Cyclodextrin polymer clean-up method for the detection of ciguatoxins in fish with cell-based assays

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Abstract

Ciguatoxins (CTXs) are marine toxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*, which are transferred through the food webs, reaching humans and causing a poisoning known as ciguatera. The cell-based assay (CBA) is commonly used for their detection because of its high sensitivity and the provided toxicological information. However, matrix effects may interfere in the CBA. In this work, γ-cyclodextrin-hexamethylene diisocyanate (γ-CD-HDI), γ-cyclodextrin-epichlorohydrin (γ-CD-EPI) and γ-CD-EPI conjugated to magnetic beads (γ-CD-EPI-MB) have been evaluated as clean-up materials for fish flesh extracts containing CTXs. The best results were achieved with γ-CD-HDI in column format, which showed a CTX1B recovery of 42% and 32% for *Variola louti* and *Seriola dumerili*, respectively, and allowed exposing cells to at least 400 mg/mL of fish flesh. This clean-up strategy provides at least 4.6 and 3.0-fold higher sensitivities to the assay for *V. louti* and *S. dumerili*, respectively, improving the reliability of CTX quantification.

Keywords: cyclodextrin (CD) polymer, ciguatoxin (CTX), cell-based assay (CBA), matrix effects, *Variola louti*, *Seriola dumerili*.

1. Introduction

Ciguatoxins (CTXs) are marine toxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*, which are transferred through the food webs, from herbivorous to carnivorous fishes, potentially reaching humans (Ledreux et al., 2014; Litaker et al., 2017). The consumption of fish containing CTXs causes a foodborne poisoning known as ciguatera (Lewis, 2001; Dickey and Plakas, 2010). Although ciguateric fishes are endemic in some tropical and subtropical areas, their presence in more temperate regions has been reported. In Europe, ciguateric fish has been found in the Canary Islands (Spain) and Madeira (Portugal) (Pérez-Arellano et al., 2005; Boada et al., 2010; Otero et al., 2010; Costa et al., 2018).

The USA Food and Drug Administration has established a guideline level of 0.01 µg/kg for CTX1B (Pacific CTX) or 0.1 µg/kg for C-CTX1 (Caribbean CTX), since C-CTX1 is less toxic (US FDA, 2019). In Europe, fish products containing CTXs must not be placed on the market (Regulation (EC) No. 853/2004). The analysis of CTXs is highly challenging because of the complexity and variety of their chemical structures, the long and tedious protocols for their extraction from natural samples, and the extremely sensitive techniques required to detect such low toxin contents. Nevertheless, several analytical methods for the detection and quantification of CTXs have been developed. Among them, the mouse bioassay (MBA) has been traditionally used since it provides a composite toxicological response from a sample (Hoffman et al., 1983). However, its use is decaying since it suffers from low specificity and sensitivity, and ethical concerns. Receptor binding assays, based on the interaction of CTXs with voltage-gated sodium channels (VGSCs), are useful for screening but may not be sensitive enough (Dechraoui et al., 2005). Immunoassays and immunosensors, based on the structural interaction of CTXs with antibodies, are highly sensitive and easy to implement, but may not recognise all toxic CTX congeners (Tsumuraya et al., 2018; Leonardo et al., 2020; Gaiani et al., 2020). Instrumental analysis techniques, such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), allow unequivocal identification of individual CTX congeners, but are expensive and complex, and require reference materials (Lewis, Yang, & Jones, 2009). The cell-based assay (CBA) may be the most used method for the detection of CTXs. Like the MBA, it provides a composite toxicological response, since CTXs interact with the VGSCs, blocking them in an open state. Although the CBA may present interferences from other toxins (e.g., brevetoxins) and compounds, and is time-consuming, variable and difficult to harmonize, its high sensitivity and the provided toxicological information have led to its implementation in the official control for ciguatera of the Canary Islands (DG of Fisheries of the Canary Government, 2018).

The CBA is prone to suffering variability among days and samples due to the use of living cells. Therefore, the limit of quantification (LOQ) of a CBA depends on the sensitivity of the cells the day of the assay and the matrix effects coming from the fish sample. It is evident that, if possible, exposing cells to high fish tissue equivalent concentrations involves reaching lower LOQs for CTXs. However, high fish tissue equivalent concentrations may interfere in the assay by affecting or even killing cells regardless of the CTX contents. A possible solution to reduce these undesirable fish matrix effects is to clean up samples to obtain purified extracts. In this direction, solid-phase extraction (SPE) cartridges containing magnesium silicate (Florisil) and octadecyl silica (C18) have been tested (Castro et al., 2020). As a critical point, clean-up strategies may suffer from toxin losses during the different steps of the protocols, which may also compromise the detection capability of the assay providing false negative results. Therefore, the ideal clean-up material should be able to remove the undesirable fish matrix compounds and recover high toxin amounts.

Cyclodextrins (CDs) are cyclic glucose oligomers that form a conical structure with a hydrophobic internal cavity and two external hydrophilic rims decorated with hydroxyl groups. The number of glucose units, 6 in α-CD, 7 in β-CD and 8 in γ-CD, determines the size of the cavity, which allows the inclusion of a variety of organic molecules of appropriate size, shape and polarity (Villalonga et al., 2007). CDs have been used in different fields, such as drug delivery (Ramirez et al., 2007) and biosensing (Ortiz et al., 2011a, 2011b; Wajs et al., 2016). Recently, they have also been exploited as passive sampling materials for marine toxin tracking in *Prorocentrum lima* cultures as well as in harbour waters during a *Dinophysis sacculus* bloom (Campàs et al., 2021). Nevertheless, they have never been tested as clean-up materials in toxin analysis.

