

Supplementary Information

Fish extract fractionation by solid phase extraction: Investigating co-occurring ciguatoxins by LC-MS/MS and N2a-bioassay

Astrid Spielmeyer*, Vincent Blaschke, Christopher R. Loeffler

*corresponding author: astrid.spielmeyer@bfr.bund.de

S1. DNA barcoding

Amplification of the cytochrome *b* barcoding region was conducted using the primers L14735 (5'-AAAAACCACCGTTGTTATTCAACTA-3') and H15149ad (5'-GCICCTCARAATGAYATTTGTCCTCA-3'). Reactions were performed in a Master cycler gradient cycler (Eppendorf, Hamburg, Germany) within 25 µL reaction tubes. Amplicons were sequenced by Eurofins Genomics (Ebersberg, Germany). Obtained sequences were blasted against the genetic sequence database GenBank® of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

S2. Sample pretreatment for fractionation

Different sample pretreatments were tested for fractionation and found suitable for the protocol outlined in Section 2.4. and Figure 3.

If extracts were prepared according to Section 2.3., equal aliquots of the non-fractionated filtrate and eluate were combined and evaporated to dryness. Results presented in this publication were obtained by this approach.

Alternatively, eluates of the reversed phase SPE (Section 2.3., Figure 2) can be evaporated to dryness. The dried residue can be fractionated as described in Section 2.4. (Figure 3), replacing the normal phase SPE step mentioned in Section 2.3. (Figure 2).

If extracts are obtained by other sample preparation methods, an aliquot of these extracts can be reduced to dryness. Further treatment will be conducted as described in Section 2.4.

