *v*: *v*) was previously used for extraction of P-CTX-1 in fish flesh for LC-MS/MS analysis [14, 16], and hence, different ratios of M:H (1:3, 1:1, 3:1 and 1:0, *v*:*v*) were tested for their P-CTX-1 extraction efficiency using ASE. ASE extracts were injected in the LC-MS/MS system after further cleanup procedures. The results showed that the extraction efficiency of P-CTX-1 in fish flesh was improved by increasing the proportion of methanol in the extraction solvent. Methanol was the most effective solvent for extracting P-CTX-1 in flesh of moray eel using ASE with recoveries of 73±3% including cleanup (Fig. 3a). Similar results were obtained when the flesh of a ciguatoxic *G. undulatus* was extracted using these four extraction solvents, and the concentrations of P-CTX-1 were found to be 6.73±0.46 ng/g (M:H, 1:3, *v*:*v*), 6.70± 0.35 ng/g (M:H, 1:1, *v*:*v*), 7.50±0.35 ng/g (M:H, 3:1, *v*:*v*) and 7.83±0.11 ng/g (M:H, 1:0, *v*:*v*), respectively. As described in several publications, fractionation extraction using ASE can be employed for selective removal of interferences during sample extraction [21–23], and therefore, fractionation extraction with hexane prior to the extraction of the analyte of interest was tested to remove lipids. However, the recovery of P-CTX-1 in flesh of *G. undulatus* decreased to 61±7% with the additional lipid removal procedure. As P-CTX-1 is a lipid-soluble toxin, it may be extracted by hexane at elevated temperatures. Therefore, methanol alone was determined to be the best extraction solvent, and the lipid removal step was omitted.

The effect of a series of temperatures, 75 °C, 95 °C and 115 °C, on the recovery of P-CTX-1 in fish flesh was evaluated. Seventy-five degrees Celsius was chosen as the lowest tested temperature, as cooking of fish flesh at this temperature range was involved in the previous method [14, 16]. Increased temperature resulted in comparable extraction efficiencies of P-CTX-1 from fish flesh, but more interferences were extracted as indicated by the dark yellow suspension observed in the raw extracts. Because of these interferences, 75 °C was chosen for subsequent optimization. The influence of the number of extraction cycles (two or three cycles; static time, 5 min) and static time (5 or 10 min, two cycles) was examined, but negligible differences were found in the extraction efficiency of P-CTX-1 from fish flesh (Fig. 3b). Considering the low level of co-extracting interfering components, and the reduced time and cost involved, the optimized procedure for P-CTX-1 extraction employed methanol as the extraction solvent for two extraction cycles, 5 min of static time at 75 °C and 1,500 psi with 60% flush volume and 100 s of purge time. By spiking the same amount (20 ng) of P-CTX-1 in the raw extract after extraction, the recovery of P-CTX-1 was found to be 79±1% in the cleanup step (SPE) alone. Although the absolute recovery of the present method was around 73%, the estimated recovery of P-CTX-1 from fish flesh was determined to be over 90% for the ASE extraction. Although an ASE system may be expensive and additional time is required for sample preparation steps such as freeze-drying and grinding with DE, sample extraction is

Table 2 Optimized conditions for extraction of P-CTX-1 in fish flesh using accelerated solvent extraction

Extraction solvent	Methanol
Pressure (psi)	1,500
Temperature (°C)	75
Heat-up time (min)	5
Static time (min)	5
Flush volume (%)	60
Purge time (s)	100
Number of cycles	2
Cell volume (mL)	22
Total extraction time (min)[a]	20
Total solvent used (ml)[a]	40

[a] Per sample

fully automated. Each sample required only 20 min for extraction, and relatively clean extract can be obtained without the need for filtration. Additional efficiency will be obtained at larger sample sizes. Optimized ASE conditions for extraction of P-CTX-1 in fish flesh are shown in Table 2.

