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1 **Sample preparation and liquid chromatography-tandem mass spectrometry for the**
2 **analysis of selected Pacific ciguatoxins in blood samples**

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31 **Abstract**

32 Consumption of ciguatoxin-contaminated seafood can lead to ciguatera poisoning (CP). The
33 diagnosis of CP in humans is based on the clinical symptoms after eating the fish from tropical
34 or subtropical areas because no confirmatory clinical tests are available. One of the challenges
35 for ciguatoxin analysis is their extremely low but toxicologically relevant concentration in
36 biological samples. We previously reported a method using acetonitrile to precipitate proteins
37 and extract the ciguatoxins simultaneously in whole blood samples from animals for toxin
38 quantification by N2A cell-based assay. However, a test method for unambiguous confirmation
39 of exposure of marine animals or humans to ciguatoxins is still needed. In the present study, we
40 adopted the acetonitrile extraction method and added sample clean-up in the sample preparation
41 for the determination of Pacific ciguatoxins CTX1B (aka P-CTX-1), 52-*epi*-54-deoxyCTX1B
42 (aka P-CTX-2), and CTX3C (aka P-CTX-3C) in blood plasma by LC-MS/MS. We investigated
43 sample clean-up, LC mobile phases, LC solvent programming, and settings of the two mass
44 spectrometers (4000 Q TRAP and AB SCIEX Triple Quad 5500) in order to improve the ability
45 to detect the Pacific ciguatoxins at ppt level. Rat blood plasma was used for the method
46 development. Average recoveries of the three toxins in the rat plasma samples ranged from 90%
47 to 116% with relative standard deviations of less than 15%. The method detection limits were
48 still not low enough for the determination of the Pacific ciguatoxins in individual blood samples
49 from Hawaiian monk seals with the two LC-MS systems. The methods were applied to a pooled
50 sample of blood plasma collected from Hawaiian monk seals for confirmation of toxin exposure.
51 This study will benefit monitoring of Pacific ciguatoxins in marine mammals and potentially
52 humans by LC-MS/MS.

53 *Keywords*: ciguatera; Pacific ciguatoxins; blood; LC-MS; Hawaiian monk seal.

54 **1. Introduction**

55 Ciguatera poisoning (CP) is a seafood-associated disease caused by the consumption of tropical
56 and subtropical fish or gastropods contaminated with ciguatoxins. The reported incidence of CP
57 in the United States is the highest among the diseases attributed to finfish [1]. Ciguatoxins are
58 polyether toxins, and their chemical structures vary with the area of their origins such as
59 Caribbean ciguatoxins and Pacific ciguatoxins. The marine dinoflagellate *Gambierdiscus spp.*
60 has been confirmed the origin of Pacific ciguatoxins [2, 3]. Pacific ciguatoxins from algae are
61 polycyclic ethers consisting of thirteen fused rings grouped into two types of congeners, which
62 differ in backbone structure and a side chain at A ring. One group has an oxepene in E ring and
63 a side chain at A ring represented by CTX4B [2] and its epimer CTX4A [3]. The other group
64 has an oxocene in E ring and lacks the A-ring side chain represented by CTX3C [4] and its
65 epimer CTX3B. Other congeners from these two toxin groups have also been detected in
66 *Gambierdiscus spp.* [5,6]. Algal ciguatoxins enter the food web through predation by
67 herbivorous fish and then to higher trophic levels via carnivorous fish, along with their oxidation
68 modifications in fish [2,7,8]. One of the important Pacific ciguatoxins in fish, CTX1B (P-CTX-
69 1) was isolated from moray eel (*Gymnothorax javanicus*) for structural elucidation, and is
70 believed to be a fish metabolite from CTX4A and CTX4B [2,8]. Both groups of ciguatoxins
71 including the oxidized congeners have been detected in fish [9,10]. Most oxidized Pacific
72 ciguatoxins are more potent than algal toxins [2-4,10-14]. CTX1B is one of the most potent

73 ciguatoxins [2,10-13] and is often present in ciguatera fish from Pacific Ocean [9,10,15]; spike-
74 and-recovery tests were performed for sample preparation and LC-MS/MS determination of
75 CTX1B in fish [15-19] and was extended to 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B [15].
76 Due to insufficiency or lack of toxin standards, only limited groups covered the detection of a
77 wider range of Pacific ciguatoxin congeners in fish by LC-MS/MS but without the spike-and-
78 recovery evaluations of the whole process including sample preparation and LC-MS
79 determination [9,10,20]. The United States Food and Drug Administration (FDA) has
80 recommended a guidance level of 0.01 ppb CTX1B equivalent toxicity in fish from Pacific
81 regions [1]. Ciguatoxins may undergo further transformations in marine mammals and humans,
82 which makes the detection of individual toxins by LC-MS difficult with even lower
83 concentration.

84 The diagnosis of CP in humans is based on the clinical symptoms associated with consumption
85 of fish determined or recognized to accumulate ciguatoxins. No confirmatory clinical tests are
86 available for the diagnosis of CP [1,21-23]. Our laboratory has demonstrated that the N2A cell-
87 based assay could be used for directly monitoring ciguatoxin exposure in the blood of
88 experimental and naturally exposed animals [22,24-26]. However, LC-MS/MS is generally the
89 tool used for unambiguous confirmation of the presence of toxins in animal samples thus toxin
90 exposure. In this study, we used rat plasma to develop sample preparation and LC-MS methods
91 for the determination of Pacific ciguatoxins in blood samples and the protocols were applied to
92 Hawaiian monk seal plasma samples for confirmation of toxin presence in the animals. For
93 blood sample extraction, we adopted our previous toxin extraction method using acetonitrile to
94 precipitate proteins and extract the ciguatoxins simultaneously from blood [24]. For clean-up of
95 blood extracts, we applied two solid-phase extractions (SPE; C18 and silica), similar to those
96 reported [16-18,27], but with modifications to determine not only ciguatoxin fish metabolites
97 (CTX1B and 52-*epi*-54-deoxyCTX1B) but also the unmetabolized algal ciguatoxins CTX3C. For
98 LC-MS detection of ciguatoxins, we evaluated the LC mobile phases, LC solvent programming,
99 and MS settings of two mass spectrometers (4000 Q TRAP and AB SCIEX Triple Quad 5500)
100 with a slight difference in MS interface structure for the detection of CTX1B and 52-*epi*-54-
101 deoxyCTX1B from the group of Pacific ciguatoxins carrying CTX4A and CTX4B backbone
102 structure and CTX3C from the other group in the absence and presence of blood plasma matrix.
103 Only three Pacific ciguatoxins were used in the quantification method development due to the
104 limited availability of specific ciguatoxin congeners.

