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Fish Extract Fractionation by Solid Phase Extraction: Investigating Co-Occurring Ciguatoxins by LC-MS/MS and N2a-Bioassay

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Abstract: Ciguatoxins (CTXs) are neurotoxic marine biotoxins capable of contaminating marine organisms. Approximately 30 CTX analogues have been described; however, only a few have been documented in ciguatera poisoning (CP) outbreaks. Detecting CTXs from complex matrices at CP-relevant concentrations (<1 µg per kg seafood tissue) is analytically challenging. Analytical standards for CTXs are rare. Even after multi-step sample preparation (including liquid–liquid partition, defatting, and solid-phase extraction (SPE)), extracts can contain undesirable co-eluting matrix components. These limitations can exacerbate discrepancies between results obtained by LC-MS/MS and the N2a-bioassay, which are two common CTX detection methods. Herein, a rapid and simple fractionation method, based on normal phase SPE (silica), is demonstrated. Target CTXs were eluted using solvent mixtures of ascending polarity, passed through the column, and separated into eight fractions. To challenge the method, extracts with eleven naturally incurred CTX analogues among different structural CTX groups (e.g., CTX3C, CTX4A, and C-CTX group) were used. The most complex tissue matrix tested (viscera) was improved the most for extract purity and CTX detection, enhancing the correlation between LC-MS/MS and N2a-bioassay results. This workflow represents an advancement for characterizing CTXs in seafood products and CP outbreaks, irrespective of the responsible CTX analogue and where standards are lacking.

Keywords: ciguatera poisoning; neuro-2a bioassay; mass spectrometry; fish poisoning; matrix effects; marine biotoxin

Citation: Spielmeyer, A.; Blaschke, V.; Loeffler, C.R. Fish Extract Fractionation by Solid Phase Extraction: Investigating Co-Occurring Ciguatoxins by LC-MS/MS and N2a-Bioassay. *Separations* **2024**, *11*, 238. https://doi.org/10.3390/separations11080238

Academic Editor: Sara Cunha

Received: 5 July 2024
Revised: 26 July 2024
Accepted: 26 July 2024
Published: 1 August 2024

1. Introduction

Ciguatoxins (CTXs) (Figure 1) are potent marine neurotoxins produced by dinoflagellates of the genera *Gambierdiscus* [1–5] and possibly *Fukuyoa* [6]. The human consumption of seafood contaminated with CTXs can result in ciguatera poisoning (CP), the most commonly reported non-bacterially related seafood illness globally [7].

Historically, CTXs and their analogues were classified according to the region in which they were found with P-CTXs reported from the Pacific Ocean, I-CTXs from the Indian Ocean, and C-CTXs from the Caribbean Sea [8]. However, P-CTXs are now organized into two group descriptions, namely the CTX4A and CTX3C group, based on their chemical structure [9]. Replacing a regional-based description (i.e., P-CTX) to a structural-based nomenclature (i.e., CTX3C group) adheres to the modern evidence that CTXs are contained in globally traded seafood products and to the cosmopolitan nature of *Gambierdiscus* (e.g., *G. balechii* isolated from the Pacific Ocean producing putative 'Caribbean' or 'Indian ocean' CTXs) [5,10,11]. To date, more than 30 distinct CTX analogues have been described [9]. The structural information for analogues of the I-CTX group are not yet available.

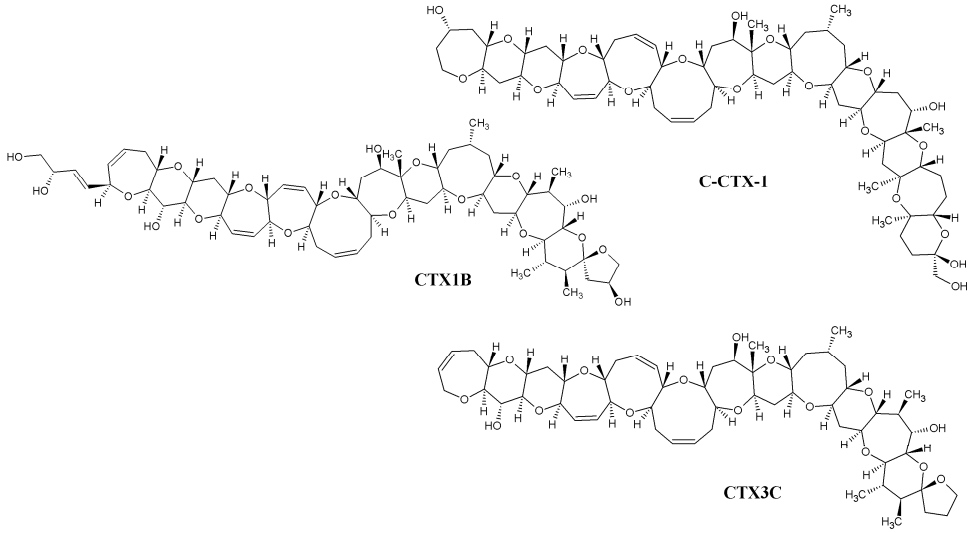

Figure 1. Structures of selected CTX analogues; stereochemistry according to [9].

CTXs produced at the base of the food web can be found intra- and extracellularly in the environment [12,13], accumulating in marine animals among trophic levels [3,4,14]. Marine animals can contain single or mixed group CTX profiles, which is dependent on the CTXs produced by the *Gambierdiscus* species present in the local environment [11,15–17]. Once ingested or absorbed, CTXs can undergo biotransformation (mainly oxidation), leading to a further diversification of CTX analogues [18,19].

Investigating samples for CTXs is most commonly conducted using a two-tier analysis approach [20]. In tier-one of the analysis, sample extracts are screened by an in vitro cell-based assay (CBA) using mouse neuroblastoma cells (N2a bioassay). The effective concentration response reported by the assay is a representation of a composite sample toxicity, i.e., representing all compounds in the extract that are capable of acting biologically on voltage-gated sodium channels, in all their various contributions. Qualitative information (e.g., which CTXs were present in the sample) from the assay is not possible. Therefore, samples deemed 'positive' are further analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and low or high resolution (HR) MS for the potential identification of CTX analogues. Most often, higher CTX concentration equivalents are reported for a sample by the cell assay, although in the case of algal cell analysis, higher contents have been reported for LC-MS/MS, indicating variability in detection and reporting [21–23]. The results obtained by the N2a-bioassay and LC-MS/MS can differ considerably, but they are considered complementary, and to date, they are mostly used independently.

Both analytical approaches are susceptible to matrix interferences, which can affect the accuracy, precision, and sensitivity of toxin quantification within and among detection methods. Matrix interferences can be reduced with intensive sample clean-up, using several liquid–liquid partition and solid phase extraction(s) (SPE) steps (for examples see reviews [24,25]). However, for LC-MS/MS analysis, signal suppression can still occur for the final extract. Complex tissue matrices such as liver or viscera or high fat content samples (e.g., fillet samples of high fat fish) can present matrix interferences that are particularly difficult to overcome. A first step to attempt to resolve matrix problems can be reducing the tissue equivalent (TE) amounts and increasing solvent volume per TE ratios. However, using less sample material for extraction also reduces the amount of the analyte being recovered from the matrix and thus influences the sensitivity of the method. Matrix effects depend on the interaction of the analyte with co-eluting matrix compounds in the ion source of the LC-MS/MS, and this interaction can be enhanced or inhibited by the eluent composition. Moreover, the extent of matrix effects can depend on the injection volume and

can be different for the respective ion transition(s) used for analysis [24–27], emphasizing the complexity of this issue.

Fractionation-based separation processes can be used for separating target analytes from unwanted matrix components. This methodology has successfully been applied towards the identification of potential CTX analogues [1,22,28,29]. Sample extract constituents can be separated using a solvent gradient through an HPLC column, collecting the effluent at defined time ranges (e.g., every minute). Fractions can be investigated for bioactivity using the N2a-bioassay (i.e., bioassay-guided fractionation) [5,28,30–32], providing feedback for LC-MS/MS CTX analysis regarding the retention time of potential toxins. However, using the same setup for fractionation and analysis would not create the necessary separation of analytes and co-eluting matrix components. HPLC analytical columns cannot handle high tissue loads, instead requiring time intensive multi-injection methods to obtain sufficient toxin amounts in one fraction.

Herein, a simple fractionation method for CTXs in fish extracts using SPE is demonstrated. This method allows the application of high tissue loads (results reported here refer to 2.5 g wet fillet tissue, 0.5 g dry fillet tissue, and 0.25 g dry viscera) while minimizing organic solvent usage (approx. 60 mL per sample, including solvents for SPE column conditioning). This approach was tested for different matrices (wet and dry tissue, fillet and viscera), and analogues of different CTX groups (CTX3C, CTX4A, and C-CTX group), ensuring its global applicability. Analyses included both LC-MS/MS and the N2a-bioassay, which enabled a direct comparison of the data obtained by these two analytical methods commonly utilized for CTX analysis.

2. Materials and Methods

2.1. Standards, Reagents and Materials

CTX3C (100 ng, lot APK4222 and ESN0667) was obtained from FUJIFILM Wako Chemicals Europe GmbH (Neuss, Germany). The standard was reconstituted in 1 mL methanol and stored in glass vials at −20 °C. CTX1B, 54-deoxyCTX1B, and 52-*epi*-54-deoxyCTX1B were purchased from Professor R. J. Lewis (The University of Queensland, Australia, prepared 17 November 2005). Methanol solutions containing 4, 2, and 1 µg mL^{-1}, respectively, were stored in glass vials at −20 °C. The dilution of standard solutions was performed with methanol.

