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Article

Selective and Efficient Capture and Release of *vic*-Diol-Containing Pacific and Caribbean Ciguatoxins from Fish Extracts with a Boronate Affinity Polymer

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ABSTRACT: Ciguatera poisoning can occur following the consumption of fish contaminated with trace levels of ciguatoxins (CTXs). These trace levels represent an analytical challenge for confirmation by LC−MS due to matrix interferences and the high instrument sensitivity required. Sample preparation procedures are laborious and require extensive cleanup procedures to address these issues. The application of a selective isolation technique employing boronate affinity polymers was therefore investigated for the capture of *vic*-diol-containing Caribbean and Pacific CTXs from fish extracts. A dispersive SPE procedure was developed where nearly complete binding of CTXs in fish extracts occurred with boric acid gel in less than 1 h. Release of the bound CTXs resulted in >95% recovery of C-CTX1/2, C-CTX3/4, CTX1B, 54-deoxyCTX1B, and 52-*epi*-54-deoxyCTX1B from the extracts. This selective extraction tool has the potential to greatly simplify both analytical sample preparation and preparative extraction and isolation of CTXs for structure elucidation and production of standards.

KEYWORDS: *boric acid gel, ciguatoxin, LC−HRMS, vic-diol, boronate*

1. INTRODUCTION

Ciguatera poisoning is caused by the consumption of finfish that are contaminated with ciguatoxins (CTXs) (Figure 1), typically from tropical and subtropical regions. Ciguatera poisoning causes a myriad of potentially severe gastrointestinal, neurological, and psychological symptoms.[1] CTX bioaccumulation and biotransformation in fish occurs via consumption of the dinoflagellates of the genera *Gambierdiscus* and/or *Fukuyoa* that produce CTX algal precursors, although there remains some uncertainty regarding the precursors and organisms responsible for some of the CTXs found in fish.[1,2] CTXs are temperature-stable, lipophilic, ladder-shaped polyethers with molecular weights of 1000−1200 Da. Regional variations in CTX congeners have been observed, with CTX1B and related congeners having traditionally been detected in fish from Pacific regions, while C-CTX1/2 have been detected in fish from the Caribbean Sea and, more recently, around the Canary Islands.[3] Toxicity and symptoms related to CTXs can occur in humans after consumption of fish containing as little as 48 ng/g CTX1B equivalents.[4] Current guidelines and recommendations are for limits of less than 10 ng/g CTX1B eq. in fish flesh for human consumption.[5,6] The need to detect such low levels of CTXs in fish flesh makes detection and quantitation difficult.

Unlike many known shellfish toxins that can bioaccumulate to high levels in filter feeders, CTXs are present at very low levels in mobile vectors (invertebrates and fish), requiring trace-level analysis due to their high potency at biological targets. Based on this need, sample preparation and extraction of CTXs from fish flesh requires several cleanup steps to remove interferences, including liquid−liquid partitioning, solid-phase extraction (SPE), and evaporation. These process steps can have significant impacts on sample throughput and analyte recovery and can result in variable removal of confounding matrix components for the subsequent analytical platform.[7] With limitations on the availability of reference materials for many CTX congeners, losses observed during isolation techniques, and large solvent requirements, the development of novel isolation techniques may also improve recoveries and enable isolation from larger quantities of lower-toxicity fish flesh. Boronate affinity polymers have been used to selectively bind compounds containing *vic*-diol functional groups while not retaining compounds lacking this functional group.[8] Several recent applications of this technique have focused on marine toxin classes, including azaspiracids, tetrodotoxins, and gambierones.[9−11] Several CTXs, including fish metabolites with high toxicity such as CTX1B, 54-deoxyCTX1B, and C-CTX1/2, contain *vic*-diols in their structures and are therefore potential candidates for boronate affinity methods.