Method validation

The repeatability and reproducibility of the whole method were estimated by evaluating the intra-batch and the inter-batch precision as reflected by the coefficient of variation (CV); these were found to be 3.82% and 4.75%, respectively. The LOD (S/N>3) and LOQ (S/N>10) of the present method were 0.1 and 0.2 ppb in fish flesh for the HPLC-3200 QTRAP. As the LOQ of the 3200 QTRAP was above the minimum level (0.1 ppb) expected to cause ciguatera, a more sensitive mass spectrometer, the 5500 QTRAP, was used instead of the 4000 QTRAP that was used by previous studies [14, 16] to examine the LOD and LOQ of this method. Using 5500 QTRAP, the LOD was 0.25 ppb in methanol, while the LOQ was 0.5 ppb in methanol and 2.5 ppb in fish extract, which were equal to 0.01 and 0.05 ppb in fish muscle, respectively. A recent report calculated the level of P-CTX-1 in fish flesh considered to be safe for human consumption to be less

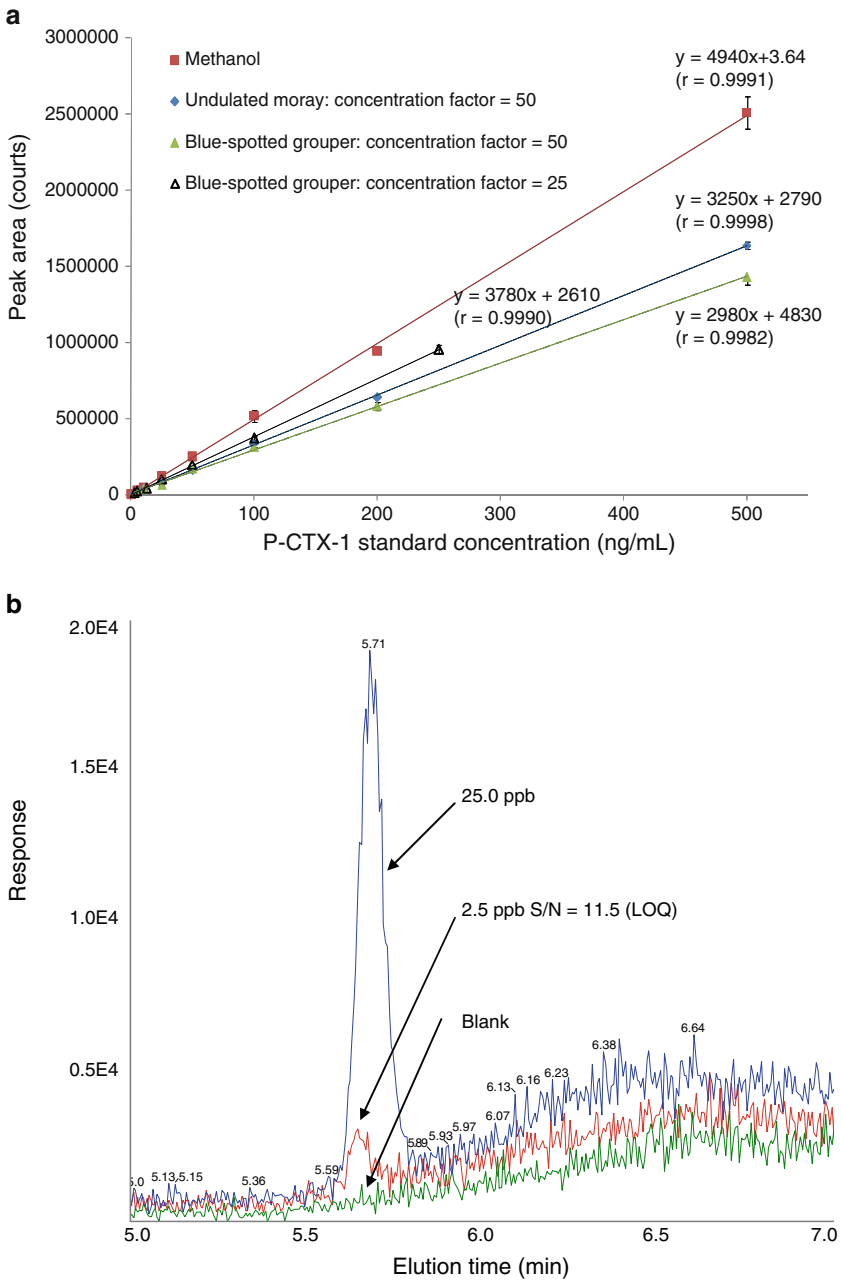

Fig. 4 **a** Calibration curves in MeOH and different matrices (accuracy, ±20%) determined by 5500 QTRAP. **b** Response detected at *m/z* 1,128.6>1,075.7 obtained for 0, 2.5 and 25 ppb of P-CTX-1 in *Gymnothorax undulatus* matrix determined by 5500 QTRAP

than 0.01 ppb, including a safety factor of 10 [8]. The LOQ (0.01 ppb) obtained from the 5500 QTRAP can thus meet this safety level for P-CTX-1 in fish flesh and thereby allows the employment of this method in routine screening and quantification of P-CTX-1 in market samples (Fig. 4b). The responses detected at *m/z* 1,128.6>1,075.7 for the blank, 2.5 ppb (LOQ) and 25 ppb of P-CTX-1 in an extract of *G. undulatus* measured by 5500 QTRAP are shown in Fig. 4b.

Matrix interference, causing either ion suppression or enhancement, is a common problem in LC-MS/MS analysis. In the detection of CTXs by LC-MS/MS, matrix effects are frequently evident, likely due to remaining fish oil content [14]. To assess matrix effects in the present method, calibration curves of P-CTX-1 in methanol and in the extract of flesh of *G. undulatus* and *C. argus* were constructed. These two fish species are an eel and a grouper, respectively, that have oily flesh and are frequently implicated in CFP. These species were chosen to represent other coral reef fish for the purposes of