Solvents and reagents were obtained from various suppliers with LC-MS grade acetonitrile, methanol, formic acid, ammonium acetate, LC grade ethyl acetate, and GC-MS grade *n*-hexane. All other reagents utilized (acetone, acetic acid, citric acid monohydrate, anhydrous sodium carbonate, and sodium chloride) were of p.a. grade. The enzyme papain was purchased from Carl Roth (Karlsruhe, Germany) (>30,000 USP-U mg^{-1} for biochemistry).

Figures of LC-MS/MS chromatograms and N2a concentration–response curves were created using SigmaPlot 14.

2.2. Fish Samples

Frozen fish fillet samples of *Lutjanus bohar* (two-spot red snapper) exported from Vietnam were obtained from a CP outbreak in Germany in 2017. Details about sample acquisition and origin can be found in [32]. Samples were not dried before extraction and were treated as "wet tissue". One sample of *Enchelynassa canina* (viper moray) was collected from the west coast of Hawai'i (USA), a location recognized as having a risk for CP [33]. The fish was eviscerated and the skin was removed. Viscera and unskinned muscle tissue were dried in a commercial dehydrator before shipment. After arrival, samples were additionally dried for 22 h using a freeze-dryer (Lyovac GT2, Amsco/Finn-Aqua, Hürth, Germany) in order to remove residual water. The same procedure was applied to a sample of *Sphyraena barracuda* (great barracuda) which was collected in the Caribbean Sea from St. Thomas (Virgin Islands, USA) (same material has been used in [5]).

Species authentication was performed via DNA barcoding as previously described [34]. Further method details are provided in Section S1. DNA extraction and barcoding were performed according to the cetyltrimethylammonium bromide (CTAB) standard protocol DIN EN ISO 21571:2013-08 [35] and DIN CEN/TS 17303:2019 [36], respectively. Sequences of the species utilized in this study are available at https://www.ncbi.nlm.nih.gov/genbank/ under the GenBank accession numbers OR447484 (*L. bohar*), OR447485 (*E. canina*), and OR447486 (*S. barracuda*).

2.3. Sample Extraction

Fish fillet (wet or dry) and viscera samples were extracted as described in [37] with small modifications to the sample pretreatment part (amount of water and enzyme added to freeze-dried material) (Figure 2). In brief, 5 g wet fillet, 1 g dry fillet, or 0.5 g dry viscera were enzymatically digested using papain. In the case of dry tissue samples, 4 mL of deionized water (independent of the sample weight) was added to the sample material before digestion to enable the enzymatic reaction. The digested sample was extracted using acetone, saturated sodium chloride solution, and ethyl acetate. The obtained raw extract was washed with a saturated sodium chloride solution and the organic phase was reduced to dryness in a stream of nitrogen at 40 °C. The residue was reconstituted in 80vol% methanol in water. Defattening was performed in three steps using *n*-hexane under neutral, basic, and acidic conditions (see Figure 2 for details). Further clean-up was conducted by a reversed phase (Chromabond EASY polystyrene–divinylbenzene copolymer with a weak anion exchanger), 200 mg, 3 mL; Macherey-Nagel, Düren, Germany) and normal phase SPE (Bond Elut SI (silica), 500 mg, 3 mL; Agilent, Waldbronn, Germany). SPE steps were conducted at atmospheric pressure. The final clean-up step delivers two fractions (filtrate and eluate, see [37] and Figure 2 for details). Both were separately reconstituted using two times 250 µL methanol. Samples were transferred into glass vials and stored at −20 °C. During method development and optimization, the filtrate was found to contain parts of CTX3C. More polar analogues (starting from the 49-epimer of CTX3C, 49-*epi*CTX3C) are transferred to the eluate. In the following, the terms "non-fractionated filtrate" or "non-fractionated eluate" refer to the samples obtained by the protocol described in this section. The term "full extract" refers to a combination of filtrate and eluate, e.g., utilized in the N2a-bioassay.

2.4. Fractionation

Samples were prepared according to Section 2.3 (Figure 2). For fractionation, 250 µL of both the non-fractionated eluate and filtrate were combined and reduced to dryness. This corresponds to 2.5 g wet fillet tissue, 0.5 g dry fillet tissue, and 0.25 g dry viscera being applied to the SPE column. Alternative sample pretreatments for fractionation are provided in Section S2 in the Supplementary Information. A detailed protocol of the fractionation method is provided in Figure 3. Fractionation by SPE was conducted at atmospheric pressure. In the following, the term "acidified ethyl acetate" refers to ethyl acetate containing 0.1vol% acetic acid.

The dried extract residue was reconstituted in 2 mL acidified ethyl acetate. A total of 2 mL *n*-hexane was added, and the mixture was applied to a pre-conditioned silica SPE (Bond Elut SI (silica), 500 mg, 3 mL) (details provided in Figure 3). The sample vessel was rinsed twice with 1 mL acidified ethyl acetate/*n*-hexane (1:1 *v*/*v*) and the volume was applied to the SPE column. Afterwards, another 4 mL of acidified ethyl acetate/*n*-hexane (1:1 *v*/*v*) was used for elution. These applied volumes (4 mL sample, 2 mL rinse, and 4 mL elution solvent) add up to fraction 1 (Figure 3). Afterwards, a sequential elution with ascending solvent polarity was conducted using seven different solvent mixtures with 5 mL each. Details concerning the solvent composition are provided in Figure 3. The column was not allowed to run dry between the respective fractions. After the complete passage of the solvent for fraction 8, the remaining solvent in the column bed was removed by applying pressure at the column inlet and the liquid was added to the eluate of fraction 8. All fractions were reduced to dryness in a

stream of nitrogen and reconstituted two times with 250 µL methanol. Samples were stored at −20 °C in glass vials until usage.

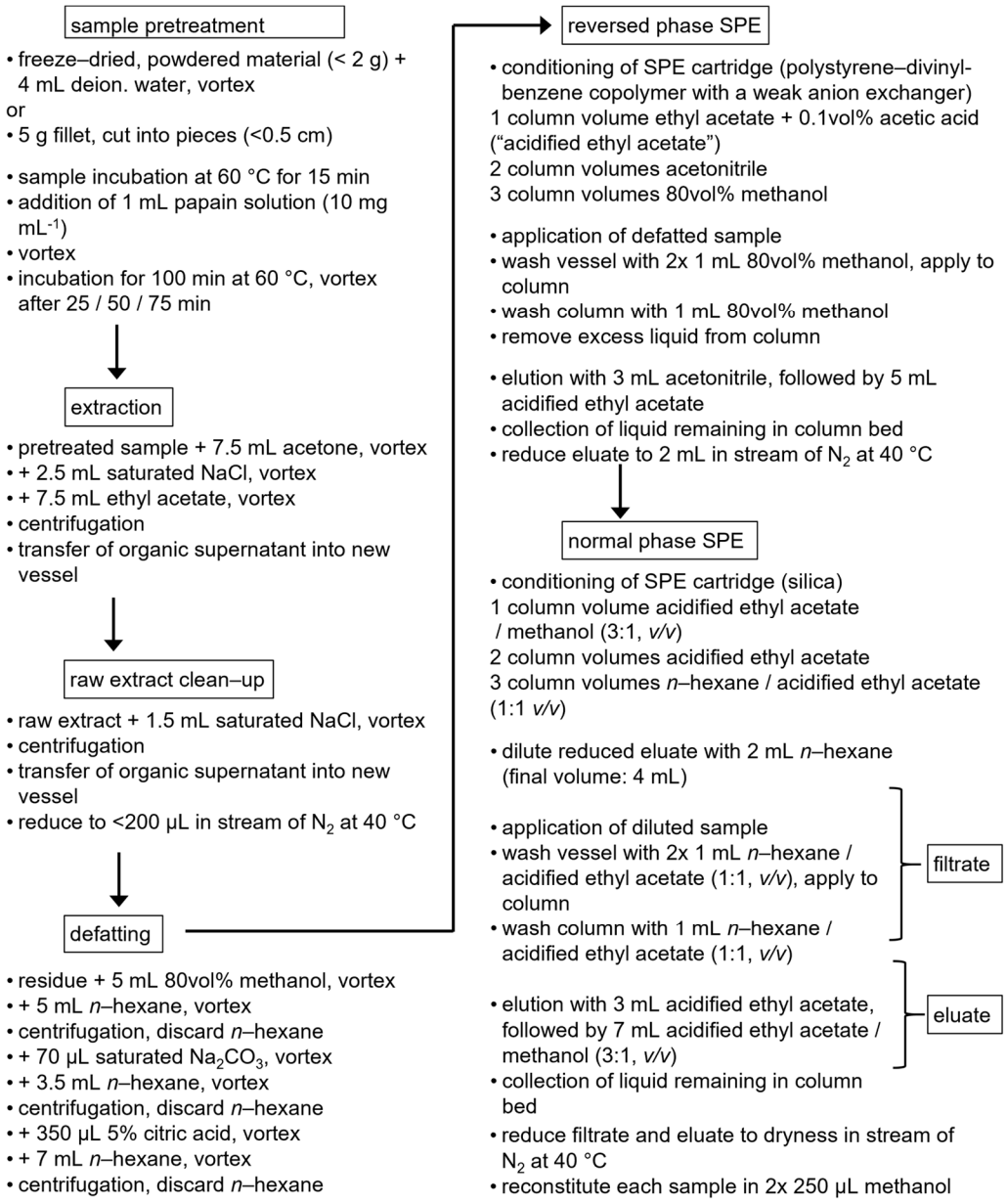

Figure 2. Flow chart of sample extraction; figure taken from [37] with modifications for the sample pretreatment part.

