Supplementary Information

Analytical Size Exclusion Chromatography Coupled with Mass Spectrometry in Parallel with High-Throughput Venomics and Bioassaying for Venom Profiling

Table of content

S1. Plasma Coagulation Assay Protocol

A HTS (high-throughput screening) coagulation assay was utilised in this research to measure the effect on plasma coagulation caused by snake venom toxins [1]. Bovine plasma incubated with Ca^{2+} results in a kinetic coagulation profile measured by spectrophotometric instrumentation as upon coagulation the plasma becomes less transparent hence resulting in higher absorbance. Coagulation bioactivity of crude venoms and venom toxins can be measured by testing the influence on the coagulation profile kinetically (i.e. by measuring the coagulation curve in-time).

The assay was performed at room temperature on transparent flat-bottom polystyrene 384-well plates (781185, Greiner Bio One, Alphen aan den Rijn, The Netherlands) filled with pre-nanofractionated and freeze-dried venom separated by SEC. First, 20 µl of 20 mM $CaCl_2$ was added to the wells, whereafter the plate was incubated for 10 minutes on a shaker at low shaking velocity. In the meantime, bovine plasma at room temperature was centrifuged for 4 minutes at 2000 rpm to get rid of particulate matter (e.g., some remaining white blood cells). After 10 minutes of incubation, 20 µl of the citrated plasma was added to the wells. The plate was then transferred to the plate reader and the measurement program was started within 1 minute. Pipetting calcium chloride and plasma was performed by a robotic pipetting system, ThermoScientific™ Multidrop™ 384.

The platereader, a Thermo Fisher Scientific Laboratory Varioskan™ LUX Multimode Microplate Reader with data analysis program SkanIt 4.1., was set to absorption at 595 nm. All 384 wells were read 80 times, resulting in a total reading time of approximately 100 minutes. Whether a compound is activating or inhibiting the coagulation process, depends on the velocity of the plasma clotting. A high velocity, meaning a fast increase in absorbance and steep slope, is caused by a procoagulant while an anticoagulant is responsible for a slow increase in absorbance. To display anticoagulation, the absorption of the 80^{th} reading was taken and plotted. At this point the non-anticoagulant wells have reached the full coagulation endpoint while anticoagulation reached a lower absorbance endpoint measurement. For assessment of procoagulation, the steepness of the coagulation slope was used. For this, the average velocity of the first 15 measurements were used. If there is activation of coagulation in the first 15 readings, the sample will be labelled as procoagulant. Approximately 80 seconds pass between each measurement, meaning procoagulation is measured within 20 minutes.

Coagulation is not specifically caused by one toxin family. Russell's viper venom for instance contains a metalloproteinase causing procoagulation and PLA_2s causing anticoagulation. When both inhibition and activation of coagulation is present in the same well, the procoagulant compound can cancel out the anticoagulation [2]. For this reason the pharmaceutical compound marimastat which inhibits the potential procoagulant activity of metalloproteinases was used in an additional assay on SEC-fractionated venom. A total of 10 µl of marimastat was added to the fractionated and vacuum centrifuged wells, followed by centrifuging the well plates for 1 minute at 1000 rpm before incubation of 30 minutes at room temperature. Afterwards, the same steps were performed as for the assay without marimastat. The concentration of marimastat was chosen according to the research of Xie *et al* 2020, which concluded in an optimal end concentration in the wells of 4 µM [2].

S2. Protein Mix separation

A protein mix made from 1 mg/ml BSA, 1 mg/ml Ribonuclease A and 0.1 mg/ml uracil was used to test the separation efficiency and capacity of the SEC column during the method optimisation steps. Figure S1 shows a chromatogram of the SEC separation of the protein mix using a mobile phase of 20% ACN + 0.1% TFA at a flow rate of 0.2 ml/min with an injection volume of 20 µl. UV detection was done at a wavelength of 280 nm. Separation of the compounds in the mixture including the BSA dimer formed in solution was achieved. Identification of the individual peaks was verified by measuring all compounds individually.