The aim of this study was to evaluate the feasibility of using boronate affinity polymers to selectively bind CTXs and to develop methodologies to release bound CTXs from these polymers. Such methodologies have the potential to improve recoveries and sensitivity and to reduce matrix effects in downstream analytical methods for CTXs, and could lead to

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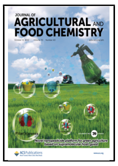

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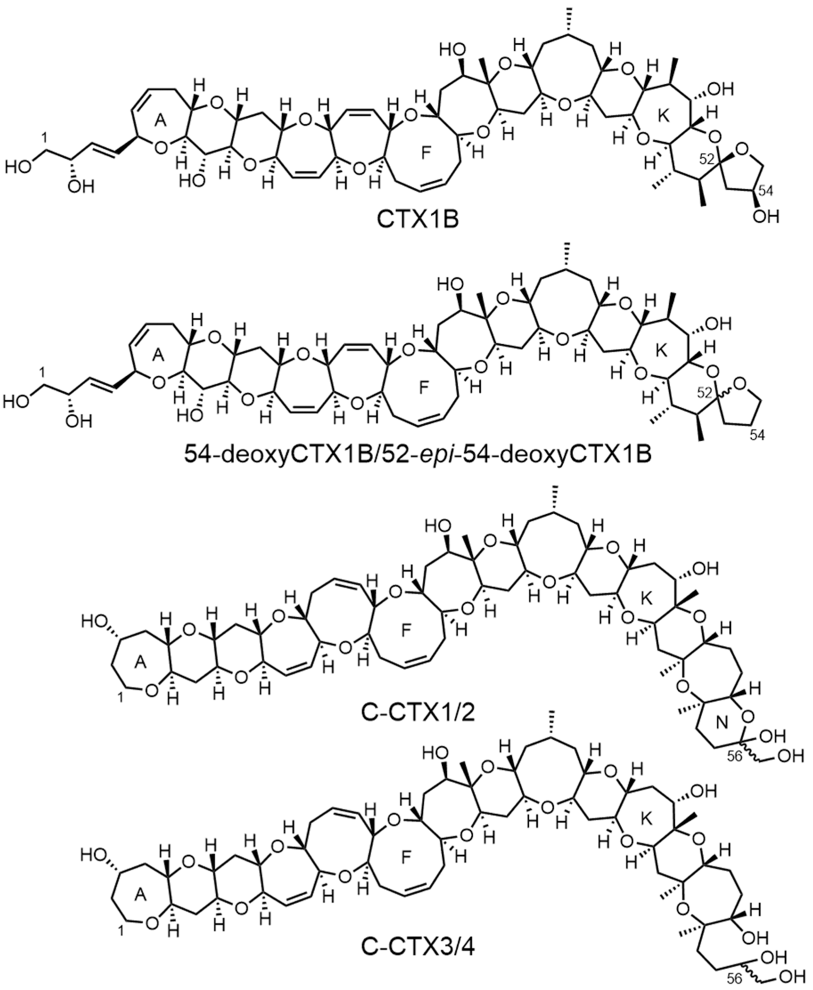

Figure 1. Structures of selected CTXs that have been identified in fish flesh and which were tested in this study. Note that C-CTX1/2 and C-CTX3/4 are each a pair of epimers at C-56, while 54-deoxyCTX1B and 52-*epi*-54-deoxyCTX1B are epimeric at C-52. CTX1B analogues have *vic*-diols at C-1, C2 and C-CTXs at C-56, C-57.

simplified preparative isolation procedures. Furthermore, such methods may facilitate discovery of novel CTXs by LC−MS methods via selective concentration of diol-containing congeners and reduction of matrix interferences.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents. MeCN, MeOH, THF, and formic acid (∼98%) were LC−MS grade from Fisher Scientific (Ottawa, ON, Canada). Reagent grade acetone, $CHCl_3$, CH_2Cl_2, hexane, diethylamine (Et_2NH), hydrochloric acid (HCl; 37%), sodium hydroxide (NaOH), and boric acid gel (0.1−0.4 mm particle size) were from MilliporeSigma (Oakville, ON, Canada). Molecular sieves (3 Å, 8−12 mesh) were from Fisher Scientific (Ottawa, ON, Canada). A stock solution of ciguatoxin CTX1B (Wako Chemicals, Richmond, VA) was prepared in MeOH at 100 ng/mL for use in subsequent experimental procedures. An extract of muscle tissue from moray eel originally sourced from Samoa and containing CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B was provided by Cawthron Institute (Nelson, New Zealand).[12]