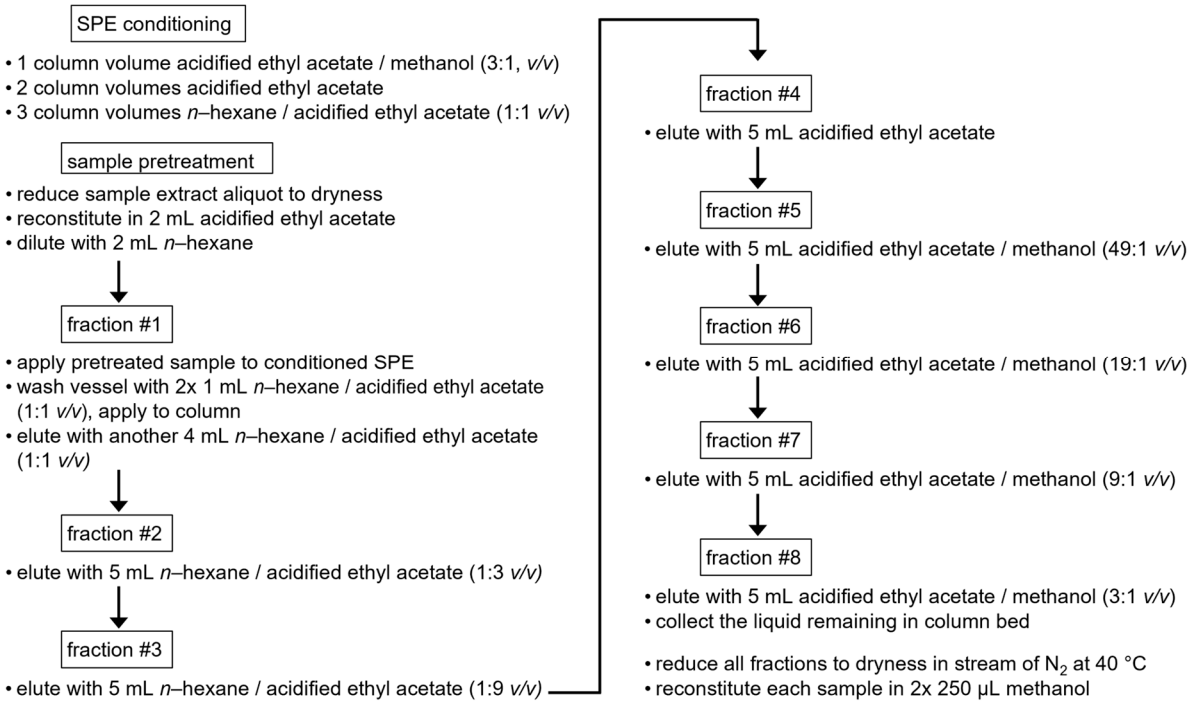

Figure 3. Flow chart of the fractionation via normal phase SPE (silica).

2.5. LC-MS/MS Analysis

Sample extracts and fractions were analyzed using a Sciex QTrap 6500+ mass spectrometer (Sciex, Darmstadt, Germany) connected to an Agilent 1290 Infinity II UHPLC (Agilent, Waldbronn, Germany). Separation was performed on a reversed-phase column (Gemini NX-C18; 150 × 2 mm, 3 µm; Phenomenex, Aschaffenburg, Germany) using (A) 1 mM ammonium acetate containing 0.5vol% formic acid and (B) methanol/acetonitrile (3:1, *v*/*v*) as mobile phases. In order to obtain more intense signals, eluent (B) was changed to methanol in the case of the C-CTX group analogues (detailed discussion provided in Section 3.3). The identification of C-CTX-1 was further supported by HRMS analyses using a Sciex TripleTOF 6600+ connected to an Agilent 1290 Infinity II UHPLC. LC column parameters and eluents were the same as those used for the QTrap system. Details concerning the LC-MS/MS conditions as well as information on LOD, LOQ, linearity range, and precision are provided in [37]. For the low-resolution analysis (QTrap), twenty pseudo ion transitions were monitored (multiple reaction monitoring, MRM), selecting the corresponding sodium adducts ($[M+Na]^+$) of all reported CTX analogues in Q1 and Q3. For high resolution analysis (TripleTOF), both full scan and product ion scans (high sensitivity mode) were performed. Further details are provided in [37]. The quantitation of analogues was performed by a one-point calibration using CTX3C (2 µg L^{-1}) and CTX1B (1 µg L^{-1}) as a reference for analogues of the CTX3C and CTX4A group, respectively. Non-fractionated samples were diluted 1:2 with methanol before analysis to obtain the same TE per mL for both non-fractionated extracts and fractions.

2.6. N2a-Bioassay

Fish tissue and viscera extracts (prepared according to Section 2.3), as well as the fractions of these samples (Section 2.4) were tested using the N2a-bioassay. In all cases, the same extracts and fractions were utilized for LC-MS/MS and in the N2a-bioassay to enable a direct comparison of the results.

Mouse (*Mus musculus*) neuroblastoma type cells of the American Type Culture Collection (N2a CCL-131, American Type Culture Collection, Rockville, MD, USA) were obtained from LGC Standards GmbH (Wesel, Germany). Cells were maintained and dosed according to [20,38,39]. Cell line modifications for ouabain (O) and veratridine (V) desensi-

tization were performed as described in [40]. For analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the incubated cells 22–24 h after dosage and the percentage of formazan color development was determined, as previously described [39]. Plate reading was performed using the Cytation 1 imaging reader (Agilent, Waldbronn, Germany). The results are expressed as µg CTX3C equivalent (eq.) per kg fish tissue eq. Fish sample weight eq. is either reported in dry weight eq. (DTE) or wet weight (WTE) depending on the source material.

For the comparison of LC-MS/MS and N2a-bioassay results, the same toxicity equivalent factors (TEFs) for all detected analogues were assumed in the bioassay. TEFs were not included in the content calculation, as these factors are not available for all analogues detected in the samples investigated in this study.

3. Results and Discussion

3.1. Development of Fractionation Protocol

The fractionation method presented here should enable the fast separation of CTX analogues of different polarities and the application of high matrix loads, while reducing matrix components, lowering material use (consumables, solvents), and saving time. Furthermore, it should be capable of integration into existing sample preparation workflows. For these reasons, SPE was considered as a suitable tool for performing extract fractionation, as it is also part of most existing sample clean-up protocols for CTX analysis (see reviews [24,25]).

Normal phase SPE (silica) was chosen as the stationary phase, based on the sample preparation protocol initially developed for fish fillet extracts [37]. This stationary phase already enabled a separation of matrix components, CTX3C, and more polar CTX analogues in two fractions ('filtrate' and 'eluate', see Section 2.3 and Figure 2 for details). Furthermore, normal phase SPE, which is used for fractionation, provides an orthogonal separation principle compared to the reversed phase column (C18) typically used for LC-MS/MS analysis. The solvents utilized for the normal phase SPE in the initial sample preparation protocol (*n*-hexane/acidified ethyl acetate (1:1, *v*/*v*), acidified ethyl acetate, and acidified ethyl acetate/methanol (3:1, *v*/*v*)) were extended by other solvent mixtures of different polarities (Section 2.4, Figure 3). The three mentioned solvent compositions were considered as 'start', 'middle', and 'end' points of the fractionation, also according to other methods using normal phase SPE for CTX sample preparation (stationary phase Florisil, sample application with 30vol% water saturated ethyl acetate in *n*-hexane or 100% ethyl acetate, final elution with 10 to 25vol% methanol in ethyl acetate, [26,41]).

A detailed method validation could not be performed due to the lack of commercially available reference material. Therefore, optimizing the solvent compositions was conducted using an extract of a naturally contaminated fish fillet (*L. bohar*, previously involved in a CP outbreak [32]) that contains several analogues of the CTX3C group with different polarities (see also Section 3.2). Based on this material, it was discovered that the portion of methanol was an important factor for the elution, and even small vol% of methanol impacted the elution profile. Estevez et al. used Florisil SPE for the sample clean-up. In their approach, the columns were washed with ethyl acetate after sample application and CTXs were found to elute using 10vol% methanol in ethyl acetate [26]. In the method presented here, silica SPE and acidified ethyl acetate were utilized and the elution of CTX analogues was observed for solvent mixtures of lower polarity, starting with CTX3C in *n*-hexane/acidified ethyl acetate (1:3, *v*/*v*). Using acidified ethyl acetate, followed by an elution with 5vol%, 10vol%, and 25vol% methanol in acidified ethyl acetate, resulted in a co-elution of several hydroxylated CTX analogues (an example is provided in Figure S1). Therefore, an additional elution step with 2vol% methanol in acidified ethyl acetate was introduced. For the final method, the volumetric percentage of methanol in acidified ethyl acetate of 2, 5, 10, and 25 (vol%) was used to achieve a better separation of the mono-, di-, and trihydroxyCTX analogues (see corresponding fractions 4 to 7 in Figure 4).