In this work, two CD polymers, *γ*-cyclodextrin-hexamethylene diisocyanate (*γ*-CD-HDI) and *γ*-cyclodextrin-epichlorohydrin (*γ*-CD-EPI), as well as *γ*-CD-EPI-modified magnetic beads (*γ*-CD-EPI-MB) (Fig. 1) have been used to clean up fish flesh extracts before their analysis with a CBA for the evaluation of CTX-like toxicity, and compared with octadecyl silica (positive control). Firstly, *γ*-CD-HDI and *γ*-CD-EPI were synthesized by polycondensation of the native CD with bifunctional cross-linkers, while *γ*-CD-EPI-MB was prepared by coprecipitation of *γ*-CD-EPI in the presence of iron salts. Secondly, the capacity of the CD polymers, used in different formats (suspension and column), to capture CTX1B was tested in the absence and in the presence of fish flesh. Afterwards, their ability to decrease or even remove the fish flesh matrix effects was evaluated, as well as their effect on the CTX1B calibration curve for quantification purposes. Finally, naturally contaminated fishes were cleaned up and analysed, and results were compared with those obtained with non-purified extracts.

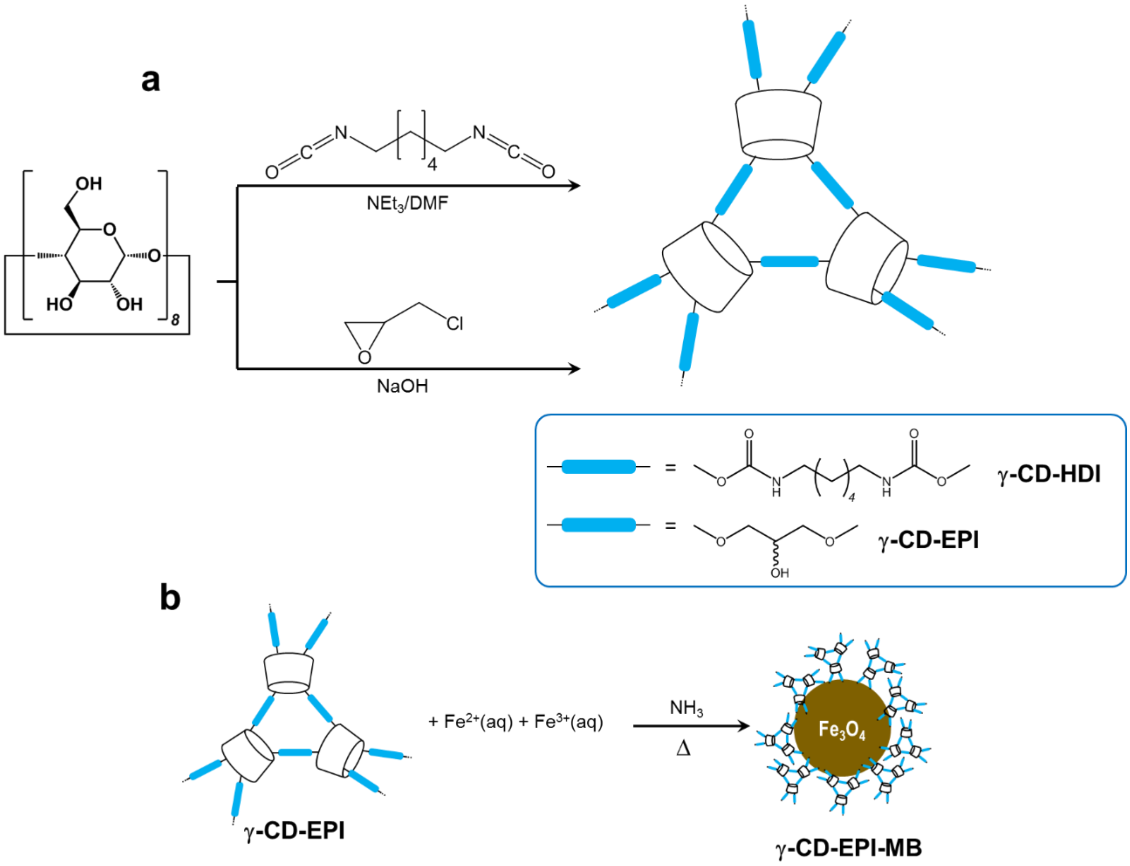


Figure 1. Preparation and structures of γ-CD-HDI and γ-CD-EPI polymers (a), and γ-CD-EPI-modified magnetic beads (γ-CD-EPI-MB) (b).