105 **2. Materials and methods**

106 2.1. Chemicals

107 CTX1B (P-CTX-1) and 52-*epi*-54-deoxyCTX1B (P-CTX-2) were from Dr. R. Lewis (The
108 University of Queensland, Australia), CTX3C was from Prof. T. Yasumoto (Japan Food
109 Research Laboratories, Japan). CTX3B, CTX4A, and CTX4B were from Dr. M. Chinain
110 (Laboratoire des Micro-algues Toxiques, Institut Louis Malardé, French Polynesia). Chloroform,
111 methylene chloride, acetone, and water were of HPLC grade manufactured by Burdick &
112 Jackson (Muskegon, MI, USA). HPLC grade acetonitrile and methanol were either from Burdick
113 & Jackson or from Fisher Scientific (Pittsburgh, PA, USA). Isopropanol of LC-MS Chromasolv
114 grade was purchased from Sigma (St. Louis, MO, USA). Formic acid (Guaranteed Reagent,

115 EMD brand, minimum 98%) was purchased from VWR International (Atlanta, GA, USA).
116 Ammonium formate was purchased from Sigma. For sample preparation, Milli-Q water was
117 used in place of HPLC water. Rat plasma (containing K2EDTA additive) from SD male rats was
118 purchased from Hilltop Lab Animals, Inc. (Scottdale, PA, USA).
119
120 2.2. Rat blood plasma extraction
121
122 Acetonitrile was used to precipitate plasma proteins and extract Pacific ciguatoxins spiked in rat
123 blood plasma (3:1, v/v), similar to previously reported [24] with modifications. Acetonitrile (600
124 µl) was added to a 15 ml polypropylene centrifuge tube (BD Falcon) containing 400 µl plasma.
125 After briefly vortex mixing, another 600 µl of acetonitrile was added to the plasma. The sample
126 was vortex mixed for about 1 min then stored at – 20 °C for 15 min. The sample was centrifuged
127 at 2750 $\times g$ and at 4 °C (IEC Centra CL3; Thermo Scientific, Waltham, MA, USA) for 8 min.
128 The supernatant was collected into a glass test tube (13 × 100 mm). An amount of 1200 µl of
129 acetonitrile was added to the plasma pellet for a second extraction, following the same procedure
130 as used for the first extraction. The pellet was broken down into the solvent through the use of a
131 small scoopula or a disposable polypropylene pipette. The combined supernatant of both
132 extractions was dried under nitrogen using a Caliper/Zymark TurboVap LV at 40 °C.
133
134 2.3. Sample clean-up
135
136 The dried extract was cleaned using two solid-phase extraction (SPE) cartridges: C18 SPE (Bond
137 Elut C18, 500 mg, 10 ml; Agilent, USA) and Si SPE (Bond Elut Si, 500 mg, 3 ml; Agilent). The
138 dried residue from extraction was dissolved in 2.5 ml methanol by vortex mixing and brief
139 sonication in a water bath. The extract was then brought to 50% methanol/water (50:50, v/v) by
140 adding 2.5 ml water with vortex mixing and then loaded on a C18 SPE cartridge conditioned
141 with 1 column volume of methanol and then 1 column volume of water. The sample tube was
142 washed with 5 ml 50% methanol then the washed solution was transferred to wash the SPE
143 cartridge. The sample tube was further washed with 6.5 ml 65% methanol/water (65:35, v/v) and
144 then the washed solution was transferred to the SPE cartridge. The toxins were eluted from the
145 cartridge with 6 ml 95% methanol/water (95:5, v/v). The C18 SPE eluate was dried under
146 nitrogen using a TurboVap LV at 40 °C. The dried residue from the C18 SPE was dissolved in 3
147 ml 2% isopropanol in chloroform (2:98, v/v) and loaded on a Si SPE cartridge conditioned with
148 about 2 column volume of chloroform. The sample tube was washed with 5 ml 2% isopropanol
149 in chloroform and the washed solution was transferred to the Si cartridge. The toxins were eluted
150 with 8 ml 10% methanol in chloroform into a glass test tube (13 × 100 mm). The Si SPE eluate
151 was dried with nitrogen using a TurboVap LV at 25 °C or below (i.e., room temperature). The
152 dried residue from Si SPE was dissolved in 200 µl methanol and centrifuged at 2750 $\times g$ at 4 °C
153 for 5 min. The supernatant was collected into an LC vial for analysis.
154
155 2.4. Hawaiian monk seal plasma samples
156
157 Plasma samples previously tested positive for ciguatoxins (0.07 to 0.27 ng CTX3C equiv./ml) as
158 analyzed by N2A assay were pooled (total volume about 6.5 ml from 11 plasma samples) for
159 extraction using procedures described by Bottein Dechraoui et al [24]. The dried extract (using

160 TurboVap LV for drying) was subjected to C18 SPE (Bond Elut C18, 500 mg, 10 ml) clean-up.
161 The residue was dissolved in 2 ml methanol, then 2 ml water was added to the solution. After
162 vortex mixing, the sample solution was loaded on the C18 cartridge conditioned with 1 column
163 volume of methanol and then 1 column volume of water. The sample tube was washed with 5 ml
164 65% methanol/water and the wash solution was transferred to the cartridge. The cartridge was
165 further washed with 6 ml 10% methanol in water and 1.5 ml 65% methanol. The toxins were
166 eluted with 5.5 ml methanol. The dried C18 eluate was dissolved in 100 µl of methanol and 10 µl
167 was injected for LC fractionation (Section 2.5). One-minute fractions (the first-minute fraction
168 was in Tube 2 or fraction 2) were collected and tested by N2A assay for CTX activity [1]. The
169 remaining sample solution after LC fractionation was dried and then subjected to C18 and Si
170 SPE clean-up as described in Section 2.3. The cleaned sample residue was dissolved in less than
171 80 µl for LC-MS/MS analysis.
172
173 2.5. Liquid chromatography-tandem mass spectrometry
174
175 Separation and detection was performed on two LC-MS platforms; both equipped with an
176 Agilent 1100 LC systems containing the same modules including a quaternary pump for each
177 system (Agilent Technologies, USA). One MS was 4000 Q TRAP and the other was AB SCIEX
178 Triple Quad 5500, both equipped with a TurboIonSpray (TIS) source (AB Sciex, USA). Toxin
179 separation was achieved on a Luna C8(2) column (150 × 2 mm, 5 µm; Phenomenex, USA) at a
180 flow rate of 0.2 ml/min and at a column temperature of 40 °C. Mobile phase A was water
181 containing 0.2 mM ammonium formate and 0.1% formic acid. Mobile phase B was acetonitrile
182 containing 0.02% formic acid. Mobile phase C was methanol containing 0.02% formic acid.
183 Mobile phase D1 was water containing 0.1% formic acid. Mobile phase D2 was water containing
184 2 mM ammonium formate and 0.1% formic acid. Mobile phase D3 was water containing 0.2%
185 formic acid. Two LC elution gradients (Gradient 1 and 2) as shown in Table 1 and 2 were
186 applied to the toxin detection in blood samples using the LC-4000 Q TRAP with the Gradient 1
187 as the final protocol. Gradient 1 was slightly modified when samples were processed using the
188 LC-Triple Quad 5500 (Gradient 3, Table 3).
189 The LC-MS method was initially set up on the 4000 Q TRAP and subsequently applied to the
190 Triple Quad 5500 (the Triple Quad 5500 was available after almost all analyses had been done
191 on the 4000 Q TRAP). The LC-MS method was transferred and modified to work with the Triple
192 Quad 5500 because Sciex 5500 mass spectrometers are reported by the manufacturer to have
193 improved sensitivity over the Sciex 4000 series. Detection of ciguatoxins was achieved by
194 multiple reaction monitoring (MRM) in positive ion mode: CTX1B with MRM transitions of *m/z*
195 1128.6 → 1057.6, 1075.6, and 1093.6 (for the Triple Quad 5500, m/z 1093.6 was replaced with
196 *m/z* 1039.6), 52-*epi*-54-deoxyCTX1B with MRM transitions of *m/z* 1112.6 → 125.1 (for Triple
197 Quad 5500, *m/z* 125.1 was replaced with *m/z* 155.1), 1059.6, and 1077.6, CTX3C with MRM
198 transitions of *m/z* 1023.6 → 125.1, 155.1, and 1005.6. The temperature of the turbo gas (TEM)
199 was set at 320 °C for the 4000 Q TRAP and 390 °C for the Triple Quad 5500. The curtain gas
200 value (CUR) was set at 20 psi. The collision gas CAD parameter was set at medium for the 4000
201 Q TRAP and 7 for the Triple Quad 5500. Other parameters were adjusted to optimum values. For
202 the 4000 Q TRAP, when acetonitrile was the only organic solvent used in the LC elution
203 gradient, the declustering potential (DP) value for CTX3C was set at 60 V; when methanol was
204 added into the LC elution gradient, the DP value for CTX3C was set at 100 V (Section 3); for the