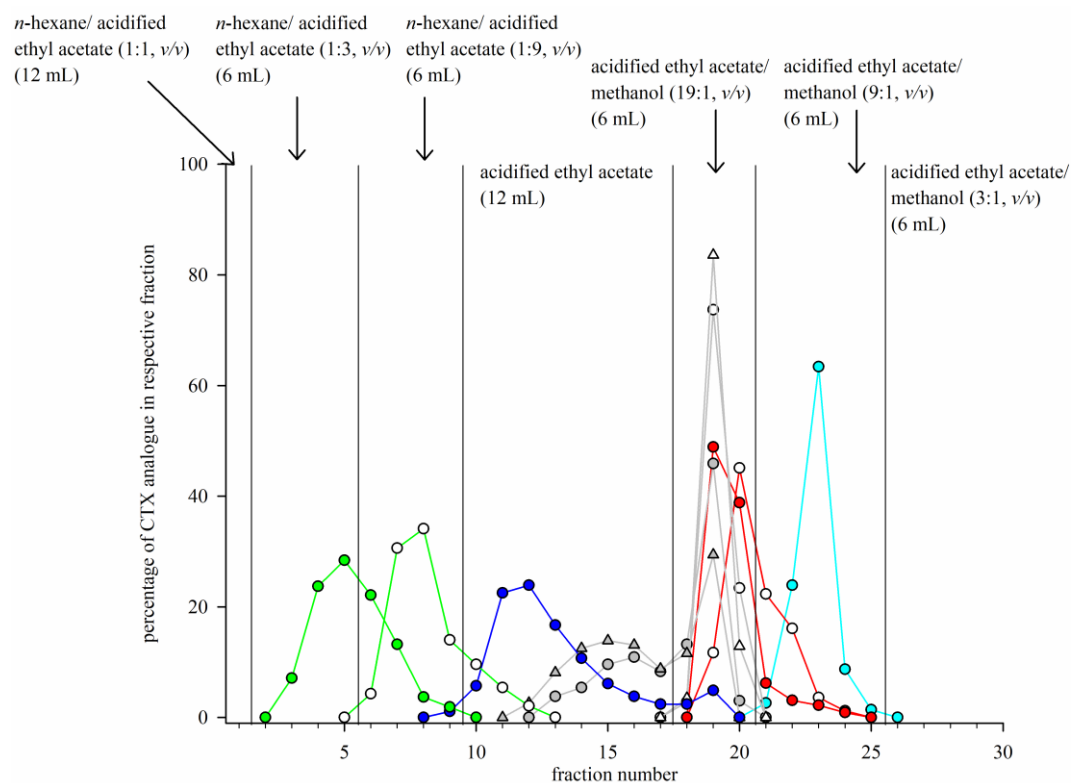


Figure S1. Example for SPE fractionation method development using an extract of *L. bohar* containing several CTX3C-group analogues; with exception of fraction 1, fractions of 1.5 mL were collected, all fractions were analyzed by LC-MS/MS; data points refer to percentage of the respective CTX analogue in the fraction compared to the total peak area of this analogue in all fractions; first and last data point of each graph refer to peak area of 0 (<LOD); data points represent CTX analogues (solid symbols) and their respective 49-epimer (open symbols) for CTX3C (green), 51-hydroxyCTX3C (blue), monohydroxyCTX3C #1 (grey, circle), monohydroxyCTX3C #2 (grey, triangle), 2,3-dihydroxyCTX3C (red) and 2,3,51-trihydroxyCTX3C (turquoise); the first fraction included the sample application (4 mL), sample vessel wash (2 mL) and additional 6 mL elution volume.