2. Materials and methods

2.1. Reagents and materials

Octadecyl-functionalized silica gel was purchased from Merck KGaA (Darmstadt, Germany). *γ*-CD-HDI was synthesized by crosslinking *γ*-CD (≥98%, Wacker Chemie AG, Burghausen, Germany) with hexamethylene diisocyanate (≥99.0%, Merck KGaA) (1:8 molar ratio) in dimethylformamide containing triethylamine (Mohamed et al., 2011). *γ*-CD-EPI was prepared by reaction of the native CD with epichlorohydrin (≥99.0%, Merck KGaA) (1:16 molar ratio) in NaOH (Crini et al., 1998). The products were purified by Soxhlet extraction in refluxing ethanol to remove unreacted starting materials and low molecular weight polymers, followed by refluxing with H_2O to rehydrate the polymer network. The γ-CD-EPI-MB was prepared by the one step co-precipitation method (Singh et al., 2011) using Fe^{2+}/Fe^{3+} precursor salts in the presence of carboxymethylated γ-CD-EPI (Fragoso et. al, 2009). Briefly, $FeCl_2{\cdot}4H_2O$ (1 mmol), $FeCl_3{\cdot}6H_2O$ (0.5 mmol) and γ-CD-EPI (1 g) were dissolved in 40 mL of de-aerated Milli-Q H_2O with vigorous stirring followed by addition of concentrated ammonia (5 mL). The reaction was continued for 1 h at 90 °C under constant stirring and nitrogen atmosphere. The resulting magnetic beads were washed with Milli-Q water (5×) and dried in a vacuum oven at 40 °C (yield 1.8 g).

CTX1B standard solution was obtained from Prof. Richard J. Lewis (The Queensland University, St Lucia, Australia) and calibrated (correction factor of 90%) in relation to the NMR-quantified CTX1B standard solution from Prof. Takeshi Yasumoto (Japan Food Research Laboratories, Japan). Low frequency polyvinyl chloride (LPVC) plastic filtration columns and frits (Supelco) were obtained from VidraFoc (Barcelona, Spain) and 1 µm nylon mesh was purchased from Sefar Maissa (Cardedeu, Spain). Acetone and diethyl ether were obtained from Chem-lab (Zedelgem, Belgium), and methanol (MeOH), *n*-hexane and dimethyl sulfoxide (DMSO) from Honeywell (Barcelona, Spain). Milli-Q H_2O (Millipore, Bedford, USA) was used to prepare solutions. Neuroblastoma murine (N2a) cells were purchased from ATCC LGC standards (Manassas, VA, USA). Foetal bovine serum (FBS), L-glutamine solution, ouabain, veratridine, phosphate buffered saline (PBS), penicillin, streptomycin, Roswell Park Memorial Institute (RPMI) medium, sodium pyruvate and thiazolyl blue tetrazolium bromide (MTT) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Fish sample extraction and clean-up

Variola louti specimens were obtained from La Réunion (France) in March 2013 (CTX negative) and April 2004 (CTX positive). *Seriola dumerili* specimens were obtained from the Canary Islands (Spain) in November 2016, August 2017 and May 2018 (CTX negative) and in May 2016 (CTX positive). CTX positivity and negativity was concluded according to CBA results. Fish samples were extracted and purified as follows: 10 g of fish flesh was heated at 70 °C for 15 min in a water bath. After cooling, 20 mL of acetone was added, the sample mixture was homogenized with an

Ultraturrax blender for 2 min and centrifuged at 3,000 × *g* for 15 min to obtain the supernatant. The pellet was re-extracted with acetone, and supernatants were pooled, passed through 0.2-µm PTFE filters, rotary evaporated to a small volume, and adjusted to 4 mL with Milli-Q H_2O. The sample was partitioned twice with 16 mL of diethyl ether. The water phases were discarded, and the diethyl ether phases were pooled and evaporated to dryness. The dried extract was resuspended in 2 mL of aqueous MeOH (80%) and partitioned three times with 4 mL of *n*-hexane. The *n*-hexane phases were discarded, and the aqueous MeOH phases were pooled and evaporated to dryness with N_2. The dried extract was then resuspended in 4 mL of HPLC-grade MeOH (100%), passed through 0.2-µm PTFE membrane filters, and stored at −20 °C until analysis. For calculation purposes, 1 mL of fish flesh crude extract contains 2.5 g equivalents of fish flesh. Whereas the CTX-positive *V. louti* and *S. dumerili* specimens were analysed individually, the CTX-negative ones were pooled to have enough material for all experiments.

Three clean-up protocols were tested (Supplementary Material, Fig. S1). Suspension format: 1) 50 mg of γ-CD-HDI, γ-CD-EPI and octadecyl (positive control) were introduced into Eppendorf tubes; 2) for the activation, the materials were incubated with 1 mL of MeOH, centrifuged for 2 min, rinsed with 1 mL of Milli-Q H_2O, centrifuged for 2 min, incubated with 1 mL of MeOH:H_2O (60:40) and centrifuged for 2 min; 3) 1 mL of CTX1B solution at 100 pg/mL in MeOH:H_2O (60:40) or fish flesh extract at 1,500 mg/mL in MeOH:H_2O (60:40) (prepared with 600 µL of methanolic fish flesh extract at 2,500 mg/mL and 400 µL of H_2O) was added and incubated overnight; 4) samples were centrifuged for 2 min and the supernatant was transferred to a vial; 5) a washing step was performed adding 1 mL of MeOH:H_2O (60:40), mixing, centrifuging samples for 2 min, and removing the supernatant, which was pooled with the supernatant obtained in step 4; 6) for the CTX1B elution, 1 mL of MeOH:H_2O (90:10) was added and incubated for 2 h; 7) samples were centrifuged for 2 min and the supernatant was transferred to a vial; 8) a second sequential extraction was performed adding 1 mL of MeOH:H_2O (90:10), mixing, centrifuging samples for 2 min and removing the supernatant, which was pooled with the supernatant obtained in step 7.