Triple Quad 5500, the DP value for CTX3C was the same for both acetonitrile and methanol mobile phases. The MS scans for an LC–MS run were divided into 4 periods accompanied with the use of a 2-position diverter valve (VICI, Valco Instruments CO. Inc., Houston, USA for the 4000 Q TRAP; a Triple Quad 5500 has a built-in diverter) to improve detectivity and sensitivity of MS and to reduce MS contamination, similar to the description reported previously [28]. The LC eluants of the first and the last periods were directed to a waste container with the ion spray voltage (IS) of 0 kV and the eluants of the middle periods covering the elution of the toxins were sent to the MS with IS of 5.5 kV. The second period was for CTX1B monitoring and the third period was for monitoring of 52-*epi*-54-deoxyCTX1B , CTX3C, their isomers, and other CTX congeners if included in the MRM scan. The LC-4000 Q TRAP was controlled by Analyst 1.4.1 software and the LC-Triple Quad 5500 was controlled by Analyst 1.6.2 software.

For analysis of cytotoxicity of LC fractions, previously described LC-MS conditions [25] were used to accommodate the existing N2A assay and fractionation was performed on the LC-4000 Q TRAP. The LC mobile phase was water (A)/acetonitrile (B) with both containing 0.1% formic acid additive. The LC gradient was: 1 min of 50% B, linear gradient to 80% B at 20 min, 95% B at 40 min, held at 95% B for 5 min, returned to 50% B at 46 min, and held for 9 min. For MRM method setup to locate the retention time of toxins in the LC fractions, protonated molecular ions of the ciguatoxins were the precursors, *m/z* 125 was the fragment for toxins with L and M ring having the same structure as that of CTX3C and a protonated molecular mass minus 36 Da as a fragment for other ciguatoxins.

3. Results and Discussion

3.1. SPE methods

We adopted the 2-column-SPE clean-up methods of Lewis et al. [16], Stewart et al. [17], and Mak et al. [27] with modifications. 95% methanol/water, instead of 80% methanol/water was used to elute both groups of ciguatoxins from 500 mg C18 cartridges. For Si SPE, we found the solubility of CTX1B was low in chloroform and chloroform could not be used to transfer CTX1B from test tubes to SPE cartridges in the absence of sample matrix (e.g., fish tissue or blood matrices). Blood samples collected from marine mammals are generally small in volume so a small quantity of isopropanol (2%) in chloroform was used to solubilize CTX1B in the dried eluates from the C18 cartridges and also as a loading solvent for the Si SPE cartridges. Greater than 50% of CTX3C was eluted from the Si SPE cartridge with 2% methanol in chloroform (~ 6 ml) as the loading and wash solvents. When acetone was mixed with methylene chloride as the loading and washing solvents for the Si SPE cartridges, the majority of CTX1B was lost during the Si SPE process. No CTX1B was detected in the loading and washing solvents eluted from the cartridge and trace or no P-CTX-1 was detected in the elution solvent (i.e., 8 ml of 10% methanol in chloroform). The loss of P-CTX-1 in the Si SPE process with the use of acetone and methylene chloride solvent mixture might be one of the reasons that we did not observe CTX1B in Hawaiian monk seal liver samples [25]. Florisil cartridges (500 mg, 4 ml; Alltech) were tested as an alternative to the Si SPE using the same solvent scheme as reported [9], but the injections of Florisil-cleaned blood extracts caused the contamination of the LC-MS and resulted in a MS signal loss of CTX3C injected from the subsequent standard solutions.

3.2. Liquid chromatography–Mass spectrometry

3.2.1. Selection of MRM transitions for Pacific ciguatoxins

CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C can form protonated adducts $[M+H]^+$, ammonium adducts $[M+NH_4]^+$, and sodium adducts $[M+Na]^+$ for MS detection. The relative intensity of the three adducts for each toxin in the LC-MS chromatograms monitored by selected ion monitoring (SIM) scan was affected by LC elution conditions (e.g., type of organic solvent and its composition, additives and their concentration in the LC mobile phases) and MS interface conditions. Ciguatoxin sodium adducts could be detected as the prominent ions by 4000 Q TRAP, but they were not prone to being dominant adduct ions using an AB SCIEX Triple Quad 5500 under common LC-MS conditions. Ciguatoxin sodium adducts do not fragment well in mass spectrometers with low collision energy (e.g, triple quadrupole mass spectrometers) and they were accompanied by interfering ions of the same mass in LC-MS chromatograms. Therefore, ciguatoxin sodium adducts were not adopted for MS determination in blood matrices as they were used for the analysis of fish samples [9]. Ammonium adducts were adopted as precursor ions for CTX1B and 52-*epi*-54-deoxyCTX1B detection [16-18,20,27]. For CTX3C, the intensity ratio of $[M+H]^+$ to $[M+NH_4]^+$ with the 4000 Q TRAP usually could be about or higher than 1 when both formic acid and ammonium formate were the mobile phase additives, lower than 1 when only ammonium formate was the additive, and higher than 1 when only formic acid was the additive; this ratio with the Triple Quad 5500 could be about or lower than 1 when the additives included ammonium formate and higher than 1 when formic acid was the only additive. The ammonium adduct of CTX3C could be fragmented to protonated adduct when an increased declustering potential (DP) was applied to the 4000 Q TRAP, especially with methanol mobile phases. However, this phenomenon was not observed or not obvious on the Triple Quad 5500 due to the difference between the interfaces in the two MS instruments. Therefore protonated adduct was generally used for CTX3C detection with the 4000 Q TRAP while protonated or ammonium adduct for CTX3C detection was used with the Triple Quad 5500 depending on the organic modifier and the additives in mobile phases. Product ions with one or two water molecules lost from the ciguatoxin precursors (e.g., m/z 1023.6 → 1005.6 for CTX3C) generally showed much higher signals than non-water-loss fragments and were selected for MRM transitions as reported [16-18,27]. However, they generally carried much higher noises than other fragments with blood matrix in LC-MS chromatograms, non-water-loss fragments (e.g., m/z 125 and 155 for CTX3C) and fragments with three water molecules lost from precursors (e.g., m/z 1128.6 → 1093.6 for CTX1B was replaced with m/z 1128.6 → 1039.6 with the Triple Quad 5500) were added into MRM settings to improve MS detection.