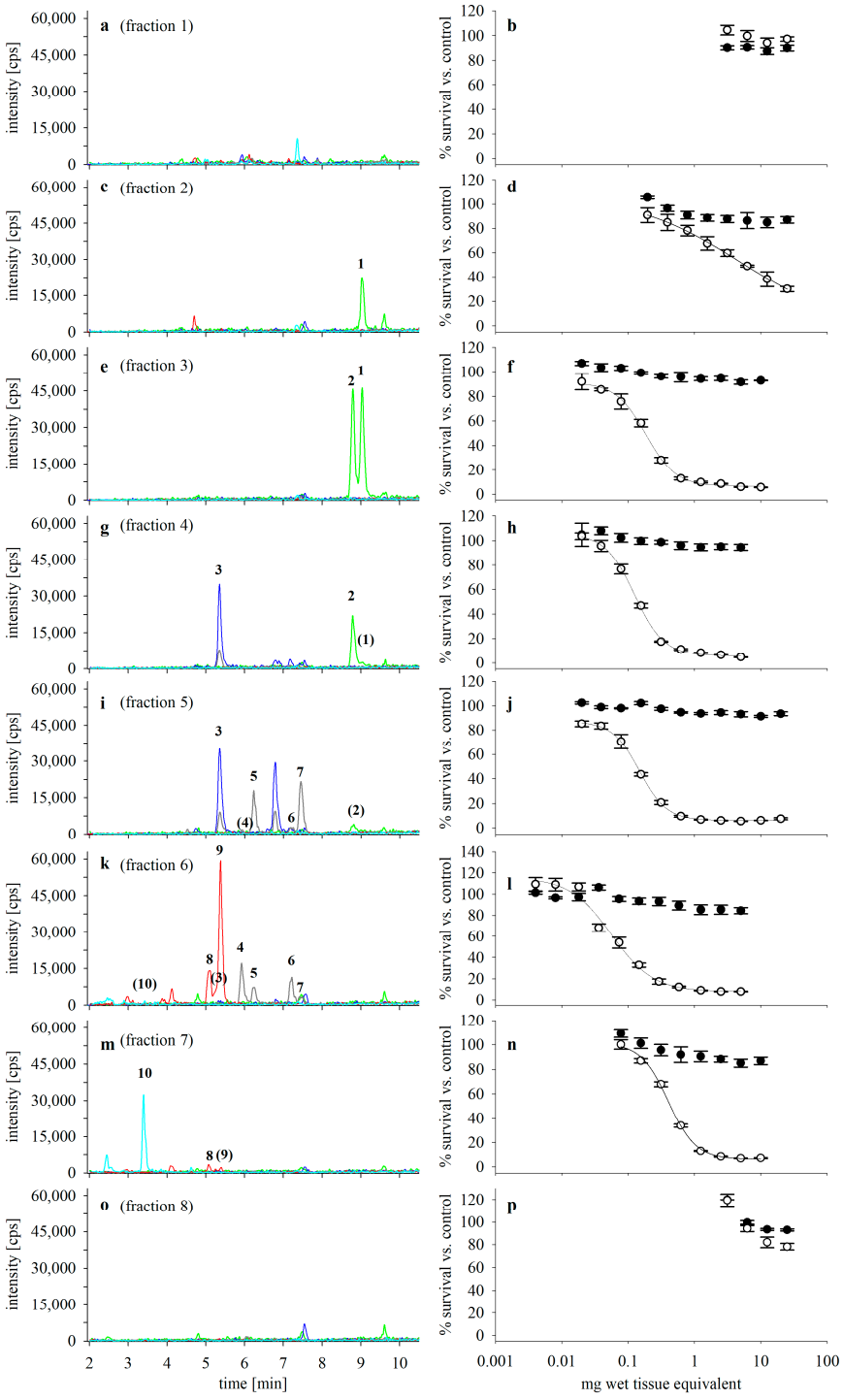

Figure 4. LC-MS/MS chromatograms (left column) and concentration–response curves obtained from the N2a-bioassay (right column) for the analysis of *L. bohar*; panels show (from top to down) chromatograms and curves for fractions 1 to 8; the respective solvent compositions of the fractions are provided in Section 2.4, Figure 3; chromatograms of the non-fractionated filtrate and eluate are provided in Figure S2i,j; the isolated ion traces of the LC-MS/MS chromatograms refer to the sodium adducts ($[M+Na]^+$) of CTX3C (1) and its 49-epimer (2) (green), 51-hydroxyCTX3C (3) (blue), monohydroxyCTX3C #1 (5) and its 49-epimer (4) (grey), monohydroxyCTX3C #2 (7) and its 49-epimer (6) (grey), 2,3-dihydroxyCTX3C (9) and its 49-epimer (8) (red), and 2,3,51-trihydroxyCTX3C (10) (turquoise); peak labelling in () indicates peaks contributing less than 10%

to the total peak area of the analogue; non-highlighted peaks correspond to either matrix compounds or potential unknown CTX analogues, possessing the same low-resolution *m/z* as the target analytes; signals correspond to 5.0 g wet tissue equivalents per mL; concentration–response curves show the results for the incubation without ouabain (O) and veratridine (V) (-OV conditions, solid symbols) and for +OV conditions (open symbols) when exposed to increasing concentrations of fractions 1 to 8; data represent the mean ± standard deviation of multiple microplates with triplicate determination per plate; concentration–response curve of the full extract is provided in Figure S2k.

The fractionation elution profile of CTXs was reproducible and CTX analogues were grouped among fractions according to their different polarities. Individual fractions were found to be suitable for identifying toxic fractions from non-toxic (matrix) fractions using the N2a-bioassay. This supported the peak annotation in the LC-MS/MS analysis, specifically when unknown CTXs are present that have no corresponding standards or reference material (see Sections 3.2 and 3.3). It also aided the performance of the LC-MS/MS analysis through the better separation of analytes and the matrix, allowing an enhanced detection performance for CTXs by reducing matrix interferences (see Section 3.4).

Among eight fractions only, a separation of each CTX analogue was not obtained (Figure 4, see also Sections 3.3 and 3.4). Using the solvent mixtures outlined in Figure 3, congeners were distributed in two or a maximum of three fractions. In the case of an analogue's elution over three fractions, the majority of the analyte was found in two primary fractions, with the third fraction contributing <10% to the total peak area (see following sections). This multi-fraction elution performance was due to the resolution of an SPE column which cannot compete with LC columns. This was the case even when attempts were made to collect smaller volume fractions from the SPE (e.g., 1.5 mL instead of 5 mL, example provided in Figure S1). Additionally, methods optimized for specific CTX analogues (e.g., CTX3C group) might not be suitable for other congeners (e.g., CTX4A or C-CTX group). Therefore, the method developed was designed based on the workflow best suited for analysis by the N2a-bioassay. One microplate for the N2a-bioassay (with triplicate replicates) can accommodate up to eight fractions, enabling a fast and reliable screening for CTX-like toxic fractions.

3.2. Application to Fish Fillet Containing CTX3C Group Analogues

A fish fillet (*L. bohar*) containing several CTX3C group analogues was used for extraction and an aliquot of the extract was fractionated (details about the sample provided in [32]). The non-fractionated filtrate and eluate as well as the fractions were analyzed by LC-MS/MS and tested in the N2a-bioassay to compare CTX contents and composite toxicity (Figure 4, non-fractionated samples shown in Figure S2).

In total, ten potential CTX3C analogues were identified, namely CTX3C (R-configuration at C-49) and its 49-epimer (49-*epi*CTX3C, S-configuration at C-49), 51-hydroxyCTX3C, 2,3-dihydroxyCTX3C and its 49-epimer, 2,3,51-trihydroxyCTX3C, as well as two potential monohydroxyCTX3C analogues (e.g., 2-hydroxyCTX3C, M-*seco*-CTX3C) and their 49-epimers (all analogues of the CTX3C group previously reported in [19], NMR data for some analogues provided in [3,42]). Information concerning peak annotation based on retention times, fragmentation of ammonium adducts, and HRMS data are provided in [11,37]. Individual analogues were not transferred into a single fraction but split up over two or three fractions (Figure 4). Fractions 1 and 8 were negative for CTX-like toxicity and did not reveal any peak of any known CTX analogues (Figure 4a,b,o,p).

In the following, the contents of the respective analogues in the fractionated and non-fractionated samples are discussed. The contents were determined by using a CTX3C standard solution (Section 2.1) as a reference for all analogues, assuming similar ionization efficiencies and matrix effects in the case of LC-MS/MS and the same TEF for all analogues in the N2a-bioassay. Given these limitations, the contents should be carefully considered. For LC-MS/MS, peak areas would be a less biased parameter. However, due to the complexity of the data set provided, contents were chosen for better comprehensiveness.

Furthermore, the calculation of contents allows the comparison of the results obtained by the two analytical methods.

Contents determined by LC-MS/MS before and after fractionation were in the same order of magnitude, indicating no major losses during fractionation (Table 1). The quantitative results showed no improvement of the sample purity in the fractions compared to the non-fractionated extract. The extraction method and clean-up procedure was initially developed for both wet and dried fillet tissue [37]. For the LC-MS/MS-based quantitation of CTXs in a low-fat fillet, the additional sample preparation step using fractionation does not appear reasonable considering the extra workload and required resources.