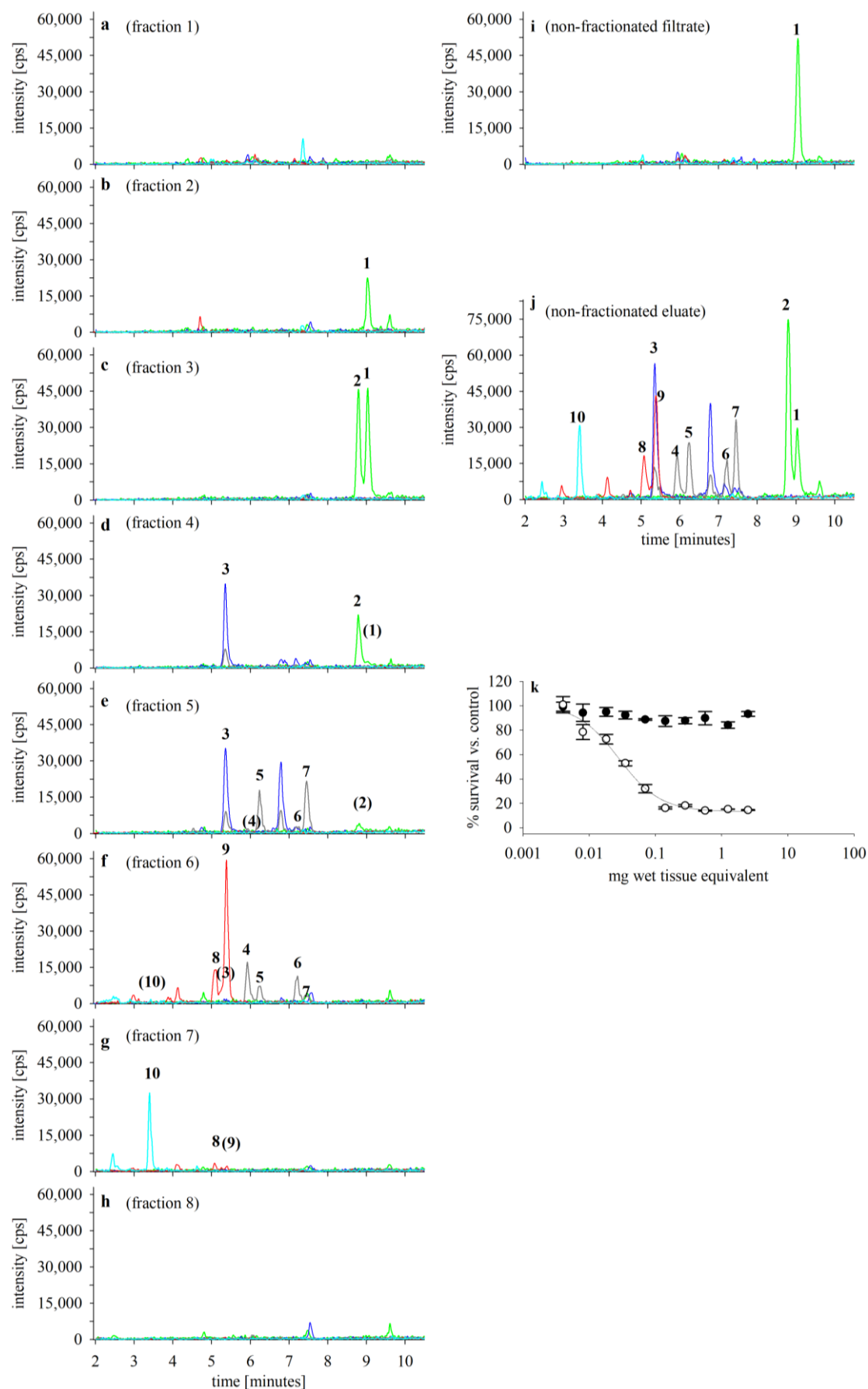


Figure S2. LC-MS/MS chromatograms and concentration-response-curve for the analysis of *L. bohar*; panels show chromatograms for (a-h) fractions 1 to 8, (i) non-fractionated filtrate, and (j) non-fractionated eluate; the respective solvent compositions of the fractions are provided in Section 2.4., Figure 3; the size of the panel of the non-fractionated eluate was adjusted to provide the same scale as for the other panels; 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; panel **(k)** shows the concentration-response-curve for the non-fractionated sample, applied as combination of eluate and filtrate; data points show the results for the incubation for -OV (solid symbols) and +OV conditions (open symbols); error bars show the standard deviation for a triplicate determination per plate.