3.2.2. LC mobile phases and their additives for MS detection

LC mobile phases with either methanol/water or acetonitrile/water have been used for Pacific ciguatoxin quantification [9,16]. Pacific ciguatoxins are polycyclic ethers with similar molecular structure, but they showed different ionization efficiencies with respect to the type of organic solvent (acetonitrile versus methanol) in the LC eluent. Methanol was favorable to CTX3C while acetonitrile was favorable to 52-*epi*-54-deoxyCTX1B for the production of adduct ions (protonated or ammonium adducts) at the MS interface (as large as 2 to 9 fold on peak height

295 with a comparison using an isocratic elution of 88% acetonitrile/water vs. 88% methanol/water,
296 both containing formic acid and ammonium formate additives). Methanol caused more toxin
297 band broadening than acetonitrile during the migration of the ciguatoxins inside the LC column.
298 The peak height of CTX1B with an LC eluent of 88% acetonitrile/water containing 2 mM
299 ammonium formate and 0.1% formic acid (eluted at 2.10 min) was about 2-fold of that observed
300 with the corresponding LC eluent in which acetonitrile was replaced with methanol (eluted at
301 2.48 min), but with slightly less peak area as detected by the 4000 Q TRAP. The peak height of
302 52-*epi*-54-deoxyCTX1B with an isocratic elution of 88% acetonitrile containing 0.02% formic
303 acid/12% water containing 0.2 mM ammonium formate and 0.1% formic acid was about 9-fold
304 (peak area ratio about 6 fold) of that obtained with the 88% methanol/water isocratic elution
305 containing the same additives, as detected by the Triple Quad 5500. CTX3C eluted late in the
306 reversed-phase LC columns. The peak broadening caused by methanol/water eluent greatly
307 counteracted the high ionization efficiency of methanol towards this toxin especially with a
308 traditional HPLC system using a long LC column at a low flow rate compared to a UPLC system
309 using a short LC column at a high flow rate. Zorbax Eclipse Plus C18 UPLC columns (2.1 × 50
310 mm, 1.8 µm; Agilent Technologies) have been successfully used for the detection of Pacific
311 ciguatoxins in fish using methanol/water mobile phases at 0.4 ml/min [9,20]. However, the
312 corresponding HPLC column (2.1 × 150 mm, 5 µm) showed severe band broadening with
313 CTX3C due to the use of longer column for better separation at 0.2 ml/min flow rate.
314 Acetonitrile/water mobile phases containing 0.1% formic acid and 2 mM ammonium formate
315 have been reported for the quantification of CTX1B with or without 52-*epi*-54-deoxyCTX1B and
316 54-deoxyCTX1B [16,17,27]. At an LC flow rate of 0.2 ml/min, we found that the optimized
317 concentration of the mobile phase additives in an LC eluent for the detection of Pacific
318 ciguatoxins with ammonium adducts was less than 0.1% for formic acid and less than 2 mM for
319 ammonium formate. Methanol/water mobile phases generally needed slightly higher amounts of
320 the additives than acetonitrile/water mobile phases in the LC eluent. The MS response of CTX1B
321 with acetonitrile/water mobile phases varied in the range of 1 to 3 fold typically with ammonium
322 formate of 0.1 to 2 mM and formic acid of 0.03% to 2% in the LC eluents. Higher concentration
323 of mobile phase additives caused suppression of the toxin adduct formation while extremely
324 lower concentration caused less efficiency in the electrospray process. The optimized
325 concentration of the additives and their MS effects were similar between the 4000 Q TRAP and
326 the Triple Quad 5500 but with slightly variation.
327
328 CTX1B and 52-*epi*-54-deoxyCTX1B were overwhelmed by the ammonium adducts compared to
329 their protonated adducts in mass spectra using the mobile phases containing both weak acids and
330 ammonium salts as the additives. Unlike CTX1B and 52-*epi*-54-deoxyCTX1B, the ratio of the
331 ammonium adduct to its protonated adduct of CTX3C could be close to 1 in mass spectra with
332 either methanol/water or acetonitrile/water mobile phases containing weak acid and ammonium
333 salt additives. Raising the concentration of formic acid and removing the ammonium salt in the
334 LC acetonitrile /water eluent would greatly favor the production of CTX3C protonated adduct,
335 which could generate the toxin peak height comparable to that of either protonated or
336 ammonium adduct from methanol/water mobile phases containing both formic acid and
337 ammonium formate additives.
338
339 3.2.3. LC solvent programing for MS detection

340
341 3.2.3.1. LC-4000 Q TRAP
342 Based on previous sections, acetonitrile/water was chosen as the preferred mobile phase for
343 CTX1B and 52-*epi*-54-deoxyCTX1B MS detection and the amounts of ammonium formate and
344 formic acid additives in the mobile phases were reduced compared to those reported. Ammonium
345 formate was reduced from 2 mM [16,17,27] to less than 0.5 mM (e.g., 0.2 mM) in aqueous
346 mobile phase A (i.e., water containing 0.2 mM ammonium formate and 0.1% formic acid) and
347 from 2 mM [16,17,27] to 0 mM in acetonitrile mobile phase B (e.g., acetonitrile containing
348 0.03% or 0.02% formic acid). CTX3C, a common component in certain ciguatoxic fish, had
349 diminished detection sensitivity with the CTX1B optimized mobile phases A/B gradient as
350 shown in Fig.1A. Based on previous sections, there were two preferred choices for better
351 CTX3C detection: one was acetonitrile/water mobile phases with only formic acid additive and
352 the other was methanol/water mobile phases with ammonium formate and formic acid additives.
353 Therefore, the LC gradients were adjusted to accommodate the optimal conditions for MS
354 detection with each group: mobile phases A/B were used in all the LC gradients (Table 1-4 and
355 Fig. 1-2) for the CTX1B and 52-*epi*-54-deoxyCTX1B group, additional mobile phases C, D1,
356 D2, and D3 were added as choices (Table 1-4 and Fig. 1-2) for the preferred CTX3C LC eluents,
357 which varied slightly with the two mass spectrometers. Mobile phase D1 (water containing 0.1%
358 formic acid as the additive) was added to LC solvent programming (mobile phases A/B/D1) to
359 have CTX3C elute out of the LC column into the MS needle in the solvent from mobile phases B
360 and D1, the peak height of CTX3C increased almost 2.5 fold (Fig. 1B). Addition of mobile phase
361 C (methanol with 0.02% formic acid as the additive) into LC solvent programming (mobile
362 phases A/B/C) to have CTX3C elute into the MS needle in the solvent from mobile phases C and
363 A, the peak height of CTX3C (Fig. 1C) was similar to what was obtained from the conditions in
364 Fig.1B. Increasing the declustering potential (DP) from 60 to 100 V for CTX3C caused a slight
365 increase in peak height of CTX3C with the use of methanol for CTX3C MS detection while
366 almost no change on the peak height of CTX3C with acetonitrile. Replacement of mobile phase
367 D1 (water containing 0.1% formic acid) with mobile phase D2 (water containing both 0.1%
368 formic acid and 2 mM ammonium formate) in combination of LC solvent programming of
369 mobile phases A, B, C, and D2, increased the peak height of CTX3C slightly above 2.5 fold (Fig.
370 1D vs. Fig. 1C) with DP at 100 V. The conversion of CTX3C ammonium adduct to protonated
371 adduct with the use of methanol and ammonium salts in the mobile phases greatly enhanced the
372 sensitivity of CTX3C (section 3.2.1). However, the amount of ammonium formate could not be
373 increased too much in the LC elution program; otherwise, it caused ion suppression and thus low
374 sensitivity (e.g., replacement of mobile phase C methanol containing 0.02% formic acid with
375 95% methanol/water containing 0.1% formic acid and 2 mM ammonium formate). The problems
376 with the use of methanol instead of acetonitrile for the detection of CTX3C by LC-MS were
377 higher MS noises than those observed with acetonitrile and about 90% or above of methanol
378 needed in the LC eluant for eluting CTX3C out of LC column without causing significant peak
379 broadening (Section 3.2.2).
380
381 3.2.3.2. LC-AB SCIEX Triple Quad 5500
382
383 The LC conditions for the Triple Quad 5500 were similar to what were used for the LC-4000 Q
384 TRAP but with slight modifications (Fig. 2). The optimum LC conditions for the detection of