Table 1. CTX contents determined by LC-MS/MS and semi-quantification of CTX-like toxicity (CTX3C eq.) determined by the N2a-bioassay in a *L. bohar* extract before and after fractionation.

	LC-MS/MS											N2a-Bioassay
Analogue Sample	**2,3,51-Trihydroxy CTX3C (10) [a]**	**49-*epi*-2,3-Dihydroxy CTX3C (8) [a]**	**2,3-Dihydroxy CTX3C (9) [a]**	**49-*epi*-Monohydroxy CTX3C #1 (4) [a]**	**Monohydroxy CTX3C #1 (5) [a]**	**49-*epi*-Monohydroxy CTX3C #2 (6) [a]**	**Monohydroxy CTX3C #2 (7) [a]**	**51-Hydroxy CTX3C (3) [a]**	**49-*epi*CTX3C (2) [a]**	**CTX3C (1) [a]**	**Total [b]**	**µg CTX3C eq. per kg WTE [c]**
Fraction 1												
Fraction 2										0.33	0.33	0.10
Fraction 3									0.79	0.82	1.60	1.58
Fraction 4								0.55	0.38	(0.04)	0.98	2.14
Fraction 5				(0.02)	0.27	0.04	0.41	0.62	(0.07)		1.44	2.00
Fraction 6	(0.01)	0.26	1.03	0.29	0.13	0.20	0.07	(0.02)			2.02	5.00
Fraction 7	0.44	0.05	(0.04)								0.52	0.68
Fraction 8												
sum in all fractions	0.45	0.31	1.07	0.31	0.40	0.24	0.47	1.20	1.24	1.19	6.88	11.50
sum in non-fractionated filtrate and eluate	0.49	0.35	0.84	0.33	0.42	0.28	0.53	1.00	1.39	1.40	7.02	9.68

Contents provided in µg (LC-MS/MS) respective µg CTX3C eq. (N2a-bioassay) per kg wet tissue; contents for LC-MS/MS refer to a single injection; values in () correspond to peaks contributing less than 10% of the total peak area as highlighted in Figure 4; WTE—wet tissue equivalent; [a] numbers refer to peak labelling in Figure 4; [b] calculated with non-rounded values; [c] determined as composite toxicity.

Extract fractionation can aid qualitative CTX analysis in terms of peak annotation. Of the ten compounds detected in this sample, only CTX3C is available as a commercial standard. This hampers the peak annotation of other CTX analogues and can lead to discrepancies between the LC-MS/MS and N2a-bioassay quantitative results. For the non-fractionated extract of *L. bohar*, there was a good agreement between the total CTX content determined by LC-MS/MS and composite toxicity (Table 1), implying that all major compounds were captured by the LC-MS/MS method. For fractions 2 to 7, LC-MS/MS and N2a-bioassay data agreed in the detection of analogues and evidence of CTX-like toxicity in the N2a-bioassay, respectively (Figure 4, Table 1). This agreement supports the peak annotation performed for this sample, despite the lack of available standard compounds.

Four peaks of potential monohydroxyCTX3C analogues were detected (peaks 4 to 7 in Figure 4), which might be attributed to M-*seco*-CTX3C and 2-hydroxyCTX3C and their 49-epimers, which are the only CTX3C group analogues reported to date corresponding to the detected *m/z* [19]. The presence of 49-epimers is supported by the elution profile. As observed for CTX3C and its 49-epimer (peaks 1 and 2 in Figure 4), the main part of the potential 49-epimers (peaks 4 and 6) is eluted in a different fraction compared to the analogues with the potential R-configuration at C-49 (peaks 5 and 7, Figure 4).

For fraction 2, no complete concentration–response curve was obtained despite the detection of CTX3C in that fraction (Figure 4c,d). Within a previous study, the same *L. bohar* sample was extracted with a different protocol and the extract was fractionated by analytical HPLC [32]. Also, in that case, the fractions containing CTX3C revealed almost no toxicity. For the matrix-free CTX3C standard solutions used as reference (Section 2.1), concentration–response curves were obtained, proving the applicability of the bioassay for this CTX analogue. Fraction 3 of the *L. bohar* sample contained CTX3C and its 49-epimer and delivered a full concentration–response curve (Figure 4e,f). For CTX3C in the fraction 2, a concentration of 1.6 µg L^{-1} was determined. This concentration is considered to be sufficient to cause toxicity in the N2a-bioassay (EC_{50} 1.35 ng L^{-1} according to [40]). The

lack of a response in the assay might be due to other matrix compounds masking the effect of CTXs. Such effects might generally limit the applicability of bioassay-guided fractionation methods, independent of the setup. This aspect should be investigated in detail in future studies.

3.3. Application to Fish Tissue Containing C-CTX-1

One sample of *S. barracuda* from the Caribbean Sea was extracted based on the protocol initially developed for the CTX3C and CTX4A group [37] and the sample extract was found to be positive in the N2a-bioassay for CTX-like activity (1.2 µg CTX3C eq. per kg dry TE). The LC-MS/MS analysis of the non-fractionated eluate revealed the presence of potential C-CTX-1 (Figure 5c,d). C-CTX-1 standards or reference material are not commercially available; thus, the retention time could not be directly compared. Based on previous studies using similar LC-MS/MS conditions, C-CTX-1 elutes after CTX1B, but before 54-deoxy-CTX1B and its 52-epimer (52-*epi*-54-deoxyCTX1B) [22], which corresponds with the retention times determined in this study (Figure 5a–d).

After fractionation, the potential C-CTX-1 peak appeared in fractions 5 and 6, with fraction 5 containing the majority of the analogue among the two fractions (Figure 5g–j). Both fractions were positive for CTX-like toxicity in the N2a-bioassay, providing additional evidence for the presence of a CTX analogue (Figure S3). The HRMS analyses confirmed the elemental composition of the potential C-CTX-1 (Table 2). The peak showed both the corresponding sodium and the ammonium adduct in the full scan MS. MS/MS fragmentation led to the loss of several water molecules, further supporting the peak annotation (Table 2). This *S. barracuda* sample demonstrated that the sample extraction, clean-up, and fractionation can be applied to analogues of the C-CTX group. The example of C-CTX-1 shows how the fractionation method combined with LC-MS/MS analyses and the N2a-bioassay could be used for supporting peak assignments in cases where standard material is unavailable.

Table 2. HRMS data for the analysis of C-CTX-1 in *S. barracuda* in both the non-fractionated extract and fraction 5 compared to available CTX analogues in matrix free standard solution.

	Formula		**MS/MS of $[M+NH_4]^+$ [a]**				**Full Scan [a]**	**MS/MS of $[M+Na]^+$ [b]**	**Full Scan [b]**
			$[M+H-3H_2O]^+$	**$[M+H-2H_2O]^+$**	**$[M+H-H_2O]^+$**	**$[M+H]^+$**	**$[M+NH_4]^+$**	**$[M+Na]^+$**	**$[M+Na]^+$**
CTX1B [c]	$C_{60}H_{86}O_{19}$	calculated	1057.5519	1075.5625	1093.5730	1111.5836	1128.6102	1133.5656	1133.5656
		found	1057.5592	1075.5605	1093.5835		1128.6102	1133.5669	1133.5636
		(Δ ppm)	(6.9)	(−1.8)	(9.6)		(0.0)	(1.2)	(−1.7)
54-deoxy CTX1B [c]	$C_{60}H_{86}O_{18}$	calculated	1041.5570	1059.5676	1077.5781	1095.5887	1112.6152	1117.5706	1117.5706
		found		1059.5781	1077.5821	1095.5931	1112.6199	1117.5723	1117.5695
		(Δ ppm)		(9.9)	(3.7)	(4.0)	(4.2)	(1.5)	(−2.0)
CTX3C [c]	$C_{57}H_{82}O_{16}$	calculated	969.5359	987.5464	1005.5570	1023.5676	1040.5941	1045.5495	1045.5495
		found		987.5500 (3.9)	1005.5591	1023.5723	1040.5940	1045.551 (1.4)	1045.5487
		(Δ ppm)			(2.1)	(4.6)	(−0.1)		(−0.8)
C-CTX-1 non-fractionated eluate	$C_{62}H_{92}O_{19}$	calculated	1087.5989	1105.6094	1123.6200	1141.6306	1158.6571	1163.6125	1163.6125
		found	1087.5947	1105.6106	1123.6195		1158.6541	1163.6119	1163.6088
		(Δ ppm)	(−3.8)	(1.1)	(−0.4)		(−2.6)	(−0.5)	(−3.2)
fraction 5		found	1087.6000	1105.6136	1123.6233	1141.6261	1158.6563	1163.6118	1163.6096
		(Δ ppm)	(1.0)	(3.8)	(2.9)	(−3.9)	(−0.7)	(−0.6)	(−2.5)

[a] temperature of ESI-source set at 150 °C, [b] temperature of ESI-source set at 500 °C, [c] determined for a standard solution in methanol with 1 µg L^{-1} for CTX1B and 54-deoxyCTX1B, and 2 µg L^{-1} for CTX3C (Section 2.1); analyses were conducted using methanol as organic eluent.