Incubation steps were performed under agitation on a mixing wheel. Centrifugations were performed on a Spectrafuge™ Mini Lab Centrifuge. Column format: 1) 50 mg of γ-CD-HDI, γ-CD-EPI and octadecyl (positive control) were introduced into LPVC plastic filtration columns containing 1 µm nylon mesh filters and frits; 2) for the activation, 5 mL of MeOH were loaded, vacuum was applied, 5 mL of Milli-Q H_2O were loaded, vacuum was applied, 5 mL of MeOH:H_2O (60:40) were loaded and vacuum was applied (collected solutions were discarded); 3) 1 mL of CTX1B solution at 100 pg/mL in MeOH:H_2O (60:40) or fish flesh extract at 1,500 mg/mL in MeOH:H_2O (60:40) (prepared with 600 µL of methanolic fish flesh extract at 2,500 mg/mL and 400 µL of H_2O) was loaded, vacuum was applied, and the sample was collected; 4) a washing step was performed adding 1 mL of MeOH:H_2O (60:40), applying vacuum and collecting the sample, which was pooled with the sample collected in step 3; 5) for the CTX1B elution, 2 mL of MeOH:H_2O (90:10) was added, vacuum was applied, and the sample was collected. Vacuum was applied with a Vac-Elut SPE vacuum manifold (Varian, Harbor City, CA, USA). MB format: the protocol is essentially the same as in the suspension format but using 50 and 200 mg of γ-CD-EPI-MB and replacing the centrifugation steps with magnetic separations, which were performed on a MagneSphere Technology Magnetic Separation Stand (for twelve 1.5-mL tubes) from Promega Corporation (Madison, WI, USA). All experiments were performed in duplicate.

2.3. CBA

The CBA was performed as previously described (Diogène et al., 2017). Briefly, N2a cells were seeded in a 96-well microplate in 200 µL of RPMI medium (containing 5% FBS, 5% L-glutamine solution, 5% penicillin-streptomycin and 5% sodium pyruvate) at 34,000 cells/well and incubated at 37 °C in a 5% CO_2 humid atmosphere for 24 h. Before exposure to CTX1B standard solution or fish flesh extract, some N2a cells were pre-treated with ouabain and veratridine at 0.1 and 0.01 mM, respectively. CTX1B standard solution or fish flesh extract were dried (volumes depending on the concentrations to be tested in each experiment), reconstituted in 200 µL of RPMI medium, serially diluted, and 10 µL were added to the wells with and without ouabain/veratridine pre-treatment. After 24 h, cell viability was measured using the MTT assay (Manger et al., 1993). Measurements were performed in triplicate.

3. Results and Discussion

3.1. Recovery from CD polymers

3.1.1. Recovery using CTX1B in solvent

Firstly, the performance of the different CD polymers and formats was evaluated using CTX1B in MeOH:H_2O (60:40). Fig. 2 shows the CTX1B contents obtained in the eluates as well as in the supernatants, expressed as CTX1B recovery percentages. Octadecyl silica was used as a positive control because commercial C18 cartridges, commonly used for the solid-phase extraction of lipophilic marine toxins, contain this material. Although commercial C18 cartridges could have been used, we preferred to use the raw material and implement the same procedure for comparison purposes. The CTX1B recovery value obtained with octadecyl in column format was excellent (94 ± 5% and no CTX1B detected in the supernatant). However, this material was not used in suspension format, since the use of Milli-Q H_2O during the activation resulted in "sticky" suspensions impossible to be centrifuged.

Regarding CD polymers, no differences were observed when using them in suspension or in column formats. The CTX1B recovery values obtained with γ-CD-HDI were very good (82 ± 6%), and low CTX1B amounts were detected in the supernatants (21 ± 2%). However, the CTX1B recovery values obtained with γ-CD-EPI were substantially lower (31 ± 4%) and high CTX1B amounts were observed in the supernatants (69 ± 8%). These low CTX1B recovery values obtained with γ-CD-EPI are certainly not related with the elution, but with the capture. The structure of the CDs, and more precisely the bridging units, different in γ-CD-HDI and in γ-CD-EPI, may be playing a role. The higher hydrophobicity of the HDI spacer (which contains six CH_2 groups connected to the CD by O(C=O)NH groups) compared to the EPI one, provides a more lipophilic environment for the capture of CTX1B. Additionally, hydrogen bond interactions between the amide groups of the HDI spacer and the multiple OH groups of the CTX1B may contribute to the higher recovery values.

Regarding γ-CD-EPI-MB, when using 50 mg results were very similar to those obtained with γ-CD-EPI (25 ± 2% of recovery and 74 ± 4% in the supernatant), even though 50 mg of this material contains a much lower amount of CD polymer than 50 mg of γ-CD-EPI (due to the high weight of the magnetic beads). When using 4-fold higher amounts of γ-CD-EPI-MB (200 mg), the trend was practically the opposite (68 ± 15% of recovery and 29 ± 1% in the supernatant). However, the use of even higher γ-CD-EPI-MB amounts (400 mg) resulted in lower CTX1B recovery values (35 ± 5%), which seems to indicate that steric effects caused by interparticle interactions could inhibit the CTX1B capture.