385 CTX1B and 52-*epi*-54-deoxyCTX1B with the Triple Quad 5500 were the same as those applied
386 to the LC connected to the 4000 Q TRAP. For the detection of CTX3C on the Triple Quad 5500
387 with protonated adduct using acetonitrile/water mobile phases, mobile phase D1 (water
388 containing 0.1% formic acid; section 3.2.3.1) for the 4000 Q TRAP was replaced with mobile
389 phase D3 (water containing 0.2% formic acid), which showed a slight increase in sensitivity for
390 CTX3C. No difference between mobile phase D1 and mobile phase D3 for the detection of
391 CTX3C with the 4000 Q TRAP was observed. For the detection of CTX3C on the Triple Quad
392 5500 with ammonium adduct using methanol/water mobile phases, mobile phase D2 for the 4000
393 Q TRAP was replaced with mobile phase A, because conversion of CTX3C ammonium adduct
394 to its protonated adduct through an increase in DP was not observed on the Triple Quad 5500.
395
396 ### 3.3. Analysis of Pacific ciguatoxins in spiked rat plasma samples
397
398 Two LC-MS methods were applied to the LC-4000 Q TRAP system based on the optimization of
399 LC and MS using the toxin standards discussed in previous sections; they had almost the same
400 setup for the determination of CTX1B and 52-*epi*-54-deoxyCTX1B while they differed in the
401 determination of CTX3C. Both methods used acetonitrile/water mobile phases containing formic
402 acid and ammonium formate additives to elute CTX1B and 52-*epi*-54-deoxyCTX1B for MS
403 detection through ammonium adducts. In the first method (Table 1), CTX3C was still eluted with
404 acetonitrile/water while the additive was switched to formic acid only. In the second method
405 (Table 2), CTX3C migrated inside the LC column with acetonitrile/water eluant and then eluted
406 with methanol/water containing formic acid and ammonium formate for MS detection. One
407 spike level (0.18, 0.625, and 2 ng/ml of CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C,
408 respectively in plasma) in triplicate was performed using the rat plasma samples due to the
409 limited availability in toxin standards. The calibration curves derived from four calibration points
410 prepared in methanol in the range of 0.0625 to 0.5 ng/ml for CTX1B, 0.3125 to 2.5 ng/ml for 52-
411 *epi*-54-deoxyCTX1B, and 1 to 8 ng/ml for CTX3C showed linear responses of all the toxins.
412 Since the observed ciguatoxin activity (mean value between 0.001 and 0.002 ng/ml CTX1B
413 equivalent in blood for positive samples from living animals) in monk seal blood samples by
414 N2A cell cytotoxicity assay [25] was far below the LOD obtained from both the LC-4000 Q
415 TRAP and the LC-Triple Quad 5500 systems, the linear ranges of the calibration curves with
416 these two LC-MS systems were not further tested. CTX1B and CTX3C were quantified using the
417 sum of all corresponding MRM channels adopted from Lewis et al. [16] while 52-*epi*-54-
418 deoxyCTX1B was quantified using the single MRM channel *m/z* 1112.6 $\rightarrow$ 1059.6 due to the
419 noises from MRM channel *m/z* 1112.6 $\rightarrow$ 1077.6. The recoveries of CTX1B, 52-*epi*-54-
420 deoxyCTX1B and CTX3C were 90 ± 6, 106 ± 7, and 90 ± 4 % (mean ± standard deviation),
421 respectively, using the LC-4000 Q TRAP with LC Gradient 1, and were 92 ± 3, 116 ±12, and 96
422 ± 10, respectively, using the LC-Triple Quad 5500 with LC Gradient 3. The relative LC-MS/MS
423 responses of the toxins obtained from spiking the toxins into rat plasma before extraction (one of
424 the above spike-and-recovery samples) and after clean-up (the toxins were spiked into the
425 control extracts as matrix-matched standards) were 125% for CTX1B, 102% for 52-*epi*-54-
426 deoxyCTX1B, and 94% for CTX3C with each toxin concentration in the matrix-matched
427 standard (MMS) 1.5 fold of that in the sample measured on one day using the LC-Triple Quad
428 5500 and were 85% for CTX1B and 88% for CTX3C with each toxin concentration in the MMS
429 about 2.5 fold of that in the sample measured on the other day (52-*epi*-54-deoxyCTX1B was not