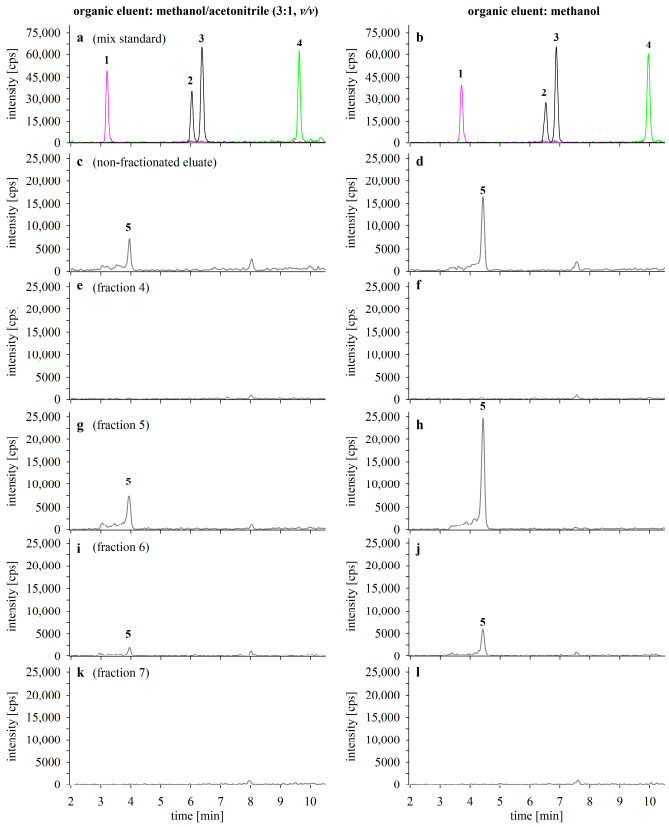

Figure 5. LC-MS/MS chromatograms for the analysis of *S. barracuda*; panels show chromatograms of (**a**,**b**) a mixed standard solution (**c**,**d**) the non-fractionated eluate (**e**,**f**) fraction 4, (**g**,**h**) fraction 5, (**i**,**j**) fraction 6, and (**k**,**l**) fraction 7 for methanol/acetonitrile (3:1, *v*/*v*) (left column) and methanol as organic eluent (right column); fractions 1–3 and 8 did not reveal any CTX peaks; the isolated ion traces refer to the sodium adducts ($[M+Na]^+$) of CTX1B (1) (pink), 54-deoxyCTX1B (3) and its 52-epimer (2) (black), CTX3C (4) (green), and C-CTX-1 (5) (grey); for chromatograms of the fish sample, the signals correspond to 2.0 g dry TE per mL; concentrations of the standards were 1 µg L^{-1} for CTX1B, 54-deoxyCTX1B and its 52-epimer, and 2 µg L^{-1} for CTX3C (Section 2.1); concentration–response curves are provided in Figure S3.

Fraction 5 showed a toxin equivalent close to the non-fractionated sample (0.8 µg CTX3C eq. per kg compared to 1.2 µg CTX3C eq. per kg), which corresponds to the distribution of the C-CTX-1 peak determined by LC-MS/MS (Figure 5). For fraction 6, no full concentration–response curve was obtained and no EC_{50} could be determined (Figure S3f). As discussed for the *L. bohar* sample, other compounds might be present that lower the impact of CTX(s) on the cells in the assay, preventing a reduction in cell viability. Traces of CTX-like toxicity were also found in fraction 4 (Figure S3d). Some parts of C-CTX-1 might have been transferred into this fraction as well, or other analogues might contribute to the composite toxicity. However, the fraction containing the majority of C-CTX-1 and contributing the most to the observed composite toxicity was fraction 5.

In the chromatogram of C-CTX-1, front peak broadening was observed close to the retention time of the CTX analogue. The shape of the peak form remained unchanged after fractionation (Figure 5c,g). As fractionation is supposed to reduce matrix effects (see also Section 3.4), it appears unlikely that impurities caused the broadening of this observed peak. Broad peaks of C-CTX-1 have been previously described by other groups [43–45]. The fast epimerization of C-CTX-1 and its 56-epimer (C-CTX-2) is considered as a reason, and could be overcome by derivatization techniques such as reductive animation [43,44].

The form of the C-CTX-1 peak was reported to be dependent on eluent additives (see Figures S5 and S6 in [43]). The initial LC-MS/MS method uses methanol/acetonitrile

(3:1 *v*/*v*) as the organic mobile phase; therefore, methanol was tested as an alternative. Methanol resulted in a slightly reduced front peak broadening, but most notably, it enhanced the signal intensity by a factor of ~2–3 (e.g., Figure 5g,h). This effect was not observed for the standards of the CTX4A group and CTX3C (Figure 5a,b). Apparently, the analogue groups are impaired by the eluent composition in different ways. This has a major impact on the quantitation of C-CTX-1 using LC-MS/MS, if other analogues (e.g., CTX1B) are used as a reference. Depending on the eluent composition, remarkably different C-CTX-1 contents might be reported even for the same extract analyzed on the same instrument. Due to the lack of standards of the C-CTX group, the extent to which this might be relevant to the entire analogue group has not been investigated. The influence of the eluent composition might be specific for the analytical instrument used in this study and might not be relevant for other LC-MS systems. This stresses the importance of method comparison studies in the field of CTX analyses to clarify fundamental analysis points and to enable the comparability of quantitative results reported.

3.4. Fractionation of Fish Fillet and Viscera—Impact on Quantitation

Samples of *E. canina* (dried viscera and dried fillet tissue) were extracted and fractionated. Both tissue types contained CTX analogues of the CTX3C and CTX4A groups. In total, nine different analogues were identified, including CTX3C and its 49-epimer, 51-hydroxyCTX3C, 2,3-dihydroxyCTX3C and its 49-epimer, 2,3,51-trihydroxyCTX3C, 54-deoxyCTX1B and its 52-epimer, and CTX1B. The viscera and fillet extracts contained the same analogues; however, only the viscera contained 2,3,51-trihydroxyCTX3C (see discussion below).

The investigated samples of *L. bohar* (origin Vietnam) and *E. canina* (origin Hawai'i, USA) were found to share several analogues. For both fish, under the same fractionation conditions, CTX analogues were eluted consistently with regard to the fraction number and their distribution among fractions, demonstrating method reproducibility for consistent analyte performance among matrices (i.e., species) and variable water content conditions (wet and dry tissue sources).

In the following, the contents of the respective analogues in the fractionated and non-fractionated samples are discussed. LC-MS/MS quantitation was performed using the CTX3C standard for the CTX3C group and CTX1B for the CTX4A group (Section 2.1), with the assumption of comparable ionization efficiencies and responses for all compounds of the respective groups. Therefore, the data provided in Table 3 should be considered conservatively. Several peaks failed to reveal a signal-to-noise ratio above 9, which would correspond to the LOQ. Nevertheless, contents instead of peak areas are provided for all samples, to allow the comparison for the estimated impact of the fractionation on the quantitation. In order to provide an impression on the peak intensity, LC-MS/MS chromatograms of the non-fractionated eluate as well as the fractions 2 to 7 are shown in Figure 6; chromatograms of all fractions are provided in Figure S4.

Table 3. CTX contents determined by LC-MS/MS and semi-quantification of CTX-like toxicity (CTX3C eq.) determined by the N2a-bioassay (last column) in freeze-dried *E. canina* depending on tissue type (fillet vs. viscera) and extract clean-up (full/non-fractionated extracts vs. fractions).

	LC-MS/MS										N2a-Bioassay
Analogue Sample	**CTX1B (8) [a]**	**52-*epi*-54-deoxy CTX1B (5) [a]**	**54-deoxy-CTX1B (4) [a]**	**2,3,51-trihydroxy CTX3C (9) [a]**	**49-*epi*-2,3-dihydroxy CTX3C (7) [a]**	**2,3-dihydroxy CTX3C (6) [a]**	**51-hydroxy CTX3C (3) [a]**	**49-*epi*CTX3C (2) [a]**	**CTX3C (1) [a]**	**Total**	**µg CTX3C eq. per kg DTE [b]**
non-fractionated [c] (fillet)	0.09	0.10	0.09	ND	0.13	0.10	0.19	0.63	0.39	1.7	3.3
Fractions [d] (fillet)	0.11	0.13	0.11	ND	0.15	0.14	0.25	0.75	0.56	2.2	3.1
Increase [e] [%]	31	29	18		16	33	32	18	44	28	
non-fractionated [c] (viscera)	0.43	0.55	0.35	0.26	1.33	1.23	0.61	2.14	1.36	8.3	34.1
Fractions [d] (viscera)	0.95	0.76	0.69	0.37	1.70	1.77	1.51	2.79	2.14	12.7	15.5
Increase [e] [%]	123	39	96	43	28	44	147	31	58	53	
ratio viscera:fillet (non-fractionated [c])	4.9	5.6	3.8		10.5	11.7	3.3	3.4	3.5	4.8	10.2
ratio viscera:fillet (fractions)	8.4	6.0	6.2		11.5	12.8	6.1	3.7	3.9	5.8	5.1

Contents provided in µg (LC-MS/MS) respective µg CTX3C eq. (N2a-bioassay) per kg dry tissue; contents for LC-MS/MS were determined for single injection; ND—not detected, DTE—dry tissue equivalents; [a] numbers refer to peak labelling in Figure 6; [b] determined as composite toxicity; [c] either non-fractionated filtrate and eluate analyzed separately (LC-MS/MS) or combined as full extract (N2a-bioassay); [d] for LC-MS/MS analyses, contents refer to the sum of all fractions in which the analogue was detected; [e] refers to LC-MS/MS analyses only; calculations were performed with non-rounded values.