2.2. Caribbean CTX Extracts. C-CTXs were extracted from two toxic *Sphyraena barracuda* specimens collected during the 18th Bastille Day Kingfish Tournament (St. Thomas, US Virgin Islands) in 2016, as described previously.[13] In short, filleted muscle tissues were homogenized, and 100 g was extracted twice with acetone (3 mL/g). Pooled extracts were stored at −20 °C for at least 12 h to precipitate proteins, followed by centrifugation at 4000*g* for 5 min. Supernatants were rotary-evaporated at 40 °C, dissolved in 80% aqueous MeOH, and defatted twice with hexane. The methanolic extracts were then diluted to 50% aqueous MeOH and partitioned three times with $CHCl_3$. The $CHCl_3$ extracts were applied to silica gel SPE columns (1 g Bond-Elute Si; Agilent, Santa Clara, CA), preconditioned with three column volumes each of MeOH and $CHCl_3$, and washed with $CHCl_3$, and CTXs were eluted with $CHCl_3$−MeOH (9:1 by volume). The eluates were evaporated to dryness under a gentle stream of N_2 at 45 °C and dissolved in MeOH at 40 g/mL equivalence (relative to the original fish).

2.3. Selective Capture of CTXs with Boric Acid Gel. Boric acid gel (0.5 g) was placed in an empty glass SPE cartridge with a bottom frit and washed sequentially with 20 mL of 0.5 M HCl; water; 0.5 M NaOH; water; 1% formic acid in MeCN; H_2O; 7:3:0.1 (by volume) THF−H_2O−formic acid; H_2O; and 20 mL of 1% Et_2NH in MeCN. The gel was placed in a glass bottle, and the residual solvent was allowed to evaporate in a fume hood overnight. The prepared boric acid gel was stored at ambient temperature in a sealed bottle until use.

Ciguatoxin extracts were prepared by combining CTX1B (10 μL) and C-CTX fish extracts (25 μL) in glass vials, and the solvent was evaporated under N_2. The prepared boric acid gel (10 mg) was added, followed by the capture-solvent (1% Et_2NH in MeCN; 100 μL) and two molecular sieves, and the vials were agitated at ambient temperature on a Multitherm shaker (250 rpm, 1 h; Mandel Scientific, Guelph, ON, Canada). The solvent was carefully transferred to a vial using a narrow-ended, gel-loading pipette tip for later analysis. The residual solvent was evaporated from the gel with a gentle stream of N_2, and the molecular sieves were removed. The gel was then extracted with the release-solvent (THF−H_2O−formic acid,

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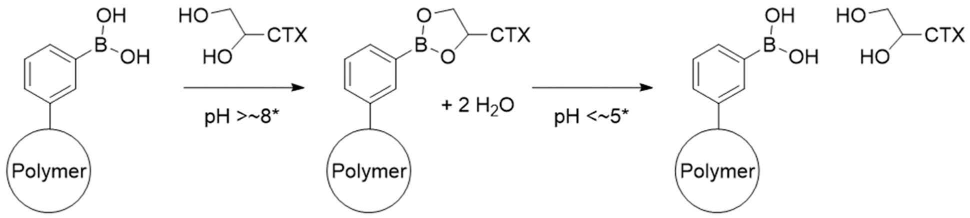

Figure 2. Scheme depicting the capture and subsequent release of a diol-containing CTX with boric acid gel. Note that the exact pH conditions required depend on the polymer-bound boronate.