430 added into the second MMS). The results of examining one spiked sample calibrated against
431 MMS indicated no obvious bad sample preparation issues. The extraction efficiency using
432 acetonitrile to extract the toxins from blood has been proved using the N2A cell-based assay [24]
433 and the efficacy of each sample clean-up step was also evaluated with the toxin standards in this
434 study. Therefore, close to 100% recoveries of the toxins in the plasma samples calibrated against
435 the toxin standards prepared in methanol were the results of the sample preparation efficacy and
436 no obvious matrix effects with LC-MS/MS measurements. The estimated limit of detection
437 (LOD, ng/ml in plasma) was about 0.036, 0.38, 0.44 with the LC-4000 Q TRAP and was 0.011,
438 0.14, 0.15 with the LC- Triple Quad 5500, for CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C,
439 respectively. The estimated limit of quantitation (LOQ, ng/ml in plasma) was about 0.046, 0.38,
440 0.82 with the LC-4000 Q TRAP and 0.028, 0.22, 0.32 with the LC-Triple Quad 5500, for
441 CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C, respectively. All three MRM monitoring
442 channels of each toxin were used to identify the toxin; the signal to noise ratio of each MRM
443 channel was 3 or above at the LOD [29]. At the limit of quantitation (LOQ) [29], the S/N ratio of
444 the quantitation MRM channel (data from one MRM channel for 52-*epi*-54-deoxyCTX1B and
445 sum of MRM channels for CTX1B and CTX3C) was 10 or above and the S/N of each MRM
446 channel was 3 or above. The LOD and LOQ were roughly estimated from the spiked plasma
447 samples because the LOD of the methods was above the average observed toxin activity in living
448 monk seal blood samples [25]. The injection volume was initially 15 µl for the LC-4000 Q
449 TRAP and was changed to 12 µl later for both the LC-4000 Q TRAP and the LC-Triple Quad
450 5500 because we observed a slight volume overload with 15 µl injections on the LC-Triple Quad
451 5500. The LOD and LOQ were slightly better with the LC-Triple Quad 5500 than the LC-4000
452 Q TRAP. The sensitivity gain from the 4000 Q TRAP to the Triple Quad 5500 varied with these
453 three toxins and individual MRM monitoring channel of each toxin. Both baseline and sample
454 matrix noises from the LC-Triple Quad 5500 were much higher than those from the LC-4000 Q
455 TRAP, which was also confirmed by switching the LC from the 4000 Q TRAP to the Triple
456 Quad 5500 and applying to the same samples. When methanol was added into the mobile phase
457 using LC Gradient 2 (Table 2) to increase the sensitivity of CTX3C, the recovery of CTX3C in
458 rat plasma was between 50% and 60% on the LC-4000 Q TRAP. The low recovery of CTX3C
459 was likely due to ion suppression from the co-eluted matrix components resulted from the use of
460 a high percentage (i.e. 92%) of methanol in the LC eluent for reduction of peak broadening
461 (Section 3.2.2).
462
463 3.4. Analysis of Pacific ciguatoxins in the plasma samples of Hawaiian monk seals
464
465 The N2A cytotoxicity assay of the LC fractions obtained from the plasma extract of Hawaiian
466 monk seals indicated several major ciguatoxin-like constituents at fractions 7, 17, 26, and 28.
467 The analysis of the toxin standards with the same LC gradient by the LC-4000 Q TRAP showed
468 the RT (min) was 6.12 for CTX1B, 25.01 for CTX3B, 26.53 for CTX3C, 27.28 for CTX4A, and
469 28.92 for CTX4B. The ciguatoxin-like constituent at fraction 7, 26, and 28 was likely
470 corresponding to CTX1B, CTX3B, and CTX3C, respectively (fraction 2 was the first-minute
471 fraction because the fraction collector automatically skipped tube 1 with the start signal from the
472 LC and the LC gradient was different from the final LC-MS protocol; see section 2.4). Analysis
473 of the monk seal plasma with the LC-4000 Q TRAP using an LC gradient similar to the one used
474 in Fig. 1A (mobile phase B was pure acetonitrile) confirmed the presence of P-CTX-1 with the

475 S/N of the two MRM channels between 3 and 10, and the third MRM channel about 10. CTX3B
476 was detected with the S/N of MRM channel *m/z* 1023.6 → 125.1 about 6, but the S/N ratios of
477 the other two MRM channels were between 1 and 3 (*m/z* 1040.6 → 1005.6 with the ammonium
478 adduct as the precursor to replace *m/z* 1023.6 → 155.1 in the final protocol). When the final
479 protocol (Gradient 1 in Table 1) was applied to the monk seal plasma sample with the LC-4000
480 Q TRAP, both CTX1B and CTX3C were confirmed, with the S/N of all the three MRM channels
481 of each toxin above 3. However, for CTX3B, the stereoisomer of CTX3C, only two MRM
482 channels (*m/z* 1023.6 → 125.1 and *m/z* 1023.6 → 155.1) showed the S/N between 3 and 10, the
483 third MRM channel (*m/z* 1023.6 → 1005.6) only had the S/N about 2. Lack of toxin standards
484 and insufficient concentration in the sample, the toxin corresponding to the second cytotoxicity
485 fraction was not able to be detected. The LC-Triple Quad 5500 was not applied to the monk seal
486 sample due to the insufficient sample solution left.
487
488 **4. Conclusions**
489 CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C vary slightly in structure, but their mass
490 spectrometric responses to LC mobile phase conditions (solvent type and mobile phase additives)
491 were not the same. The two mass spectrometers likewise detected slightly different toxin
492 ionization profiles. Since sodium adducts of the toxins were not considered for MS quantitation
493 with the blood samples, ammonium adducts of CTX1B and 52-*epi*-54-deoxyCTX1B with
494 acetonitrile/water mobile phases were the preferred choice for their determination; either
495 protonated or ammonium adduct of CTX3C was used for its determination along with the LC
496 mobile phase conditions adjusted with a quaternary pump to accommodate its different MS
497 ionization profile. The sample clean-up allowed quantification of the three ciguatoxins by LC-
498 MS/MS with average recoveries 90% or above in the spiked blood samples. However, further
499 sample clean-up may be needed to reduce the matrix noise in order to lower the LOD with Triple
500 Quad 5500. Routine monitoring Pacific ciguatoxins in individual blood samples to meet the low
501 concentration observed is still a challenge using the methods developed with these two LC-MS
502 systems. However, the sample preparation method and the strategy of LC-MS settings in this
503 study, advances the knowledge needed to monitor Pacific ciguatoxins in marine mammal and
504 humans subjects.
505
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516
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601

602 **Figure captions**

603 Figure 1. Comparison of peak heights of CTX1B (peak 1), 52-*epi*-54-deoxyCTX1B (peak 2), and
604 CTX3C (peak 3) in LC-MS total ion chromatograms obtained from different LC mobile phases
605 and their associated solvent programing with the 4000 Q TRAP. The description of mobile
606 phases A, B, C, D1, and D2 are the same as those in Section 2.5 except mobile phase B here was
607 acetonitrile containing 0.03% formic acid instead of containing 0.02% formic acid. (A) Mobile
608 phases A and B were used with the elution gradient similar to Gradient 1 in Table 1 with the
609 changes of 74% B and 26% A at 15.1 min and 90% B and 10% A at 32 min. (B) Gradient 1 in
610 Table 1 was used. (C) Mobile phases A, B, and C were used with the elution gradient similar to
611 Gradient 2 in Table 2 with the changes of 8% A and 92% C at 16.1 min and 21 min. (D)
612 Gradient 2 in Table 2 was used. CTX3C was detected using its protonated adduct as the MRM
613 precursor in all the LC-MS chromatograms in Fig. 1. The toxin amounts (pg) injected on the LC
614 column were 6.75, 22.5, and 75 for CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C, respectively.