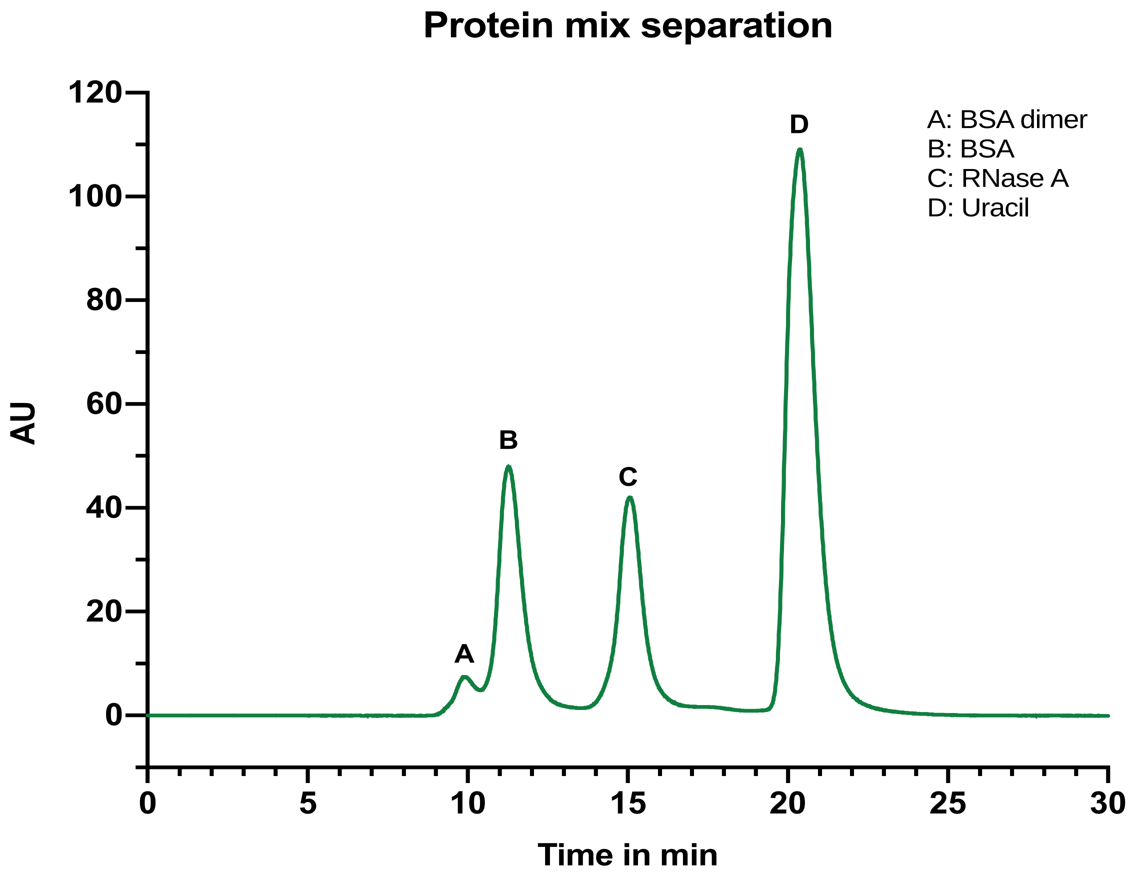


Figure S1: SEC separation of the protein mix using 20% ACN + 0.1% TFA as a mobile phase. 20 µl was of a 1 mg/ml bovine serum albumin (BSA), 1 mg/ml ribonuclease A (RNase A) and 0.1 mg/ml uracil mixture was injected.