The dried fillet sample showed a low baseline in the non-fractionated eluate and the peaks of the analogues present in the sample could be identified (Figure 6b). Fractionation resulted in a moderate increase in the analogue's contents (from 16 to 44%, Table 3). This might be due to the removal of matrix constituents which lowered the analyte ionization in the non-fractionated extract. The increase was comparable for all analogues (average 28%, median 30%) and no shift in the CTX profile was observed (Figure S5).

The non-fractionated eluate of the viscera showed remarkably higher baselines in the LC-MS/MS chromatogram than the fillet sample (Figure 6a,b), although less tissue was utilized for extraction (0.50 g viscera compared to 1.00 g fillet). Therefore, the applied clean-up protocol [37] may be less efficient for more complex matrices such as viscera where the co-eluting matrix possesses the same (low resolution) *m/z* as the investigated compounds. Nevertheless, peaks were detected in the non-fractionated extract, making the extraction protocol generally suitable for viscera type samples.

Fractionation reduced baseline intensities for the viscera extract, leading to an improved signal-to-noise ratio (Figure 6, left column) and higher contents in the fractions compared to the non-fractionated sample (Table 3). The analogues' contents increased by between 28 to 147% (average 68%, median 44%), indicating differences between the viscera and fillet tissue sample sets. For fillet, similar changes were found for all analogues (see above). The highest increase was observed for 51-hydroxyCTX3C, which showed a low peak and high baseline intensity in the non-fractionated eluate (Figure 6a,g,i). For smaller peaks, reducing the baseline intensity can have a higher impact on the absolute peak area and consequently on the content, if no matrix calibration is used. These results illustrate a problem for comparing data among published studies. Without analytical standards, aspects such as sample preparation methods, extract purity, and analytical instruments should be considered when comparing CTX contents among studies.

For both the individual analogues and the sum of all detected compounds, the contents of all CTXs determined by LC-MS/MS were five- to six-fold higher in the viscera than in the fillet (Table 3), emphasizing the outsized CP risk related to consuming viscera. Other studies analyzing the distribution of CTXs within a single animal reported up to fifty-fold differences between viscera and muscle tissue [46,47]. In Hawai'i (collection site of *E. canina*), viscera CTX contents were found, on average, to be fifty-seven times higher than in the muscle tissue (range 0.8 to 436) [33]. In the Canary Islands, three to fifty-one times higher CTX contents in liver than in the fillet were reported [23]. Data of the cited studies are reported on a wet weight basis (compared to dry weight basis here) and quantitative data were obtained by CBAs. Those methods are often less prone to matrix effects compared to LC-MS/MS methods, i.e., a higher TE can be applied per well in the N2a-bioassay (e.g., study in [48]) than that injected for LC-MS/MS analysis (see Figure 8 in [24]). Further-

more, they determine a composite toxicity, also covering low concentrated and unknown CTX analogues, which might not be covered by LC-MS/MS. Both aspects can contribute to higher CTX contents in the viscera determined by CBAs compared to LC-MS/MS analysis and, consequently, to a higher ratio of the contents in viscera and fillet for CBA methods.

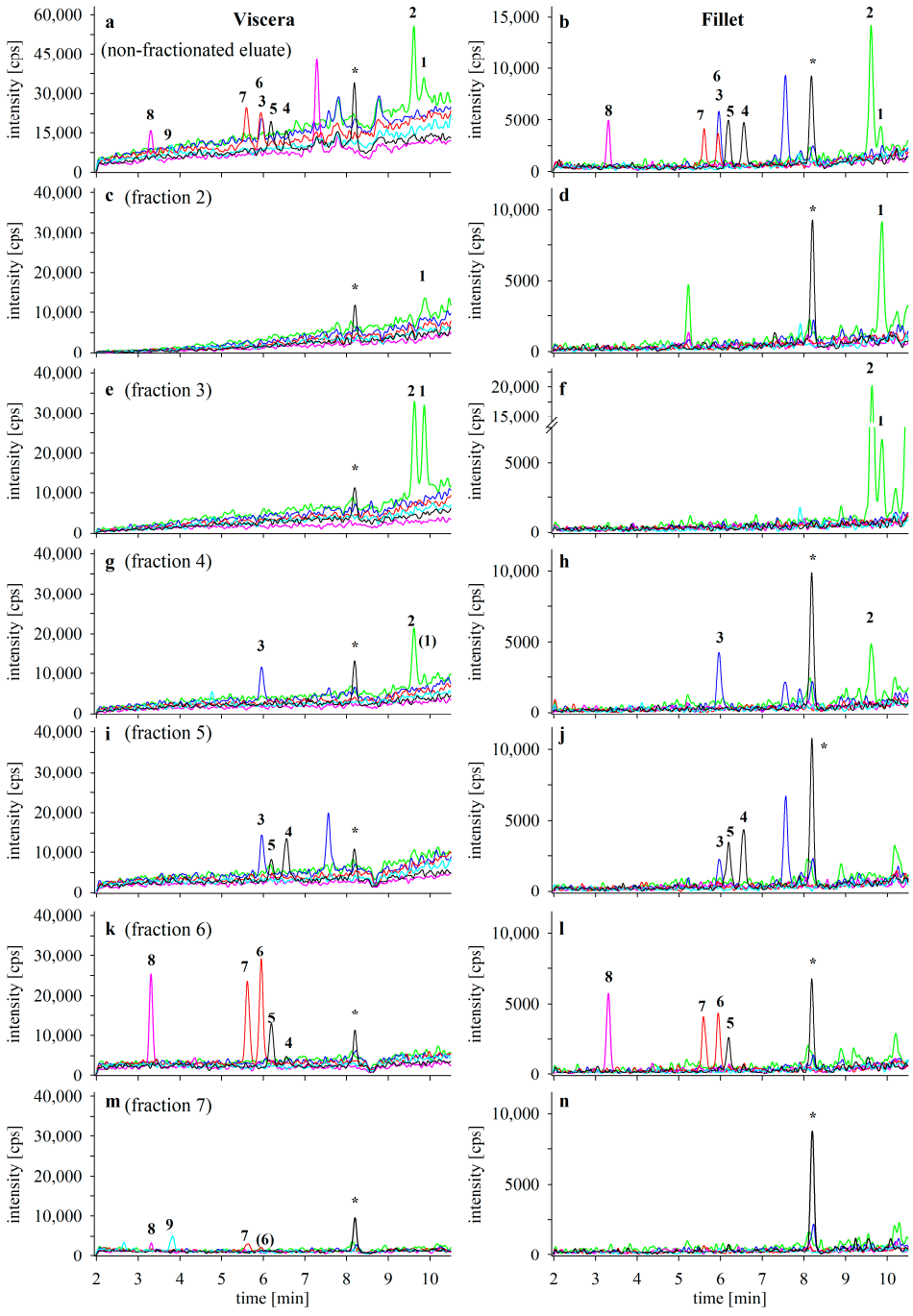

Figure 6. LC-MS/MS chromatograms for the analysis of *E. canina*; panels show chromatograms of (**a**,**b**) the non-fractionated eluate (**c**,**d**) fraction 2, (**e**,**f**) fraction 3, (**g**,**h**) fraction 4, (**i**,**j**) fraction 5, (**k**,**l**) fraction 6, and (**m**,**n**) fraction 7 for the viscera (left column) and fillet sample (right column); fractions 1 and 8 did not reveal any CTX peaks; chromatograms of these fractions are provided in Figure S4; the isolated ion traces refer to the sodium adducts ($[M+Na]^+$) of CTX3C (1) and its 49-epimer (2) (green), 51-hydroxyCTX3C (3) (blue), 54-deoxyCTX1B (4) and its 52-epimer (5) (black), 2,3-dihydroxyCTX3C (6) and its 49-epimer (7) (red), CTX1B (8) (pink), and 2,3,51-trihydroxyCTX3C

(9) (turquoise); peak labelling in () indicates peaks contributing less than 10% to the total peak area of the analogue; *—matrix interference; non-highlighted peaks correspond to either matrix compounds or potential unknown CTX analogues, possessing the same low-resolution *m/z* as the target analytes; reader is referred to the different scaling of the y-axis and the different net weights initially used for sample preparation (0.5 g for viscera, 1.0 g for fillet); for all viscera and fillet samples, the signals correspond to 0.5 and 1.0 g dry TE per mL, respectively.

To support this assumption, the same extracts and fractions were analyzed by the N2a-bioassay and the results are expressed as µg CTX3C eq. per kg DTE (Table 3, Figure S6). For the fillet sample, the LC-MS/MS and N2a-bioassay results show a good agreement with contents around 2 µg per kg for the LC-MS/MS analysis and 3 µg CTX3C eq. per kg DTE for the N2a-bioassay. The observed difference might be due to matrix effects, low concentrated analogues, and/or unknown analogues not covered by the LC-MS/MS-method. The lack of analytical standards for each analogue adds further uncertainties to the quantitative analysis. However, the results suggest that the major toxins were covered by the LC-MS/MS method. The reason for the low CTX content ratio in the viscera and fillet determined by LC-MS/MS was apparently derived from the viscera analysis (i.e., low toxin content determined in the viscera due to matrix effects).