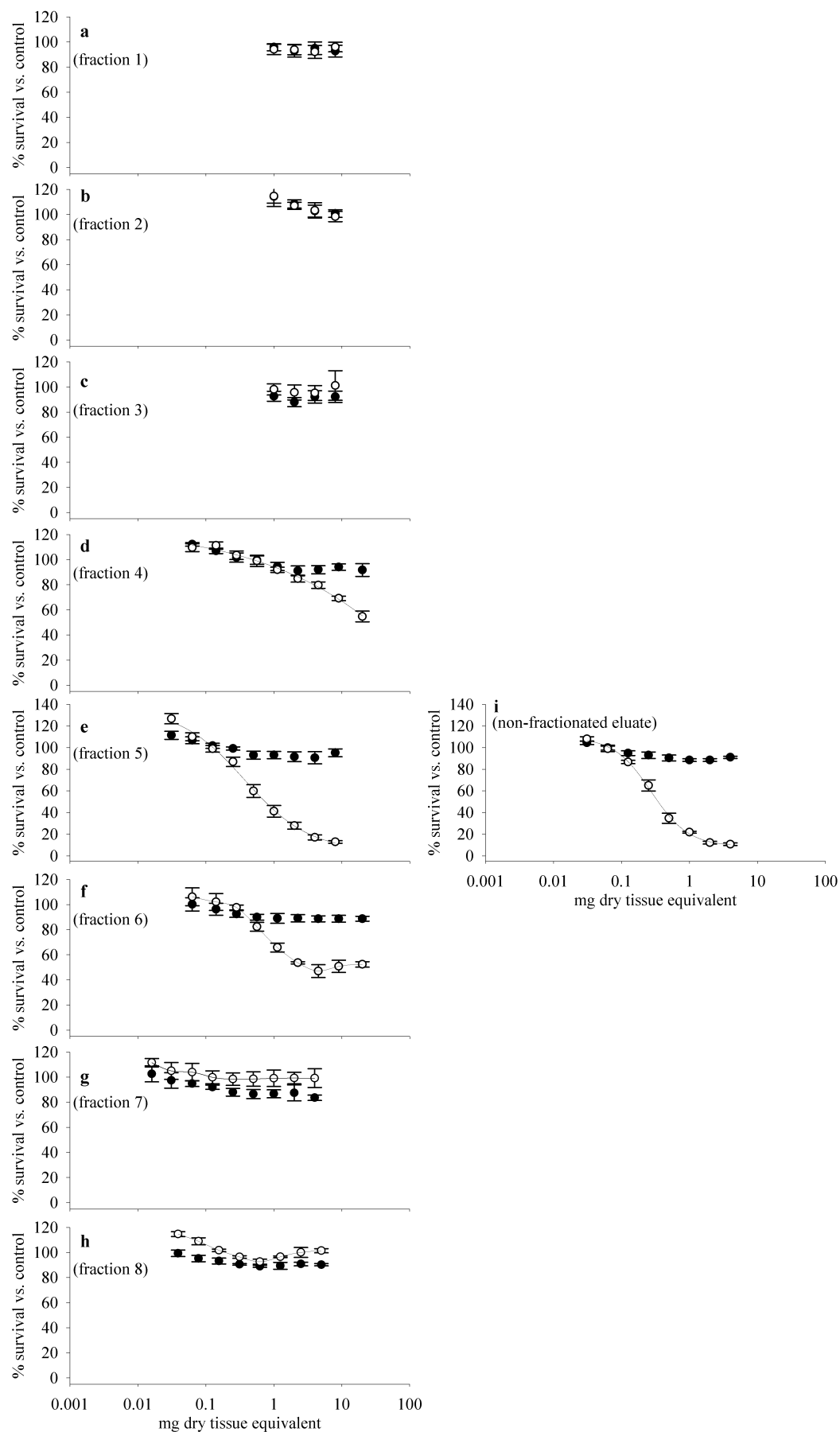


Figure S3. Concentration-response-curves obtained from the N2a-bioassay for the analysis of *S. barracuda*; panels show curves for the (a-h) fractions 1 to 8 and (i) non-fractionated eluate; the respective solvent compositions of the fractions are provided in Section 2.4., Figure 3; data points show the results for the -OV (solid symbol) and +OV conditions (open symbol); data represent the mean \pm standard deviation of multiple microplates with triplicate determination per plate.