S3. Optimisation of the SEC measurements

There are several relevant parameters that influence a SEC separation, of which many are column specifications. The column length plays a role in the resolution of the peaks and back pressure. The particle diameter and their pore size highly influence the retention range and the selectivity of the column. Other influencing non-column specific parameters are the temperature and mobile phase. The flow rate does not show an effect on the selectivity of the separation but is correlated with retention times which is important for throughput. The mobile phase composition is of influence for potential secondary column material interactions of analytes, which should be avoided at all times. Additionally, the amount of sample injected into the column must be chosen carefully to avoid overloading the column which can lead to non-repeatable results. This is specifically important to keep in mind when using highly concentrated and protein-rich snake venom samples. A selection of parameters for optimisation was made including the composition of the mobile phase, injection volume and flow rate. All optimisation steps were measured in duplicate, for which no significant differences were observed in any optimisation step.

S3.1. Flow rates

Three different flow rates, 0.1, 0.2 and 0.3 ml/min, were tested by injecting the protein mix or *B. multicinctus* venom. An injection volume of 10 µl and mobile phase composition of 20% ACN + 0.1% TFA were used. The results showed no alterations in peak shapes nor resolution. The only difference is the retention time frame in which the peaks eluted, as expected. See Figure S2 for the results.

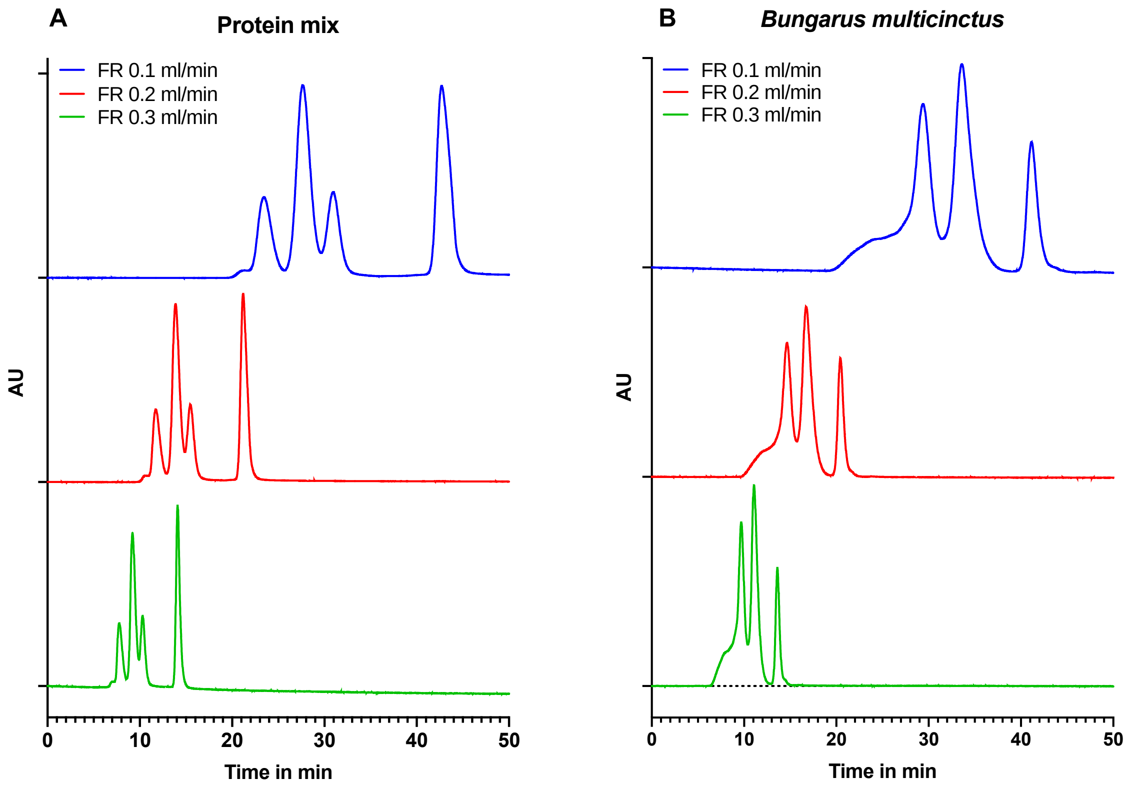


Figure S2: A; Flow rate (FR) evaluation for injection of the protein mix. B; Injection of 1 mg/ml B. multicinctus venom.