matrix-matched calibration curve construction because the oily nature of their flesh was expected to induce the greatest matrix effects. Within the tested ranges, good linear regressions with high correlation coefficients (R^2>0.99) were obtained for both calibration curves (Fig. 4a). However, comparing the slope of the matrix-matched calibration curve to that of the calibration curve constructed by standards in pure methanol showed that responses of P-CTX-1 were reduced by 34% in *G. undulatus* and by 40% in *C. argus* extracts, respectively, probably due to lipids that remained in the extract, causing ion suppression and increasing the background noise. Usually, an isotopically labelled internal standard is used in LC-MS/MS quantification because the MS is sensitive to differences among sample matrices. Because of the absence of commercially available isotopically labelled P-CTX-1 standard, other chemicals with similar structures, such as brevetoxin, were used previously as the internal standard, but the differential retention time and response to matrix effects differed from those of the analyte, and thus similar compounds such as brevetoxin are not ideal internal standards for an LC-MS/MS method [13]. Identification and development of new internal standards is urgent for the accurate quantification of CTXs. The use of internal standard is to correct for the loss of analyte during sample preparation or sample inlet and to correct the matrix effect in LC-MS/MS analysis. Instead of the addition of internal standard for P-CTX-1 analysis, QA/QC procedures such as matrix spike recovery can be carried out in each batch of analysis to check for the recovery of P-CTX-1 during sample preparation. Besides, quality control standard (P-CTX-1) can be injected after every ten sample injections to make sure the stability of the instrument. Although the matrix effect contributed to the decrease in the recovery, the degree of effect was improved compared to a previously published method in which a 75% reduction in the response of P-CTX-1 was reported [14]. Based on the matrix-matched calibration curve constructed from analysis of *C. argus* with different concentration factors (25× and 50×), ion suppression was minimized, and hence, recovery of P-CTX-1 was found to be improved by dilution.