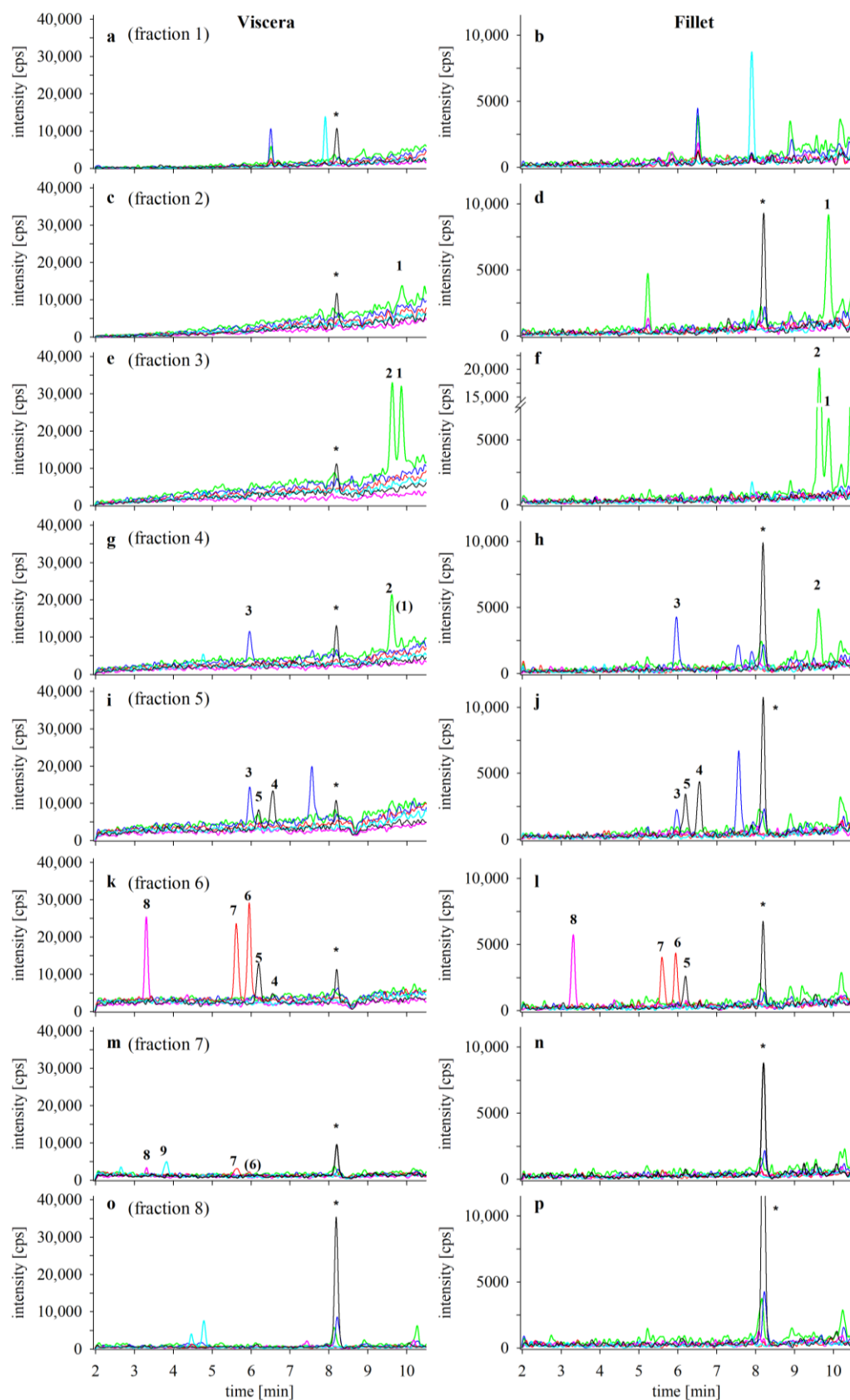


Figure S4. LC-MS/MS chromatograms for the analysis of *E. canina*; panels show chromatograms of fractions 1 to 8 for the viscera (left column) and the fillet sample (right column); the isolated ion traces refer to the sodium adducts ($[M+Na]^+$) of CTX3C (1) and its 49-epimer (2) (green), 51-hydroxyCTX3C (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 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 tissue equivalents per mL.

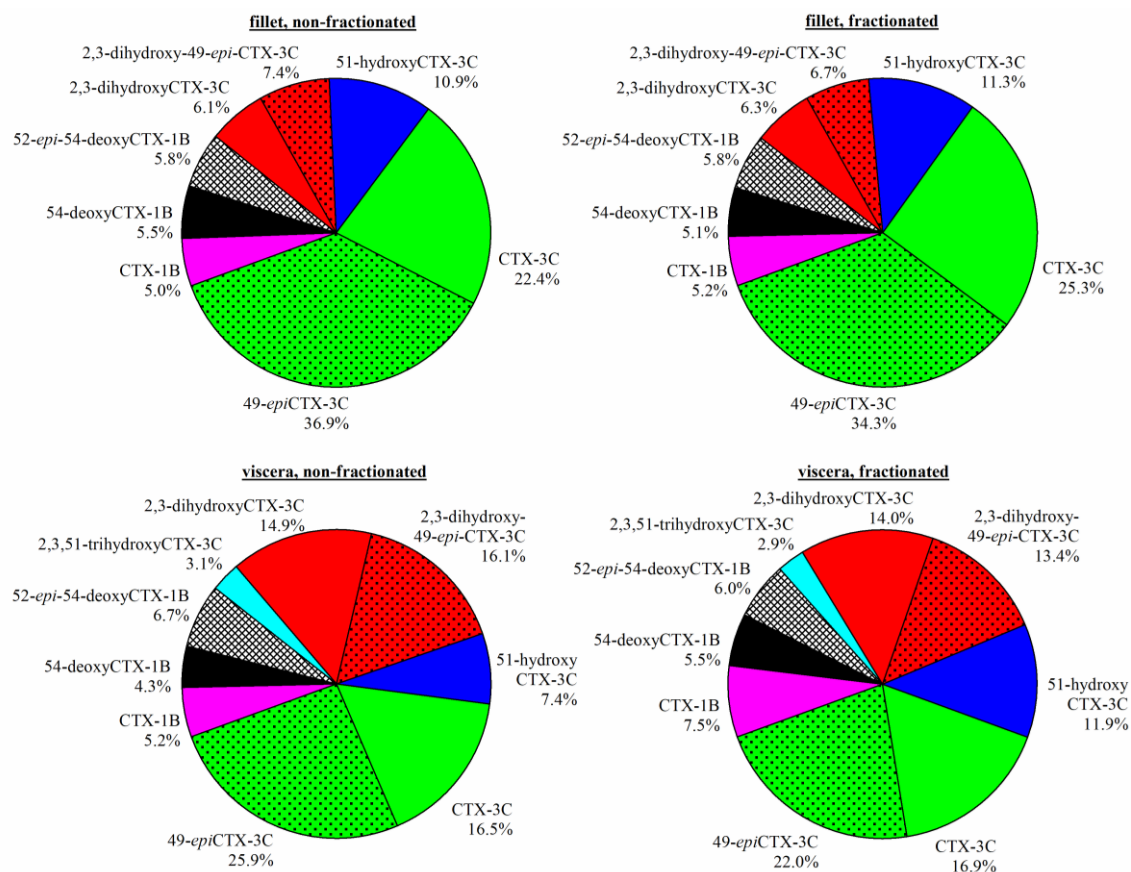


Figure S5. Pie charts representing CTX profiles of *E. canina* determined for the fillet (upper figures) and viscera sample (lower figures), both without (left) and with fractionation (right); segment colors refer to the color code of Figure 6; data are the result of a single determination; discrepancies from 100% are due to rounding.