Since no differences were observed for the CD polymers between formats, columns were chosen for the following experiments, because the process was faster than in suspension. Regarding γ-CD-EPI-MB, 200 mg was selected. Octadecyl columns were also maintained for comparison purposes.

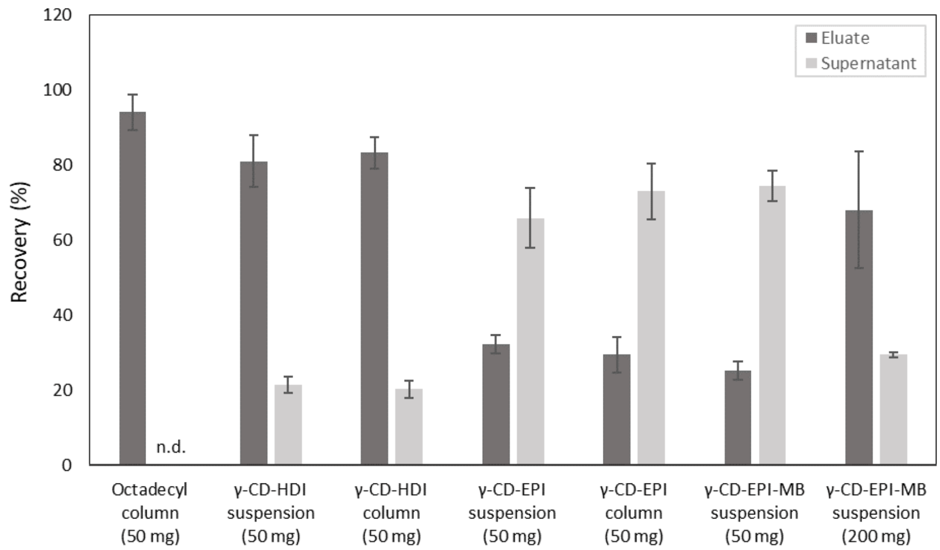


Figure 2. Recovery percentages obtained in the spiking of CTX1B in solvent.

3.1.2. Recovery using CTX1B in fish flesh extract

The performance of the different CD polymers and formats was then evaluated by spiking a CTX-negative fish flesh extract (*V. louti*) with CTX1B and proceeding with the selected clean-up strategies (Fig. 3). The CTX1B recovery value obtained with octadecyl in column format was very good (89 ± 11%). Regarding CD polymers in column format, CTX1B recovery values were lower than when working in the absence of fish flesh, and γ-CD-HDI again provided higher CTX1B recovery values than γ-CD-EPI (42 ± 2% in front of 3 ± 1 %). Whereas γ-CD-HDI could still be appropriate as a clean-up material, γ-CD-EPI should be completely discarded. Nevertheless, as observed when working in the absence of fish flesh, the use of 200 mg of γ-CD-EPI-MB provided higher CTX1B recovery values (54 ± 3%) than the polymer without magnetic beads. These results indicate that the presence of fish matrix compounds is playing a role in the interaction between the CD polymers and the toxin, certainly decreasing the capture efficiency.

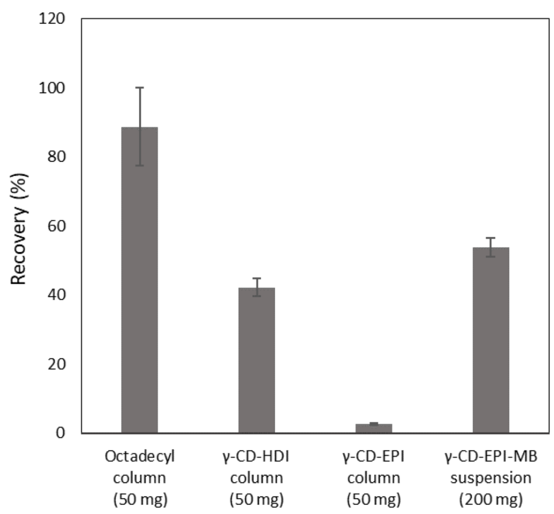


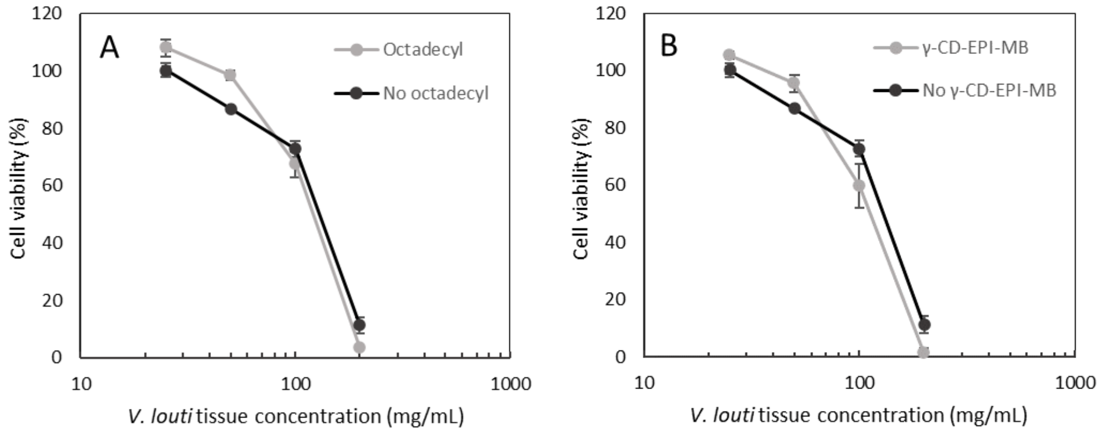
Figure 3. Recovery percentages obtained in the spiking of CTX1B in fish flesh extract.