Table 3 Matrix spike recoveries (percent) of P-CTX-1 in muscle of 17 coral reef fish species that are edible and suspected to be ciguatoxic

	Species	Common name	Diet	*n*	Recovery (%)	
					Mean	SD
1	*Gymnothorax undulatus*	Undulated moray	Carnivore	6	73	3
2	*Epinephelus spilotoceps*	Foursaddle grouper	Carnivore	4	85	3
3	*Epinephelus corallicola*	Coral grouper	Carnivore	2	77	0
4	*Epinephelus fuscoguttatus*	Black-spotted grouper	Carnivore	2	77	5
5	*Cephalopholis argus*	Blue-spotted grouper	Carnivore	2	70	1
6	*Variola louti*	Lyretail grouper	Carnivore	2	82	1
7	*Lutjanus bohar*	Two-spot red snapper	Carnivore	2	83	6
8	*Lutjanus gibbus*	Humpback red snapper	Carnivore	2	73	2
9	*Lutjanus fulvus*	Blacktail snapper	Carnivore	2	70	3
10	*Monotaxis grandoculis*	Humpnose big-eye bream	Carnivore	2	78	0
11	*Ctenochaetus binotatus*	Twospot surgeonfish	Omnivore	1	72	/
12	*Acanthurus lineatus*	Clown surgeonfish	Herbivore	2	49	3
13	*Siganus argenteus*	Forktail rabbitfish	Herbivore	2	66	3
14	*Acanthurus maculiceps*	Spot face surgeonfish	Herbivore	2	72	2
15	*Acanthurus gahhm*	Black surgeonfish	Herbivore	2	75	1
16	*Scarus ghobban*	Blue-barred parrotfish	Herbivore	2	51	3
17	*Naso lituratus*	Orangespine unicornfish	Herbivore	2	68	2

Table 4 Comparison of the concentrations and matrix spike recoveries of P-CTX-1 in fish muscle extracted by the present method and the modified method based on the methods of Lewis et al. [14] and Stewart et al. [16], but including sonication [24]

Species	Common name	Present method			Previous method [14, 16, 24]		
		Conc (ng/g)	Mean	SD	Conc (ng/g)	Mean	SD
Epinephelus spilotoceps	Foursaddle Grouper	2.67	2.70	0.0424	1.77	1.94	0.240
Epinephelus spilotoceps	Foursaddle Grouper	2.73			2.11		
Epinephelus fuscoguttatus	Black-spotted grouper	1.35	1.38	0.0354	0.870	0.833	0.0530
Epinephelus fuscoguttatus	Black-spotted grouper	1.40			0.795		
Cephalopholis argus	Blue-spotted grouper	2.92	2.86	0.0919	1.79	1.64	0.219
Cephalopholis argus	Blue-spotted grouper	2.79			1.48		
Gymnothorax undulatus	Undulated moray	7.90	7.83	0.106	4.53	5.52	1.39
Gymnothorax undulatus	Undulated moray	7.75			6.50		
Recovery (%)			73±3			51±8	

Matrix spike recoveries were determined by spiking 20 ng P-CTX-1 in 5 g of muscle of non-toxic undulated moray

The present analytical method was further applied to other reef fishes, including carnivores, herbivores and omnivores, which are the potential vectors of CFP. Among the 17 fish species analysed, recoveries of P-CTX-1 in flesh of 15 fish species ranged from 66% to 85%; two herbivorous fish, *A. lineatus* and *S. ghobban*, showed recoveries of 49% and 51%, respectively (Table 3). The coefficients of variation were lower than 7% in all cases. In order to evaluate the variability between fish of the same species, the CV was calculated based on replicate analysis of two non-toxic *E. spilotoceps* spiked with the same amount of P-CTX-1 standard, and the result showed that the variability is 3.53%. In conclusion, this method is robust and acceptable for most fish species.

Comparison to other extraction methods and application to ciguatoxic fish

The matrix spike recovery of P-CTX-1 in flesh of a non-toxic *G. undulatus* obtained by the present method was compared with that obtained by a conventional method employing homogenization as the extraction method (Table 4) [14, 16]. Dechrouai et al. [24] used sonication for extraction of CTXs from fish, so the conventional method was modified with an additional sonication step after homogenization in order to increase the extraction efficiency and ensure good overall recoveries; the values in Table 4 therefore represent a comparison between the present method and the modified method of Lewis, Stewart and Dechrouai [14, 16, 24]. The present method gave better matrix spike recovery of P-CTX-1 (73±3%) in the eel tissue, whereas that determined by the method using homogenization for extraction was found to be 51±8%. Similar results were obtained when comparing the matrix spike recovery of *Epinephelus* spp. (80% and 50%) and *L. bohar* (83% and 64%) reported here and in a previous study [16]. In addition, the CV was found to be larger when using homogenization as the extraction method. Four toxic fish of different species were extracted using both methods, and the quantification was performed using a calibration curve constructed with standards in methanol, since matrix-matched calibration curves are not very reliable due to large variations in fat content among fishes. Similarly, concentrations of P-CTX-1 extracted using the present method were relatively higher than those extracted by the homogenization/sonication method.