S3.2. Injection volume & venom concentration

Optimal injection volumes for the SEC column used as advised by the manufacturer are between 3 and 20 µl. In this study however, higher injection volumes were potentially desired due to the post-column bioassaying after fractionation. Higher injection volumes allow more sample to be injected and thus resulting in higher concentrations of fractionated toxins in the eventual bioassay. A lower sample concentration and/or a lower injection volume can result in less to no activity [3]. In previous studies with post-column bioassaying, injection volumes up to 50 µl were used, for separation by RPLC. Here, injecting venom concentrations of 1 to 5 mg/ml was normally done [1,4,5]. In this study, evaluation of injection volume was performed by injecting volumes of 5, 10, 20, 30, 40 and 50 µl of 1 mg/ml venom and by 20 µl injections of venom at concentrations of 1, 2.5 and 5 mg/ml.

Venom of *B. multicinctus* was chosen for this evaluation. As mobile phase, 20% ACN + 0.1% TFA was chosen. Separations were performed at a flow rate of 0.3 ml/min resulting in a total void time (the column volume) of 15 minutes. Variation in venom concentration as well as injection volume did not provide deviations in the separation efficiency (see Figure S3). The retention times of the peaks were the same for all separations where only the intensity of the peaks increased with increasing concentration and/or injection volume. There was no case of overloading the column or dissimilarities in peak retention times.

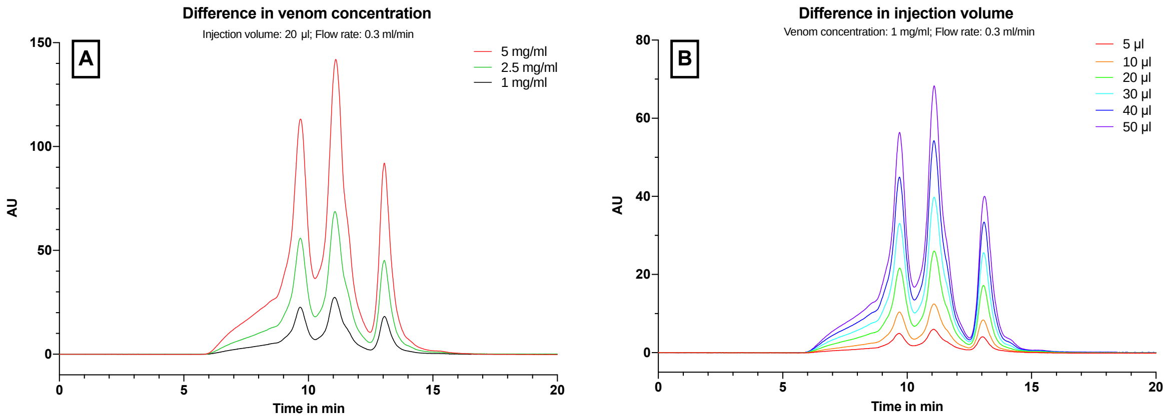


Figure S3: SEC separations of 20 µl injections of B. multicinctus venom at concentrations of 1, 2.5 and 5 mg/ml (A) and SEC separations of 1 mg/ml B. multicinctus venom with injection volumes of 5, 10, 20, 30, 40 and 50 µl (B). Separations were performed at a flow rate of 0.3 ml/min. A lower concentration venom and a lower injection volume both influenced only the intensity of the peaks. There was no overloading of the column and no differences in the separation observed from this data.

S3.3. Mobile phase composition

Different mobile phase compositions were tested by separating initially the protein mix and afterwards the venoms (see S3.4 for the venom separations). All raw data are plotted in Prism documents, see SI documents Prism S1 for the protein mix separations and Prism S2 for the venom separations.