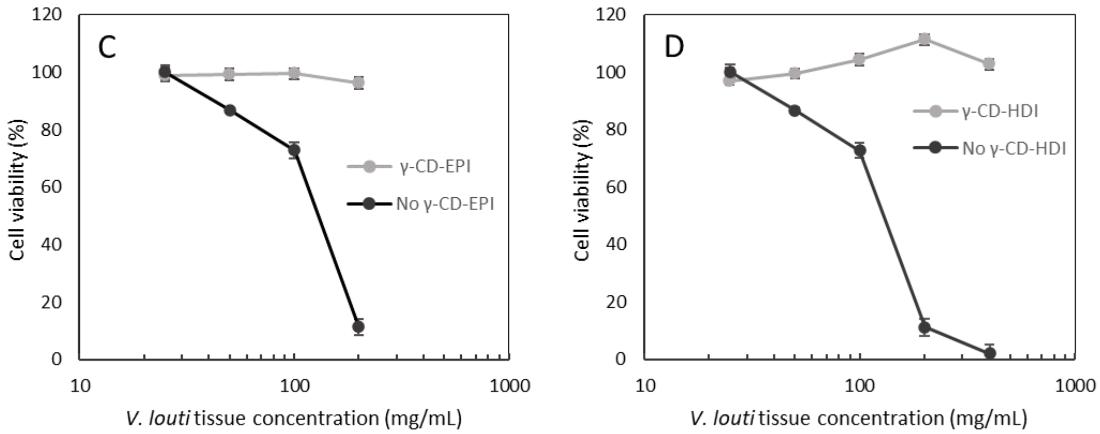
3.2. Evaluation of fish matrix effects

Besides capturing CTX1B and attaining good recovery values, the CD polymers should be able to decrease or even remove the undesirable fish flesh matrix effects on the CBA (not related with

CTXs presence). This would allow analysing fish extracts at higher tissue equivalent concentrations and, therefore, attaining lower LOQs. To this purpose, the cell viability in the absence of ouabain and veratridine with *V. louti* crude extracts and extracts that had undergone the selected clean-up processes was evaluated. The clean-up with octadecyl or γ-CD-EPI-MB did not decrease the fish matrix effects in the CBA, as cell mortality percentages were the same as with fish flesh crude extracts (Fig. 4A and 4B). In fact, no matrix effects were observed in the octadecyl or γ-CD-EPI-MB supernatants, suggesting that fish matrix compounds may still be in the cleaned-up extracts. Therefore, despite the good CTX1B recovery values obtained in the previous experiments, these two materials were put aside as they would not provide any improvement to the analysis. The result with octadecyl was surprising, since we had taken it as a positive control due to their use in commercial SPE cartridges sought for the purification of lipophilic marine toxins. However, it is necessary to take into account that most of the works that use these cartridges analyse the samples by LC-MS/MS (Gerssen et al., 2009; Estevez et al., 2019), where matrix effects are certainly different.

When using γ-CD-EPI and γ-CD-HDI in column format, the analysis of fish flesh crude extracts showed cell mortality at 50 mg/mL or higher fish flesh equivalent concentrations; on the contrary, no cell mortality was observed with cleaned-up extracts (Fig. 4C and 4D). Additionally, the analysis of the γ-CD-EPI and γ-CD-HDI supernatants showed matrix effects, evidencing the presence of fish matrix compounds in this phase. Considering together the removal of matrix effects and the recovery percentages obtained in the spiking of CTX1B in fish (3% and 42% for γ-CD-EPI and γ-CD-HDI, respectively), γ-CD-HDI in column format was selected for the following experiments. This strategy was also tested with *S. dumerili*, and the removal of matrix effects, even at 400 mg/mL, was also demonstrated (Fig. 4E). When using this fish species, the CTX1B recovery value was 32 ± 1 % (value quite close to the 42 ± 2% obtained with *V. louti*). In principle, fish flesh extracts that have undergone clean-up with γ-CD-HDI in column format could be analysed at 400 mg/mL (or may be even more), instead of 25-50 mg/mL (concentration required to analyse crude extracts), and CTX1B recovery, although not very high, would still improve the sensitivity of the assay.





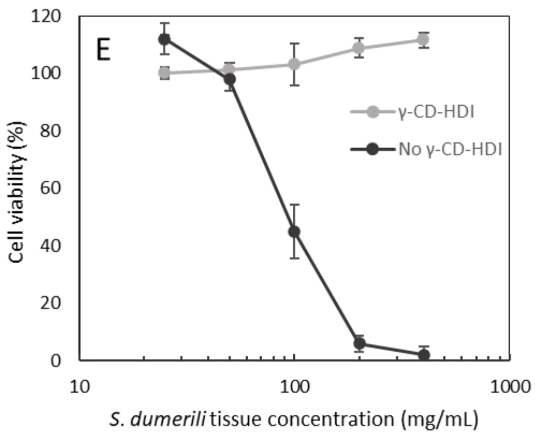


Figure 4. Cell viability for fish flesh extracts without and with clean-up with octadecyl (A), γ-CD-EPI-MB (B), γ-CD-EPI (C) and γ-CD-HDI (D and E).