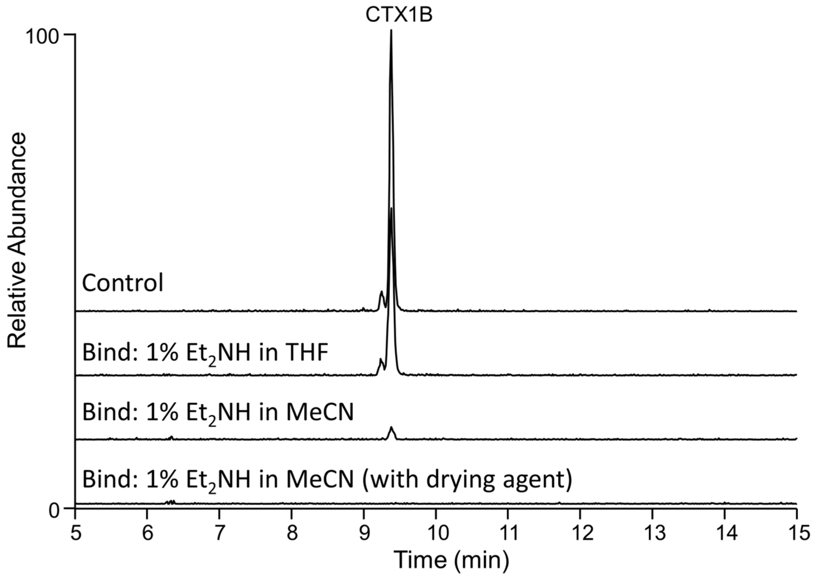

Figure 3. LC−HRMS extracted-ion chromatograms showing the effect of different solvent compositions on the residual concentration of CTX1B remaining in solution after complexation with boric acid gel.

7:3:0.1 by volume; 200 μL) at ambient temperature in the shaker (250 rpm, 2 h). The solvent was carefully transferred to a vial as described above, and a second aliquot of release-solvent (200 μL) was added, and the gel was extracted for an additional 2 h on the shaker (250 rpm). The three individual fractions of solvent collected from the gel were filtered (0.22 μm PVDF spin filters; MilliporeSigma) prior to analysis by LC−HRMS. Recovery was assessed using triplicate preparations relative to a positive control prepared by adding CTX1B (10 μL) and C-CTX fish extract (25 μL) to MeOH (165 μL).

2.4. LC−HRMS Analysis. The method employed was adapted from Kryuchkov et al.[13] Analyses were performed using an Agilent 1200 LC equipped with a binary pump, temperature-controlled autosampler (10 °C), and temperature-controlled column compartment (40 °C) (Agilent Technologies, Mississauga, ON, Canada) coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham, MA) with a heated electrospray ionization probe (HESI-II). Chromatographic separation was on a Kinetex F5 pentafluorophenyl column (100 × 2.1 mm^2, 1.7 μm; Phenomenex, Torrance, CA) using gradient elution with mobile phases composed of 0.1% formic acid in H_2O (A) and 0.1% formic acid in MeCN (B). The gradient was: 0−18 min, 30−60% B; 18−18.1 min, 60−99% B; 18.1−22 min, 99% B; followed by an 8 min re-equilibration at 30% B, with a flow rate of 0.3 mL/min and an injection volume of 5.0 μL.

Full-scan acquisition was performed with positive ionization and a mass range of m/z 1000−1250. The spray voltage of the source was 4500 V, with a capillary temperature of 340 °C, and the sheath and auxiliary gas were set at 40 and 10, respectively. The probe heater temperature was set at 150 °C, and the S-Lens RF level was set to 100. The mass resolution setting was 240,000, with an AGC target of 5×10^6 and a maximum injection time of 512 ms per scan. Extracted-ion chromatograms were used to monitor each ciguatoxin with a mass tolerance of ±5 ppm of the most abundant ion. Exact masses were as follows: C-CTX1/2 at m/z 1123.6200 $[M + H - H_2O]^+$, C-CTX3/4 at m/z 1143.6462 $[M + H]^+$. CTX1B at 1128.6102 $[M + NH_4]^+$, and 52-epi-54-deoxyCTX1B/54-deoxyCTX1B at 1112.6152 $[M + NH_4]^+$. LC−HRMS performance validation within the range of these experiments is summarized in Table S1.