Effect of Organic solvent concentration

Organic solvents acetonitrile (ACN) and isopropanol (IPA) were first tested at five concentrations: 0%, 5%, 10%, 15% and 20%, without the addition of acidifier. In all cases, no separation of the proteins within the protein mix was observed, see Figure S4. There was one peak visible which eluted at the column volume time of t=21 min. Concentrations of 5-20% ACN showed almost gaussian peaks, but when compared to separation by 100% Milli-Q, the peak showed a bit tailing. Fronting with irregular peak shape was observed for separation with IPA in the eluent. With increasing concentration of IPA, the peak became broader and showed a bump shape at the front of the peak. Under none of the investigated conditions, the compounds in the mixture were separated from each other.

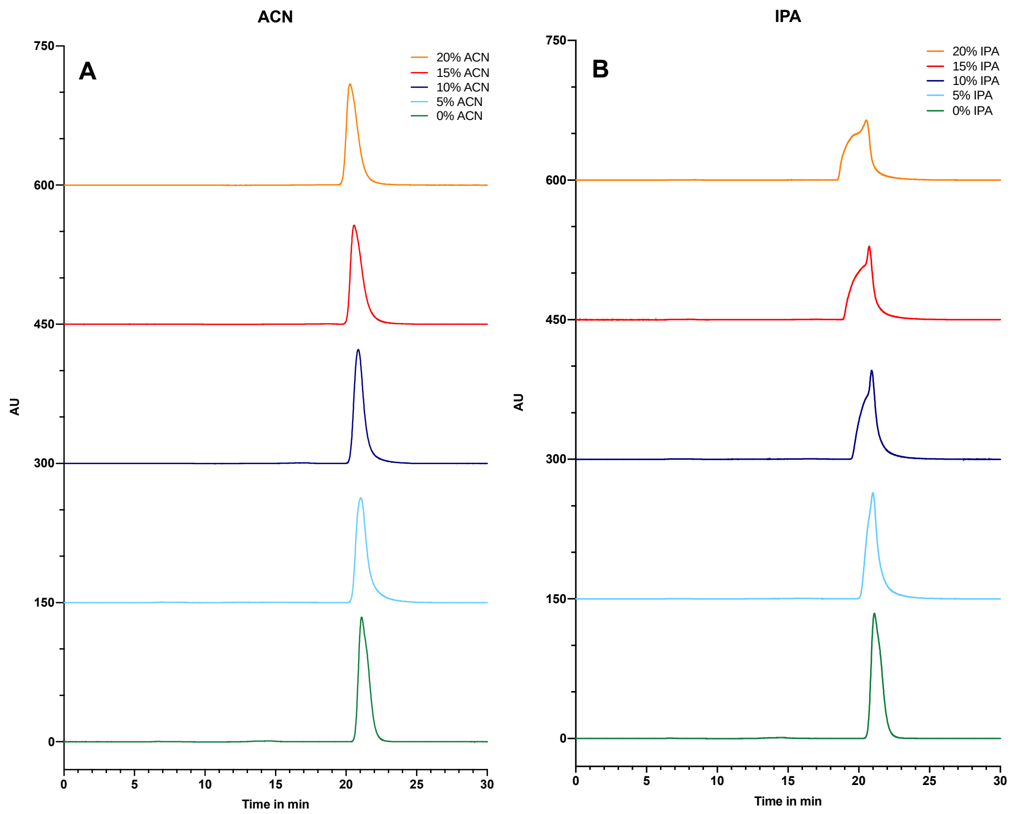


*Figure S4: Separation of the protein mix under 5 different concentrations of ACN in the eluent (**A**) or 5 different concentrations of IPA in the eluent (**B**). Under none of the investigated conditions, the compounds in the mixture were separated from each other.*

Effect of the Acidifiers

The acidifiers included in this study were first tested individually by adding a percentage of 0%, 0.05% or 0.1% to Milli-Q and using this eluent separating the protein mix, see Figure S5. Addition of TFA gave separation of two different peaks in case of both concentrations of 0.1% and 0.05% tested with a slight difference in elution time of the first peak. The difference in retention time between peak 1 and 2 was larger when a concentration of 0.05% TFA was used.