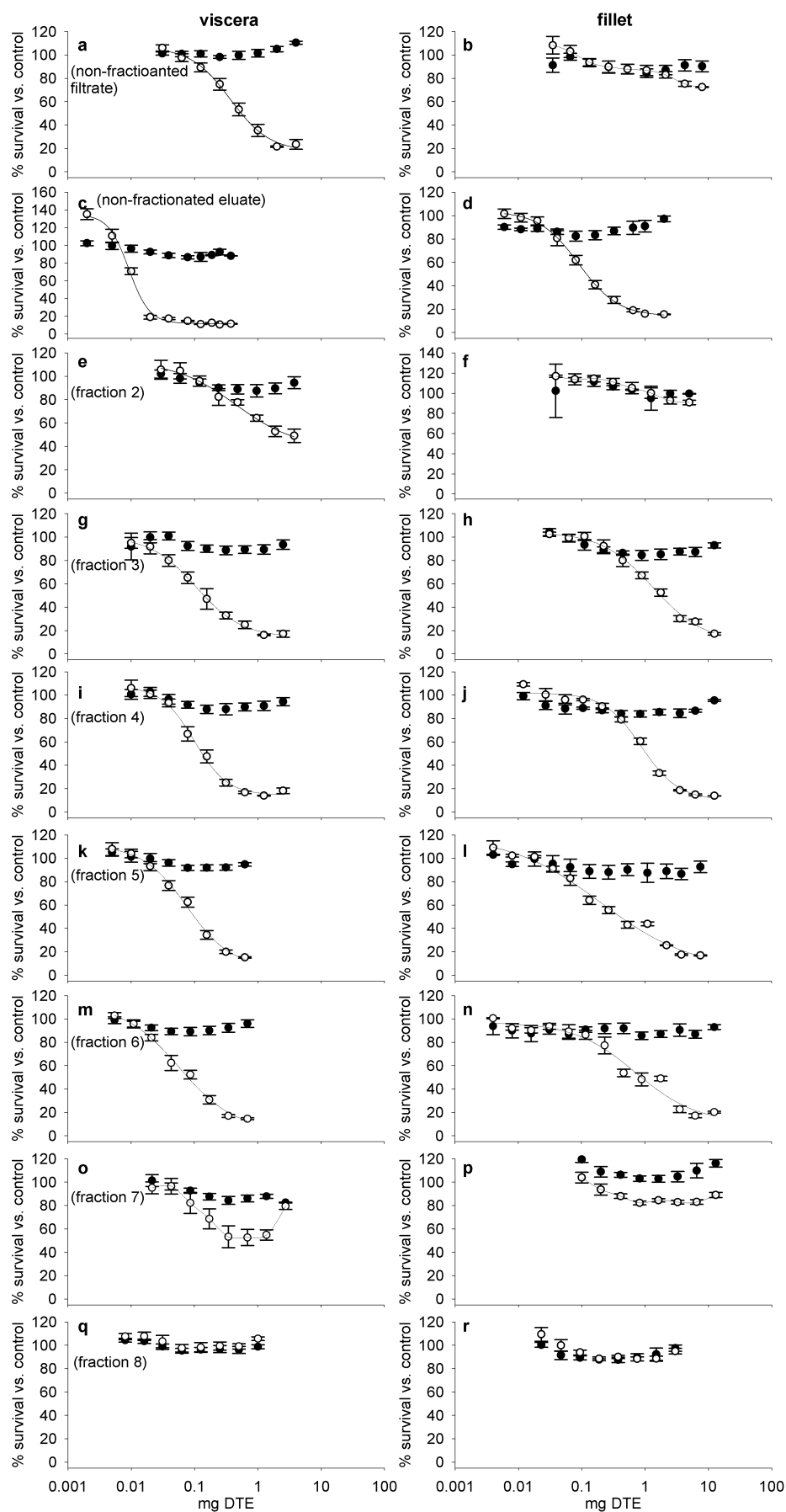


Figure S6. Concentration-response-curves obtained from the N2a-bioassay for the analysis of *E. canina*; panels show curves for the (a,b) non-fractionated filtrate, (c,d) non-fractionated eluate and (e-r) fractions 2 to 8 for the viscera (left) and fillet sample (right); the respective solvent compositions of the fractions are provided in Section 2.4., Figure 3; data points show the results for the -OV (solid symbol) and +OV conditions (open symbol); data represent the mean \pm standard deviation of multiple microplates with triplicate determination per plate.