3. RESULTS AND DISCUSSION

Boric acid gel consists of 3-aminophenylboronic acid bound to an insoluble polymer support. Traditional binding of diols (Figure 2) occurs above the pK_a of the boronic acid, followed by release under acidic conditions,[8] although other methodologies have been successfully employed.[11] A recently developed protocol, where m-aminophenylboronic acid−agarose gel was used in $CHCl_3$ to selectively bind gambierones,

Table 1. Recovery of CTX1B and C-CTX1/2 from Boric Acid Gel (BAG) Obtained under a Range of Reaction Conditions Using a Two-Level Partial Factorial Study

	treatment		CTX1B recovery (%)		C-CTX1/2 recovery (%)	
parameter	low	high	low	high	low	high
formic acid (%)	1	3	74.2	75.7	75.1	78.1
THF/H_2O	70:30	90:10	73.5	76.5	76.8	76.5
time (h)	1	3	75.2	74.7	76.2	77.1
temperature (°C)	25	50	78.2	71.1	77.0	76.3
BAG (mg)	10	25	84.6	65.3	85.0	68.3

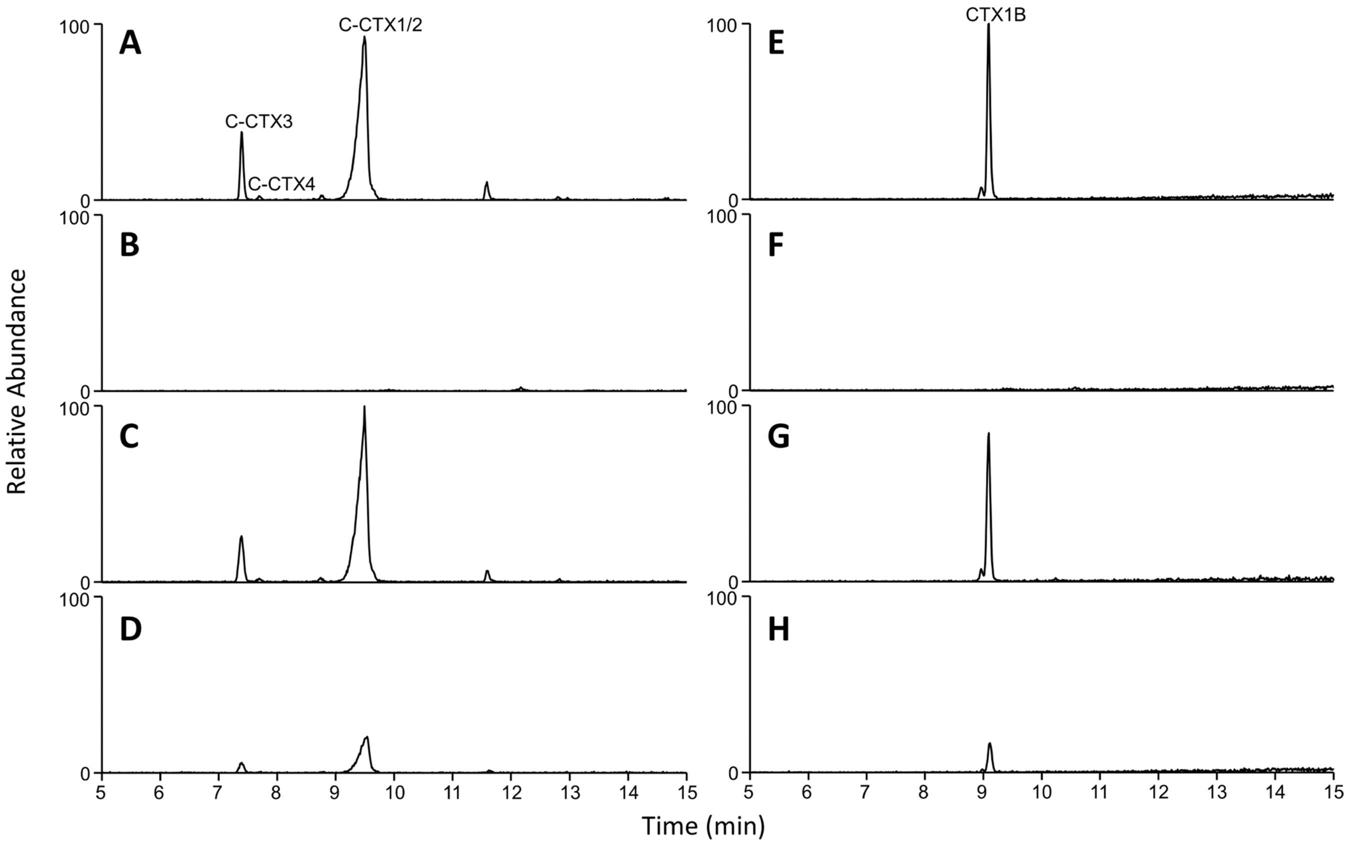

Figure 4. LC−HRMS extracted-ion chromatograms comparing the positive control preparations to the binding and release of *vic*-diol-containing CTXs from boric acid gel. Left panels, C-CTX1/2 and C-CTX3/4, and right panels, CTX1B, in (A/E) control; (B/F) binding; (C/G) release (2 h), and (D/H) second-release (2 h) extracts. The y-axis for each chromatogram is scaled relative to the maximum peak intensity observed in the corresponding control sample.

was unsuccessful when applied to CTXs;[11] so, additional studies were carried out to evaluate other conditions and immobilized boronic acids for the capture of these toxins.

Initial investigations using C-CTX-containing fish extracts showed boric acid gel had strong binding to C-CTXs in 1:1 MeOH−aqueous ammonium bicarbonate (pH 9.0), with less than 5% remaining in solution after 1 h. However, this was found to be due, at least in part, to nonspecific binding to the lipophilic polymer support rather than to specific diol−boronic acid complexation because binding of C-CTXs also occurred under acidic conditions, and reduced binding was observed with increased concentrations of MeOH (data not shown).