615 Figure 2. Comparison of peak heights of CTX1B (peak 1), 52-*epi*-54-deoxyCTX1B (peak 2), and
616 CTX3C (peak 3) in LC-MS total ion chromatograms obtained from different LC mobile phases
617 and their associated solvent programing with the Triple Quad 5500. The description of mobile
618 phases A, B, C, D3 are the same as those in Section 2.5. (A) Mobile phases A and B were used
619 with the elution gradient similar to Gradient 3 in Table 3 with the changes of 73% B and 27% A
620 at 14.1 min and 90% B and 10% A at 32 min. (B) Gradient 3 in Table 3 was used. (C) Gradient 4
621 in Table 4 was used. The MRM precursor for CTX3C was protonated adduct in Fig. 2B and
622 ammonium adduct in Fig. 2A and 2C. The toxin amounts (pg) injected on the LC column were 1,
623 5, and 16 for CTX1B, 52-*epi*-54-deoxyCTX1B, and CTX3C, respectively.

624

625 Figure 3. LC-MS/MS extracted ion chromatograms of CTX1B (sum of MRM transitions of *m/z*
626 1128.6 → 1057.6, 1075.6, and 1093.6) acquired from a standard (A) or the monk seal blood
627 sample (B) and CTX3B and CTX3C (sum of MRM transitions of *m/z* 1023.6 → 125.1, 155.1,
628 and 1023.6) acquired from a standard (C) or the monk seal blood sample (D) using the LC-4000
629 Q TRAP.