Comparison of the toxicities obtained from HPLC-MS/MS and MNA

MNA is a common bioassay method used for the detection of CTXs, and its results have been suggested to be strongly correlated with those of the in vivo mouse bioassay. In order to assess the correlation between ciguatoxicity of fish flesh measured by MNA and HPLC-MS/MS, 12 *C. argus*

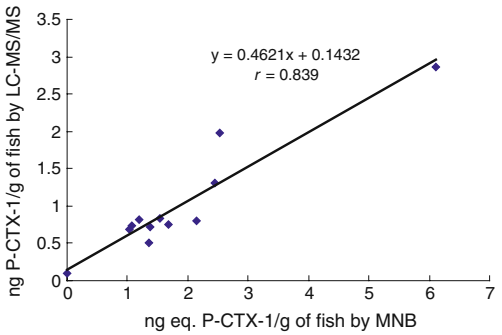

Fig. 5 The relationship between the ciguatoxicity of fish flesh measured by the MNA and the amount of P-CTX-1 quantified by HPLC-MS/MS

were extracted using the optimized method. The final extracts were both injected into the LC-MS/MS and dosed to Neuro-2a cells with appropriate dilution. A positive correlation coefficient (r=0.839; p<0.05) was found between two detection methods using Spearman rank order correlation (Fig. 5), and the ciguatoxicities (P-CTX-1 equivalents) of fish flesh determined by MNA were generally 1.5 to 2.5 times higher than the amounts of P-CTX-1 measured by HPLC-MS/MS. This difference may be due to the presence of other CTXs (P-CTX-2/3) or sodium channel activators in the sample extracts. P-CTX-1 contributed an average of 55% to the total ciguatoxicity in *C. argus* using a standard calibration curve constructed in MeOH. However, considering the ion suppression factor in HPLC-MS/MS, P-CTX-1 provided an average of 92% of the total ciguatoxicity in *C. argus* based on a matrix-matched calibration curve. This result was consistent with those of Lewis et al. [15], who showed that P-CTX-1 contributed 90% of the total lethality in mice after exposure to ciguatoxic fish extracts. The LC-MS/MS result agrees with the MNA result, and therefore, the LC-MS/MS method developed in the present study is an accurate and useful tool for quantifying ciguatoxicity.

Conclusions

In summary, a validated ASE-HPLC-MS/MS method for the quantification of P-CTX-1 in fish flesh was established in the present study. The method achieved higher recovery, sensitivity, accuracy and reproducibility for quantification of P-CTX-1 from different fish species than other analytical methods described previously. The LOQ of this method was determined to be 0.01 ng/g fish flesh, which is the level considered to be safe for human consumption [8]. Results obtained from the present analytical method were also well correlated with those measured by the MNA. Additionally, as analogues of P-CTX-2/3 were also identified in the extract of fish flesh obtained by this method, the simultaneous determination of other P-CTXs in fish flesh using ASE is feasible once appropriate standards are available. The present rapid extraction method may be useful for routine analysis of fish in the marketplace for the prevention of outbreaks of CFP by public health authorities.

The cleanup step was the most time-consuming part of the present method. Although omission of the final normal-phase SPE step has been suggested, normal-phase SPE can minimize matrix effects that may contribute to ion suppression, as well as result in a better peak shape due to the removal of residual lipids and the reduction in background noise in the chromatograph. We also tried to analyze various fish organs, such as liver and intestine, but the noticeable amount of lipid hindered the method development. Further studies can explore ways to improve the performance of the cleanup process for lipid removal.

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