To allow expanded method exploration, a commercially available CTX1B standard was used to test the feasibility of selectively binding *vic*-diol-containing CTXs to boric acid gel. Less polar solvents, including MeCN and THF, were assessed under basic conditions using 1% diethylamine to avoid the apparent nonspecific binding of C-CTXs to the polymeric support that was observed in 1:1 MeOH−buffer. Significant binding of CTX1B occurred in both solvents, with ∼50% CTX1B remaining in solution when using THF, while MeCN was superior, with only ∼10% CTX1B remaining in solution after 2 h of shaking with the gel (Figure 3). Additionally, since diol−boronic acid complexation results in the release of water into the solution (Figure 2), the effect of the removal of residual water was evaluated by addition of a drying agent (3 Å molecular sieves) to the binding solution. The molecular sieves were easily removed from the gel prior to toxin release, making this viable for analytical and preparative scale procedures. This strategy resulted in no detectable CTX1B in the binding solution (Figure 3), suggesting that the removal of water improved CTX−diol complexation.

Having developed a procedure for binding CTX1B to boric acid gel, the contact time necessary to capture CTXs was assessed. A solution of C-CTX-contaminated fish extract spiked with CTX1B was used to simultaneously evaluate the binding of different CTX structural analogues. Three time points were assessed (30 min, 1 h, and 2 h), and resultant binding percentages were 97−98% for all CTXs at all time points, indicating that a contact time of 30 min was sufficient to capture diol-containing CTXs from a fish extract using this dispersive SPE technique. Although 30 min was found to be sufficient for binding, the final protocol was established using a 1 h incubation to ensure maximal binding.

Traditional release of diol−boronate complexes is performed in the presence of strong acids, such as HCl, but C-CTX1 has been shown to undergo alkyl ketal formation under acidic conditions in the presence of alcohols.[14,15] To avoid this, the solvents evaluated for release of CTXs used a weak acid and water to hydrolyze the boronate complex, together with a high proportion of a nonhydroxylic organic solvent to extract the CTXs and to prevent their nonspecific binding to the polymeric support. Several release solvents were assessed, including 80%, 90%, and 95% aqueous MeCN, and 90% aqueous THF, each with addition of 1% formic or acetic acid. The recovery of CTX1B varied from 23−69% following dispersive SPE release, with 95% aqueous MeCN with formic acid having the lowest recovery and 90% aqueous THF with