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Figure 1

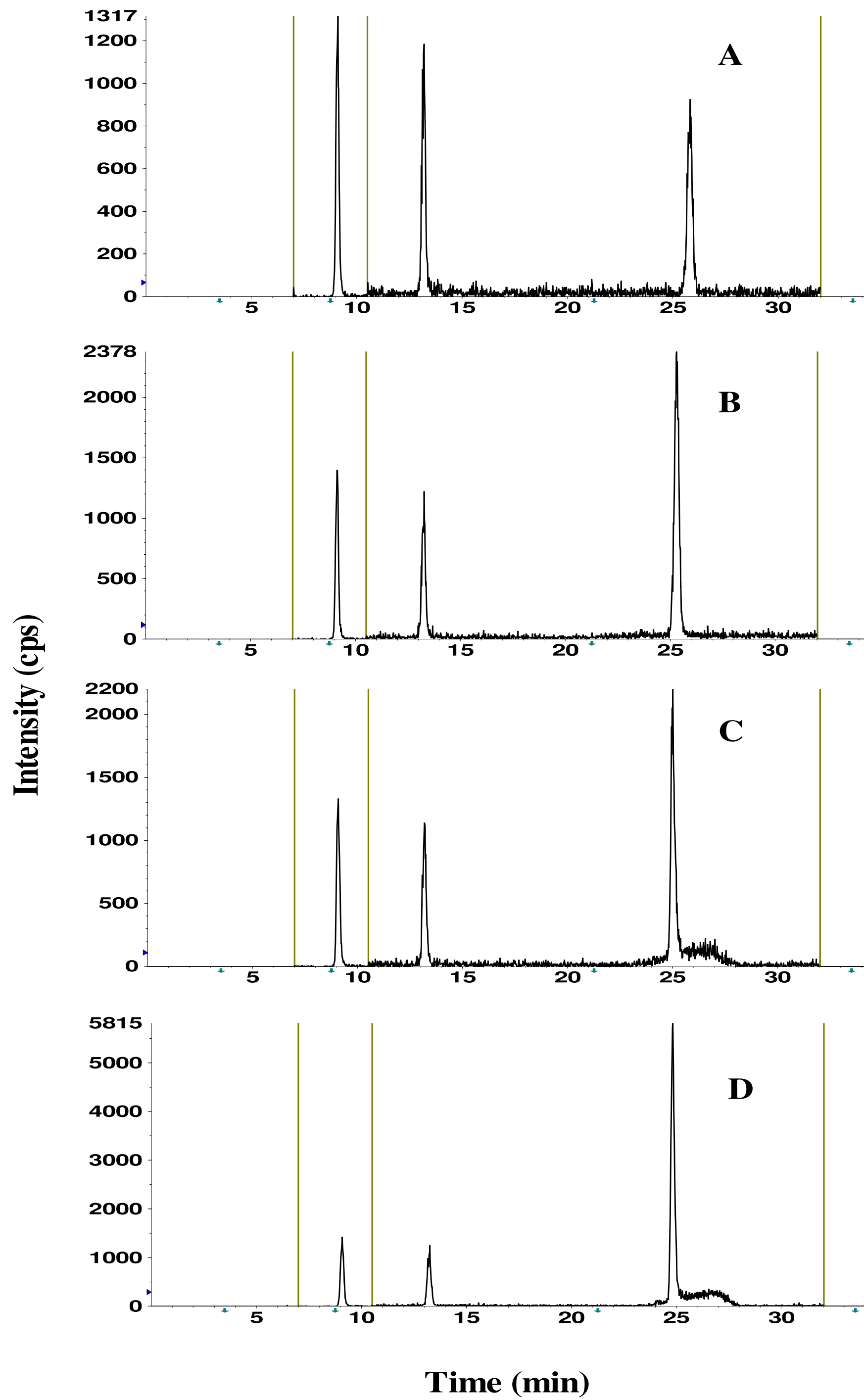


Figure 2

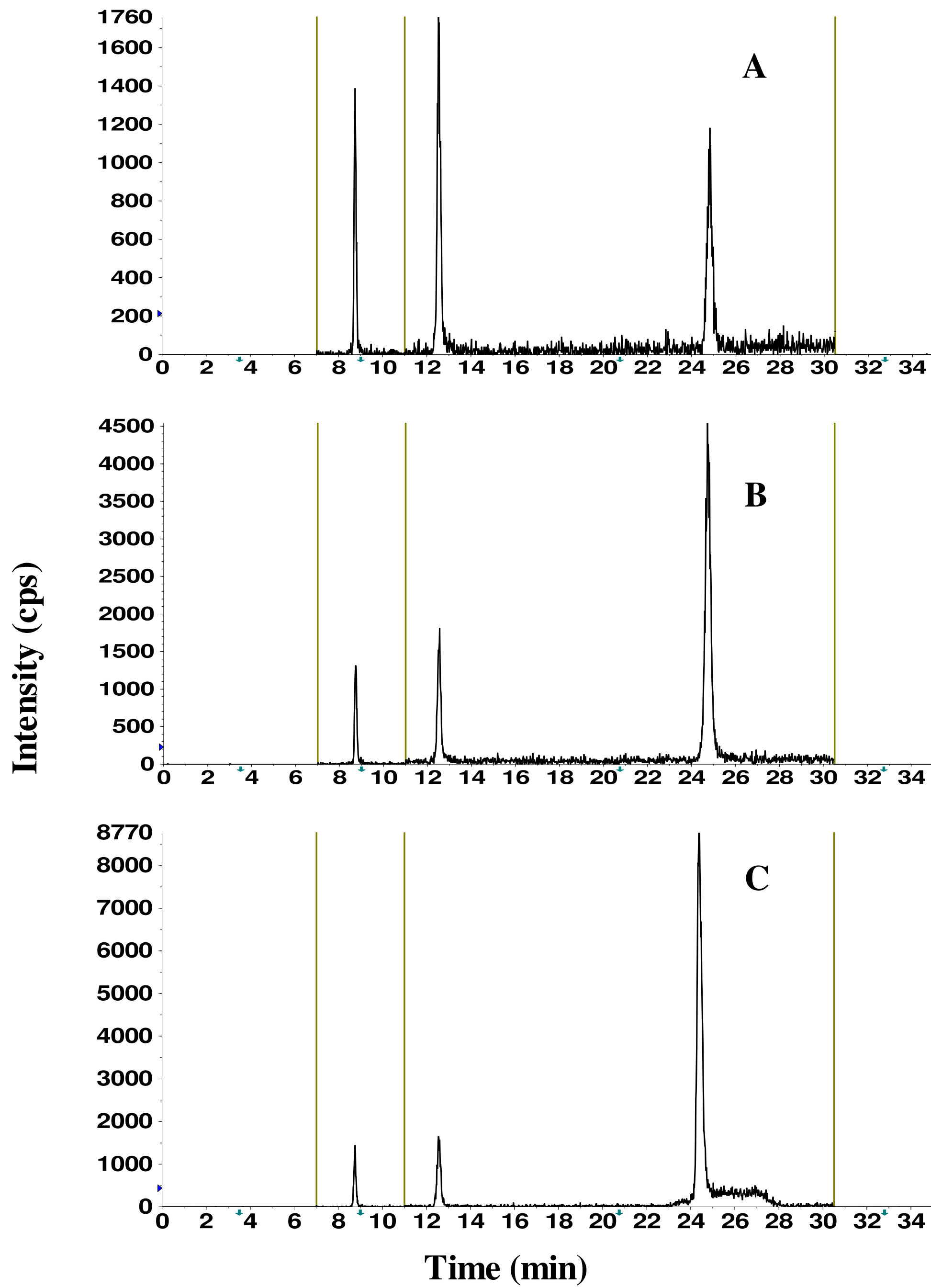
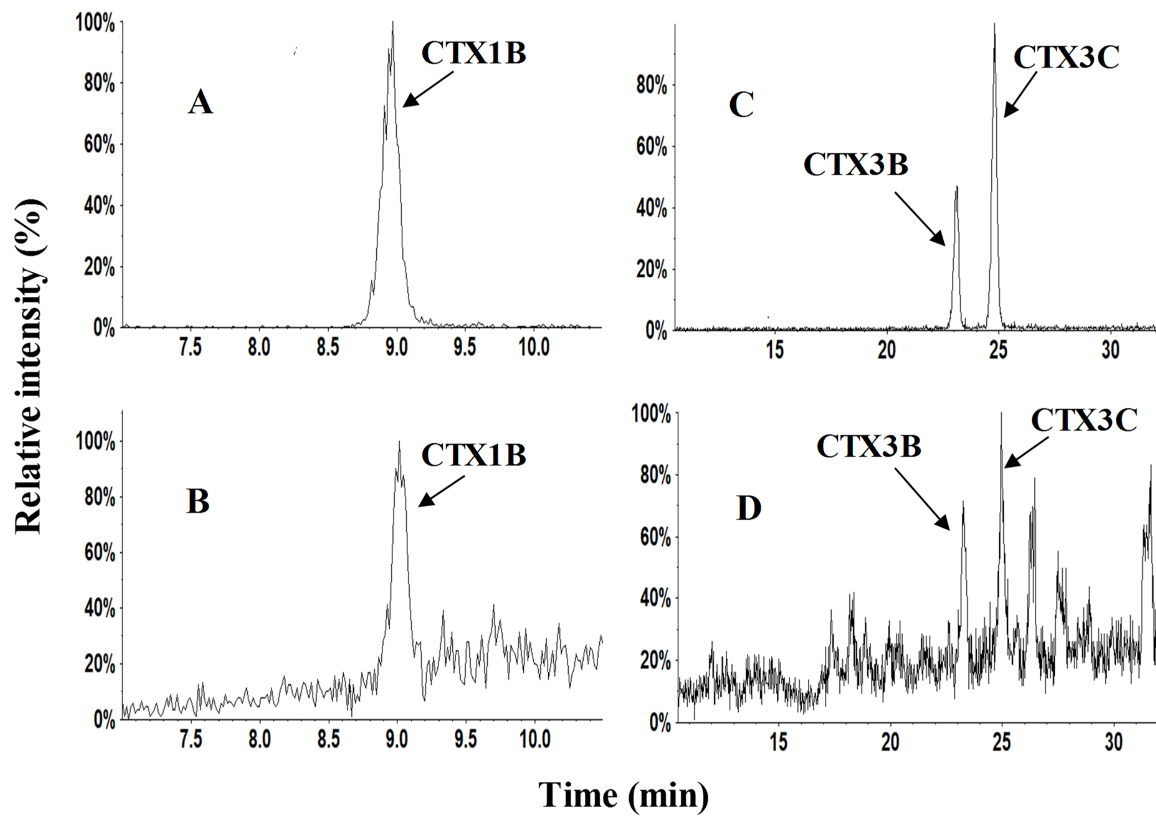


Figure 3



Tables

Table 1
LC Gradient 1 for the analysis of Pacific ciguatoxins with the 4000 Q TRAP

Time (min)	0	1	15	15.1	32	33	36	37	46
Mobile phase A (%)	62	40	26	0	0	5	5	62	62
Mobile phase B (%)	38	60	74	74	90	95	95	38	38
Mobile phase D1 (%)	0	0	0	26	10	0	0	0	0

Table 2
LC Gradient 2 for the analysis of Pacific ciguatoxins with the 4000 Q TRAP.

Time (min)	0	1	16	16.1	21	21.1	32	33	36	37	46
Mobile phase A (%)	62	40	25	0	0	20	10	5	5	62	62
Mobile phase B (%)	38	60	75	0	0	80	90	95	95	38	38
Mobile phase C (%)	0	0	0	92	92	0	0	0	0	0	0
Mobile phase D2 (%)	0	0	0	8	8	0	0	0	0	0	0

Table 3
LC Gradient 3 for the analysis of Pacific ciguatoxins with the AB SCIEX Triple Quad 5500.

Time (min)	0	1	14	14.1	32	32.1	33	35	37	46
Mobile phase A (%)	62	40	27	0	0	10	5	5	62	62
Mobile phase B (%)	38	60	73	73	90	90	95	95	38	38
Mobile phase D3 (%)	0	0	0	27	10	0	0	0	0	0

Table 4
LC gradient 4 for the analysis of Pacific ciguatoxins with the AB SCIEX Triple Quad 5500.

Time (min)	0	1	15.5	15.6	21	21.1	32	33	35	37	46
Mobile phase A (%)	62	40	26	8	8	20	10	5	5	62	62
Mobile phase B (%)	38	60	74	0	0	80	90	95	95	38	38
Mobile phase C (%)	0	0	0	92	92	0	0	0	0	0	0