3.3. Effect of the CD polymers on the CTX1B quantification

Apart from the fish flesh matrix effects on the cell viability, the presence of fish flesh matrix compounds may interfere in the CTX-like toxicity assay and shift the CTX1B calibration curves. The CTX1B calibration curve in *V. louti* and *S. dumerili* crude extracts was evaluated at 25 mg/mL (serial dilutions of the toxin, maintaining the fish flesh equivalent concentration constant). To avoid cell mortality, no higher fish flesh equivalent concentrations were used. With both fish species, the CTX1B calibration curves were displaced with respect to the CTX1B calibration curve in the absence of fish flesh extract, providing higher cell viability values, which would imply an underestimation of the CTX1B contents (see corresponding curves in Fig. 5A and 5B). It seems that fish matrix compounds are interacting with CTX1B, reducing its affinity for the VGSCs, as also observed in other works (Castro et al., 2020).

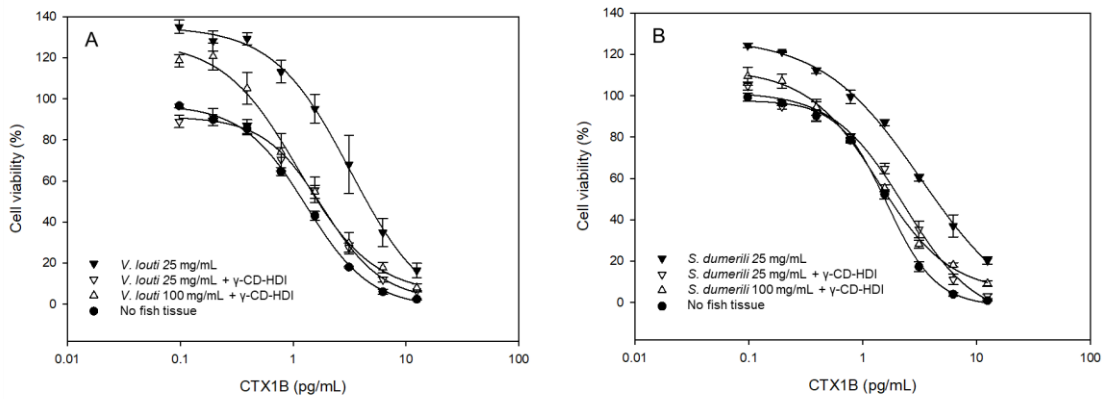


Figure 5. CTX1B calibration curves in the absence of fish flesh and in the presence of *V. louti* (A) and *S. dumerili* (B) extracts without and with clean-up with γ-CD-HDI.

To investigate if the use of γ-CD-HDI in column format could remove this fish matrix effect in the presence of toxin, cleaned-up *V. louti* and *S. dumerili* extracts were spiked with CTX1B and analysed at 25 and 100 mg/mL. The corresponding CTX1B calibration curves were obtained and compared to the ones in the absence of fish flesh extract. For both fish species, cleaned-up fish flesh extracts at 25 mg/mL recovered the CTX1B calibration curves to the original shape (see corresponding curves in Fig. 5A and 5B). When using cleaned-up fish flesh extracts at 100 mg/mL, cell viability values increased at low CTX1B concentrations, but were still lower than with fish flesh crude extracts at 25 mg/mL. It is interesting to note that this higher cell viability disappeared with increasing CTX1B concentrations, being negligible at values around the IC_{20} (80% cell viability). The LOQs (corresponding to the CTX1B concentration at the IC_{20} divided by the fish flesh equivalent concentration) for CTX1B were 0.092 µg/kg and 0.070 µg/kg for the *V. louti* and *S. dumerili* crude extracts at 25 mg/mL, respectively, and 0.055 µg/kg and 0.106 µg/kg for the cleaned-up extracts at the same fish flesh equivalent concentration (including the respective CTX1B recovery values in the calculation). The LOQs for the cleaned-up extracts at 100 mg/mL were 0.020 µg/kg and 0.023 µg/kg for *V. louti* and *S. dumerili*, respectively. As it can be observed, the clean-up process allows operating at higher fish flesh equivalent concentrations, decreasing the LOQs and providing 4.6 and 3.0-fold higher sensitivities to the assay for *V. louti* and *S. dumerili*, respectively.

Taking into account that the IC_{20} values for the curves with the cleaned-up extracts at 25 and 100 mg/mL were very similar to the one in the absence of fish flesh, and assuming a similar behaviour for curves with extracts cleaned-up with γ-CD-HDI in column format at 400 mg/mL (concentration that has been observed to not cause cell death), theoretical LOQs of 0.005 µg/kg and 0.006 µg/kg for *V. louti* and *S. dumerili* can be calculated. These values indicate that our strategy could provide at least 18.4 and 11.7-fold higher sensitivity to the assay for *V. louti* and *S. dumerili*, respectively. Although the theoretical LOQs are below the FDA guidance level of 0.01 µg/kg, the extraction process (before the clean-up) may suffer from toxin losses and compromise fish acceptance decision making. Nevertheless, this critical issue is common in all analytical techniques.