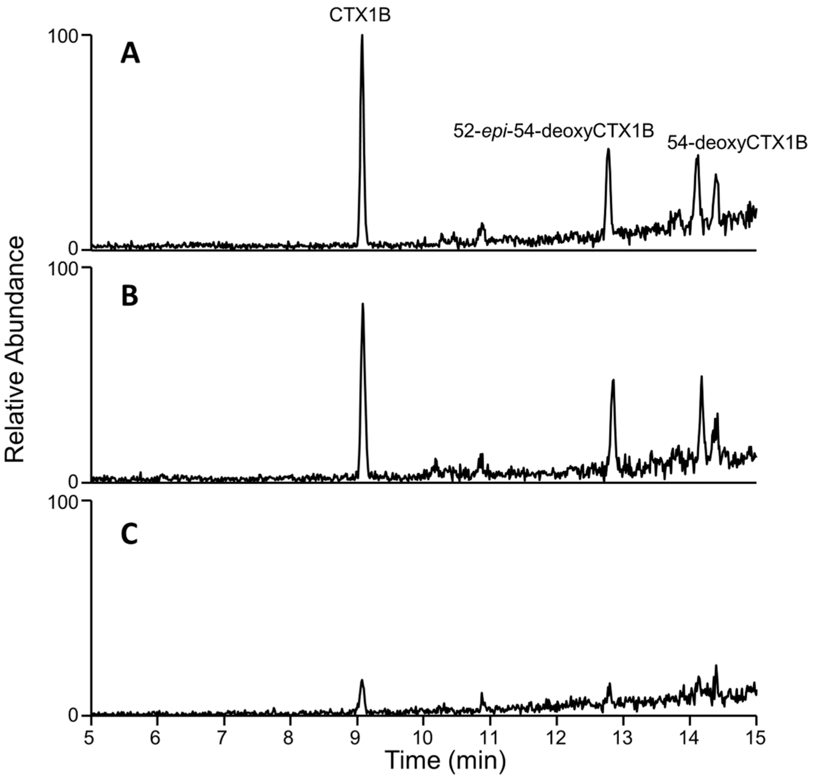

Figure 5. LC−HRMS extracted-ion chromatograms of 1,2-diol-containing ciguatoxins in the CTX4A class detected in a Samoan moray eel, comparing the control preparation to the release from boric acid gel: (A) control; (B) release (2 h), and (C) second-release (2 h). The vertical axis for each chromatogram is scaled relative to the maximum peak intensity observed in the control sample.

1% formic acid having the highest recovery. Based on these preliminary data, 90% aqueous THF with formic acid was selected for further optimization.

A two-level partial factorial design study was performed to evaluate the parameters influencing CTX recovery from the gel using the C-CTX-containing fish extract spiked with the CTX1B standard. The factors included the percentage of acid, THF:water ratio, reaction time, temperature, and the amount of gel (Table 1). These experiments indicated that the most significant factor affecting recovery was the amount of boric acid gel used, with a lower amount (10 mg) resulting in higher recoveries. This is likely due to the combination of small aliquots of solvent (100 μL) being used and relatively low concentrations of CTXs (∼10 ng/mL CTX1B) and the small amount of boric acid gel having sufficient binding sites available at this scale. Increased amounts of gel (e.g., 25 mg tested here) may have reduced solvent−gel contact and therefore reduced CTX recovery. This factorial study used 100 μL of solvent to release the CTXs, while later experiments suggested that a higher solvent-to-gel ratio (200 μL to 10 mg) further improved recovery. The percentage of acid, THF concentration, time, and temperature did not appear to significantly affect CTX recovery. LC−HRMS peak shape was significantly impacted with 90% aqueous THF due to the high volume of organic solvent being injected; therefore, 70% aqueous THF was selected since this did not reduce the recovery of CTXs. Consequently, the following parameters were selected for the final release procedure (from 10 mg gel): 1% formic acid in 7:3 THF−H_2O (200 μL), 25 °C, and 2 h shaking. The 2 h release time was selected to maximize recovery and reduce variability between replicates. This technique was developed using small volumes of CTX-incurred extracts due to limited sample availability, which required a dilution step for high recovery, but access to larger extract volumes may reduce the need for sample dilution following release from the gel if the procedure is scaled-up.