Comparing the N2a-bioassay results for non-fractionated samples of viscera and fillet, a ten times higher toxicity was found in the viscera (Table 3). This corresponds to the findings reported in the literature, as discussed above [23,46,47]. The non-fractionated full extract of the viscera revealed a four-fold higher toxicity in the N2a-bioassay compared to LC-MS/MS. Due to the good agreement of the results obtained by both analytical methods for the fillet sample, it appears less likely that several unknown analogues contribute remarkably to this higher toxicity in the viscera. Rather, it implies that matrix interferences influence the quantitation by LC-MS/MS. Consequently, the variability among both methods was reduced following fractionation (Table 3). The results show that in the situation of a high matrix load sample, such as extracts of viscera, fractionation can improve the comparability of results between LC-MS/MS and the N2a-bioassay, through an improved detection of CTX analogues in the fractions by LC-MS/MS. Furthermore, if extracts are analyzed by both methods and a high difference between the results is found, fractionation could provide hints as to what might cause the discrepancy, for example, whether the target method is sufficient at covering all the relevant analogues.

Comparing the fractions made from the viscera and fillet samples, the viscera contained a five-fold higher toxicity compared to the fillet when tested by the N2a-bioassay. This decrease in the ratio of toxicity for the viscera vs. fillet from the fractions (5x) compared to the non-fractionated samples (10x), is mainly due to the lower composite toxicity of the viscera sample (Table 3). Fraction 2 did not deliver a full concentration–response curve for the viscera and the fillet produced no observed toxicity (Figure S6e,f), although CTX3C was detected in these samples by LC-MS/MS (0.3 µg L^{-1}) (Figure 6c,d). As discussed for *L. bohar* (Section 3.2), CTX3C seems to co-elute with compounds masking the effect of CTX in the bioassay. The semi-quantitative aspect of the N2a-bioassay with the determination of concentration–response curves should be considered as well as the potential propagation of errors (e.g., if the results of a large number of fractions are compared with the result of one non-fractionated sample).

For the fractionated samples, CTX analogues and their respective epimers (e.g., CTX3C and 49-*epi*CTX3C) showed a similar ratio for the contents in viscera and fillet (Table 3). This implies that in the fish, there is a similar distribution of both configuration isomers. However, among fillet and viscera tissue types, the CTX profiles differed, with the viscera containing a higher share of higher oxidized CTX3C group analogues (Figure S5). In contrast to the fillet, the analogue 2,3,51-trihydroxyCTX3C was found in the viscera. This analogue might be present in the fillet as well, but in amounts too low for detection by LC-MS/MS. This compound has been reported from fillet samples, demonstrating that it can be transferred from viscera to this tissue type [11,17]. Viscera also possessed a higher share of 2,3-dihydroxyCTX3C and its 49-epimer than the fillet. For these two compounds,

the highest difference between viscera and fillet was observed (approx. factor 12, Table 3). In a feeding study, Li et al. found different CTX profiles in feed and among several tissue samples of the orange-spotted grouper (*Epinephelus coioides*) [49], identifying the analogues CTX1B and 54-deoxyCTX1B and its 52-epimer. Among all tissue types tested (e.g., muscle, brain, skin, liver), the highest share of the higher oxidized analogue CTX1B was found in the liver, whereas the source feed showed the lowest share of CTX1B [49]. CTXs are mainly biotransformed by oxidation. The reaction is catalyzed by enzymes, e.g., in the liver, as shown in in vitro studies with S9 mixes obtained from different fish species [18]. In feeding studies, most of the CTX body burden was found in the viscera [46] and skin [49] at the beginning of the experiment. With ongoing incubation, CTXs were transferred to the muscle and liver tissue. The differences between the viscera and fillet observed in this study are thus in agreement with results from the literature. The sample investigated here had naturally incurred CTXs, i.e., it was not part of a controlled feeding study. Therefore, whether the profiles found in the viscera and fillet would become more similar due to the transfer of the analogues from the viscera into the fillet over time could not be elucidated.

3.5. Application for the Fractionation Method

This fractionation method demonstrated the separation of co-occurring CTX analogues from a single sample with a low effort concerning materials and time. The approach also enabled the partial separation of epimers, as demonstrated for CTX3C and its 49-epimer (fraction 2 vs. fraction 3, Figures 4 and 6), and the potential monohydroxy-CTX3C analogues (fraction 5 vs. fraction 6, Figure 4). If a distinct separation of two analogues is intended, then the method could be easily modified by using other solvent compositions or through a repeated application of the fraction of interest, using the same solvent setup. For example, the repeated fractionation of fraction 5 might separate 51-hydroxyCTX3C from the other analogues present in the specific fraction (Figures 4 and 6).

The fractionation is conducted using a normal phase SPE, whereas analyses are performed on a reversed phase LC column. This approach of orthogonal separation mechanisms enhances the chance that analytes and co-eluting matrix constituents are separated, as shown for the viscera sample of *E. canina*. In addition, a separation of co-eluting analytes might be achieved. The analogue 51-hydroxyCTX3C shares the same retention time with 2,3-dihydroxyCTX3C, but with fractionation, it was eluted earlier from the SPE cartridge by two fractions (Figures 4 and 6).

In cases where reference standards are lacking, several analytical techniques are available to provide evidence of whether a peak in the LC-MS/MS chromatogram could be attributed to a CTX analogue (HRMS, detection of selective MS/MS fragments). These instrumental analyses are ideally accompanied by functional assays (e.g., N2a-bioassay) to test for CTX-like specific activity/toxicity. For this task, the best option would be fractionation, using the same setup that is used for the sample analysis. Identified toxic fractions would directly correspond to the elution time window of the analytical run, providing valuable information on the retention time of the potential CTX compound(s). Due to the limited capacity of analytical LC columns, this approach can be time-consuming, particularly if several injections are needed to obtain a comparable amount of material to the SPE fractionation method for the N2a-bioassay (e.g., 25 injections with 10 µL injection volume). The usage of (semi-)preparative LC columns and systems allow the separation of a higher matrix load and automatic fraction collection, but requires the user to have access to these systems. Fractionation by SPE could overcome some of these issues as the cartridges can deal with a higher matrix load than analytical columns and this approach does not depend on specific (expensive) instrumentation. The different approaches could also be complementary. Fractionation by SPE could be utilized to reduce the matrix and to narrow down the number of relevant fractions. Afterwards, a fractionation using the analytical LC setup could be conducted to identify the relevant retention time window of the analogue(s). Due to the reduced matrix load in the sample, higher injection volumes might be utilized.

4. Conclusions

A method using SPE for the fractionation of fish extracts containing CTXs has been developed. The method's applicability was demonstrated among different matrices and CTX analogues, analyzed by both LC-MS/MS and the N2a-bioassay. A final method validation should be performed when reference materials become available. The combination of extract fractionation with dual detection using LC-MS/MS analysis and the N2a-bioassay can support peak assignment(s), as shown for C-CTX-1. The usage of orthogonal separation mechanisms (normal phase (silica) SPE for fractionation; reversed phase (C18) for LC-MS/MS analysis) enables a fast fractionation and separation of analogues as well as analogues and matrix constituents. Reduced matrix interference can enhance LC-MS/MS analyses detection, particularly in complex samples, and can improve the correlation between LC-MS/MS and N2a-bioassay analyses results. High tissue loads can be applied to SPE cartridges, overcoming the limitations of fractionation via analytical LC systems. This method can be applied as an easy 'first-step approach' for compound isolation, to aid in efforts for further structure elucidation or analogue confirmation. Current guidance values for CTXs in seafood products focus only on the historically recognized C-CTX-1 and CTX1B analogues; however, new evidence from CP outbreaks confirm that multiple CTX analogues can be present in a sample. The method demonstrated herein provides researchers and seafood safety regulators with a fast and simple method capable of separating CTX analogues, especially from complex tissue matrices, regardless of the tissue water content or CTX-analogue, enabling a true CTX profile identification.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11080238/s1, Section S1: DNA barcoding; Section S2: Sample pretreatment for fractionation; Figure S1: Example for SPE fractionation method development using an extract of *L. bohar* containing several CTX3C group analogues; Figure S2: LC-MS/MS chromatograms and concentration–response curve for the analysis of *L. bohar*; Figure S3: Concentration–response curves obtained from the N2a-bioassay for the analysis of *S. barracuda*; Figure S4: LC-MS/MS chromatograms for the analysis of *E. canina*; panels show chromatograms of fractions 1 to 8 for the viscera and the fillet sample; Figure S5. Pie charts representing CTX profiles of *E. canina* determined for the fillet and viscera sample, both without and with fractionation; Figure S6: Concentration–response curves obtained from the N2a-bioassay for the analysis of *E. canina*.

Author Contributions: Conceptualization, A.S. and C.R.L.; methodology, A.S. and C.R.L.; formal analysis and investigation, A.S., C.R.L. and V.B.; writing—original draft preparation, A.S.; writing—review and editing, A.S., C.R.L. and V.B.; visualization, A.S. and C.R.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author/s.

Conflicts of Interest: The authors declare no conflicts of interest.

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