In the work by Castro et al. (2020), the use of Florisil cartridges removed the *Pomatomus saltatrix* matrix effects in the absence of CTXs at 50 mg/well (concentration equivalent to 217 mg/mL). The combination of Florisil cartridges with C18 cartridges was necessary to remove the matrix effects from *S. dumerili* and *Acantocybium solandri* (although for the latter, only partially). No cell death from *Epinephelus marginatus* and *Pagrus pagrus* was observed at this fish tissue equivalent concentration. Their work makes evident the differences between fish species, also observed in our work. In general terms, it seems that the clean-up strategy with γ-CD-HDI in column format is better at removing the matrix effects. For example, the assay for *S. dumerili* can be performed at a fish flesh equivalent concentration as high as 400 mg/mL using only one cartridge for the clean-up process.

Although Castro and co-workers (2020) evaluated the effect of the cleaned-up *Pagrus pagrus* extract on the C-CTX1 and CTX1B calibration curves, spiking of CTX to fish extracts before clean-up was not performed, neither ciguateric fishes were analysed. Nevertheless, in a previous work from the same group (Estevez et al., 2019), they had evaluated the recovery values for *Lutjanus malabaricus* using LC-MS/MS, which were between 57.6 and 77.2% (including the ionic suppression) depending on the clean-up protocol. As previously mentioned, toxin recovery is a critical issue in a clean-up process, and it is evident that it depends not only on the clean-up process itself but also on the sample. In our study, although CTX1B recovery was not as high as desired, it is still enough to improve the assay. Further efforts should focus on increasing the CTX1B recovery, for example tailoring the CD polymers in terms of cavity size and specific functionalities to improve their affinity for the toxin.

3.4. Analysis of naturally contaminated fishes

One *V. louti* individual and one *S. dumerili* individual, known to be positive for CTX from previous experiments, were chosen to demonstrate the applicability of γ-CD-HDI as a new and sustainable material for the clean-up of fish flesh extracts before analysis with CBA. Crude and cleaned-up extracts were analysed (Table 1). In the analysis of *V. louti* crude extract, 0.576 ± 0.036 µg CTX1B equiv./kg were obtained. The analysis of the corresponding cleaned-up extract revealed 0.251 ± 0.033 µg CTX1B equiv./kg. The application of the CTX1B recovery percentage for this fish species (42%) to the quantification resulted in 0.598 ± 0.079 µg CTX1B equiv./kg, value very similar to the one obtained in the analysis of the crude extract. In the analysis of *S. dumerili* crude extract, 0.074 ± 0.010 µg CTX1B equiv./kg were obtained. The analysis of the corresponding cleaned-up extract revealed 0.021 ± 0.002 µg CTX1B equiv./kg. The application of the CTX1B recovery percentage for this fish species (32%) to the quantification resulted in 0.067 ± 0.005 µg CTX1B equiv./kg, value again very similar to the one obtained in the analysis of crude extract. Although for these two fish individuals the analysis of crude extracts was feasible, the experiment demonstrates that γ-CD-HDI can effectively be used as a clean-up material for the detection of CTXs in fish with CBA. The removal of interfering compounds certainly improves the reliability of the CBA, reducing the probability of false positive and negative results.

Table 1. CTX1B equiv. contents detected in naturally contaminated fishes (µg CTX1B equiv./kg).

Fish species	Crude extract	Cleaned-up extract without recovery	Cleaned-up extract with recovery
V. louti	0.576 ± 0.036	0.251 ± 0.033	0.598 ± 0.079
S. dumerili	0.074 ± 0.010	0.021 ± 0.002	0.067 ± 0.005

4. Conclusions

CD polymers have been tested for the first time as new and sustainable clean-up materials to purify CTXs from fish flesh extracts that are going to be analysed with CBA. The best results, considering together removal of fish matrix effects, appropriate CTX1B recovery values and ease of the protocol, have been obtained with γ-CD-HDI in column format. The higher hydrophobicity of the HDI spacer compared to the EPI one could be key in the CTX1B capture. The removal of matrix effects with this material was evident, making possible to expose cells to fish flesh equivalent concentrations as high as 400 mg/mL (and may be even higher). Additionally, the incorporation of this clean-up step provided CTX1B calibration curves very similar to the ones obtained in the absence of fish flesh extract, improving the reliability of the CTX quantification. We have demonstrated that our clean-up strategy provides 4.6 and 3.0-fold higher sensitivities to the assay for *V. louti* and *S. dumerili*, respectively, and we hypothesise that the improvement could be even greater. This is an excellent achievement considering the extremely low CTX contents that need to be detected to guarantee fish safety and protect human health. Although more ciguateric fish individuals, containing a wide range of CTX contents, should be analysed to validate the strategy, this proof-of-concept demonstrates the suitability of CD polymers as clean-up materials. Since CD polymers can be tailor-synthetised, toxin recovery, which is a critical point of this work but common in all clean-up protocols, could be increased. The clean-up of fish flesh extracts in an efficient and easy way can certainly contribute to screen samples with high matrix effects that contain low CTX contents and, therefore, require to be analysed at high fish flesh equivalent concentrations.

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