While a small proportion of the release-solvent can be expected to adhere to the polymer, the concentration in the recovered solution, when compared with the original solution, should provide an approximation of recovery from the polymer. In this case, maximum recoveries in the partial factorial design study were about 85%. Improved recovery was obtained by ensuring the gel was completely immersed in the release-solvent by increasing the amount of solvent to 200 μL. A second extract step was added to determine the amount of CTX remaining in the gel/residual solvent, which would improve total recoveries for purification protocols. Using the optimized conditions and triplicate preparations of C-CTX-contaminated fish extracts, the average peak area responses for C-CTX1/2 and C-CTX3/4 were 96 and 95%, respectively, relative to the control after the first extraction. Triplicate preparations resulted in a repeatability of 4.7% RSD in the peak area response of C-CTX1/2 from the gel. When the gel was re-extracted for 2 h with fresh solvent, an additional ∼20% of both CTXs was detected relative to the control. The extracted-ion chromatograms for these CTXs in the extracts before and after extraction with boric acid gel are shown in Figure 4. The results show that there was high recovery of CTXs from boric acid gel and consistent behavior of both pairs of Caribbean CTX epimers with the gel. Replicate preparations of CTX1B, either spiked into the C-CTX-containing fish extract or neat, resulted in recoveries of 96% after the first extraction, with an additional 15−20% observed in the second extraction step (Figure 4). Repeatability was consistent with C-

CTX1/2 at 5.6% between triplicate preparations. These data suggest that recovery was greater than 100% following the second extraction, potentially due to reduced matrix effects from the fish extracts. However, similar results were obtained with a neat CTX1B standard and with CTX1B spiked into a fish extract, suggesting this effect is more likely due to CTXs in the residual solvent adhering to the gel particles following the first extraction, which are then extracted into the fresh solvent during the second extraction step. The second extract step provides a higher total recovery from a purification perspective and may be useful in some applications, while data suggest a single extraction is sufficient when considering its use for future analytical methodologies. The protocol was further assessed with a Samoan moray eel extract containing low levels of CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B, with similar recoveries observed (Figure 5).

The application of boronate affinity polymers allows the selective extraction of *vic*-diol-containing metabolites, including many marine biotoxins.[9−11] Studies with azaspiracids showed major reductions in matrix effects in the LC−MS quantitation after the use of boric acid gel on a mussel tissue extract.[9] This study demonstrates the successful application of this technique to CTXs from fish from both the Caribbean Sea and the Pacific Ocean, with very high recoveries. However, it should be noted that this technique has so far only been tested with processed extracts and standards due to the low concentrations of CTXs (ppb) found in highly toxic fish in ciguatera-endemic regions. Further work is needed to optimize the procedure for routine monitoring by LC−MS in smaller subsamples, crude extracts, and at levels found in field-contaminated fish. Further evaluations will be required to optimize sample throughput and to evaluate binding efficiencies and kinetics in the presence of various biological matrices. Conventional CTX sample preparation and isolation procedures employ several steps, including liquid−liquid partitioning, solid-phase cleanup procedures, and evaporation, which aim to reduce impurities and matrix interferences but which are also time-consuming and reduce overall analyte recovery.[7] Given the selectivity of boric acid gel affinity to *vic*-diols, further studies investigating the application of boronic acid complexation at different stages of CTX sample preparation and/or isolation procedures could reveal the optimal point at which to apply this technique. Ultimately, with further optimization, we hope to reduce the number of cleanup steps, time, and effort required for routine CTX analysis. Although the procedure was only tested with LC−HRMS detection, it seems likely that it could also prove beneficial when using other types of analysis, such as LC−MS/MS, immunoassays, or in vitro bioassays.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c03789.

Performance validation characteristics for analysis of ciguatoxins by LC−HRMS (PDF)

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Notes

The authors declare no competing financial interest.

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