In contrary to TFA, separation with FA as acidifier gave three peaks, which was the expected situation since there are three different proteins in the protein mix. Between the retention time frame of 9 to 13 minutes, two peaks were visible which is contradicted to the separation by TFA. Addition of an acidifier to the mobile phase for the SEC separation gave improvement in the chromatographic performance. TFA in this regard is called the golden standard when dealing with RPLC separations since it positively increases the resolution [6]. A downside of TFA is that it suppresses the ion signal in MS, causing poor MS data due to ion suppression. TFA, with a pKa value of 0.3, is also a very strong acidifier and this might affect the proteins native form. DFA was proven to be a good alternative for TFA in RPLC separations because of the small loss in resolution as compared to TFA used as acidifier [6]. DFA is a less strong acidifier, but nevertheless, still categorised as strong with a pKa of 1.34. Comparing both acidifiers as additions to the mobile phase v for SEC separations, DFA gave a better separation than TFA. The reason for this could be that TFA is too harsh on the proteins, causing alterations in their form. Studies have shown that a lower pH can cause denaturation of proteins [7]. This could also be an explanation for the better separation with FA compared to TFA.

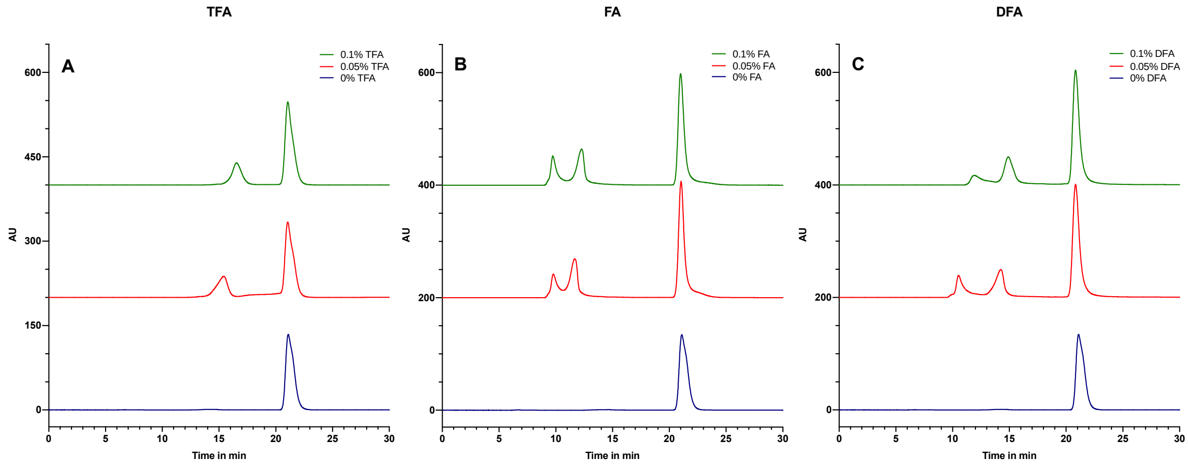


Figure S5: Separation of the protein mix tested with three different concentrations of the acidifiers TFA (A), FA (B) and DFA (C)

Effect of combinations of both organic solvent and acidifier together in the eluent

First, protein mix separation was investigated by four different concentrations of ACN in combination with 2 different concentrations of acidifier TFA, FA or DFA (see Figure S6) In most cases, the separation performance increased with increasing concentration of ACN. Between acidifiers DFA and TFA small differences were observed in peak shape and retention times. DFA gave slightly better resolution compared to TFA, and also in combination with 5% ACN was still capable of separating the (first) two eluting proteins. FA gave poorer separation when looking at the first two (protein) peaks. Also, the BSA-dimer peak was not observed for any separation with FA.

The minimum concentration of ACN resulting in a good separation depended highly on the acidifier and its concentration added. For instance, when looking at the separation by ACN + 0.05% or 0.1% TFA in Figure S6 it is seen that all proteins in the protein mix were separated with a minimum concentration of 15% ACN + 0.1% TFA and a minimum of 10% ACN + 0.05% TFA. The BSA-dimer was best separated with 0.1% TFA + 15% or 20%, and by 0.1% DFA with all concentrations ACN tested.

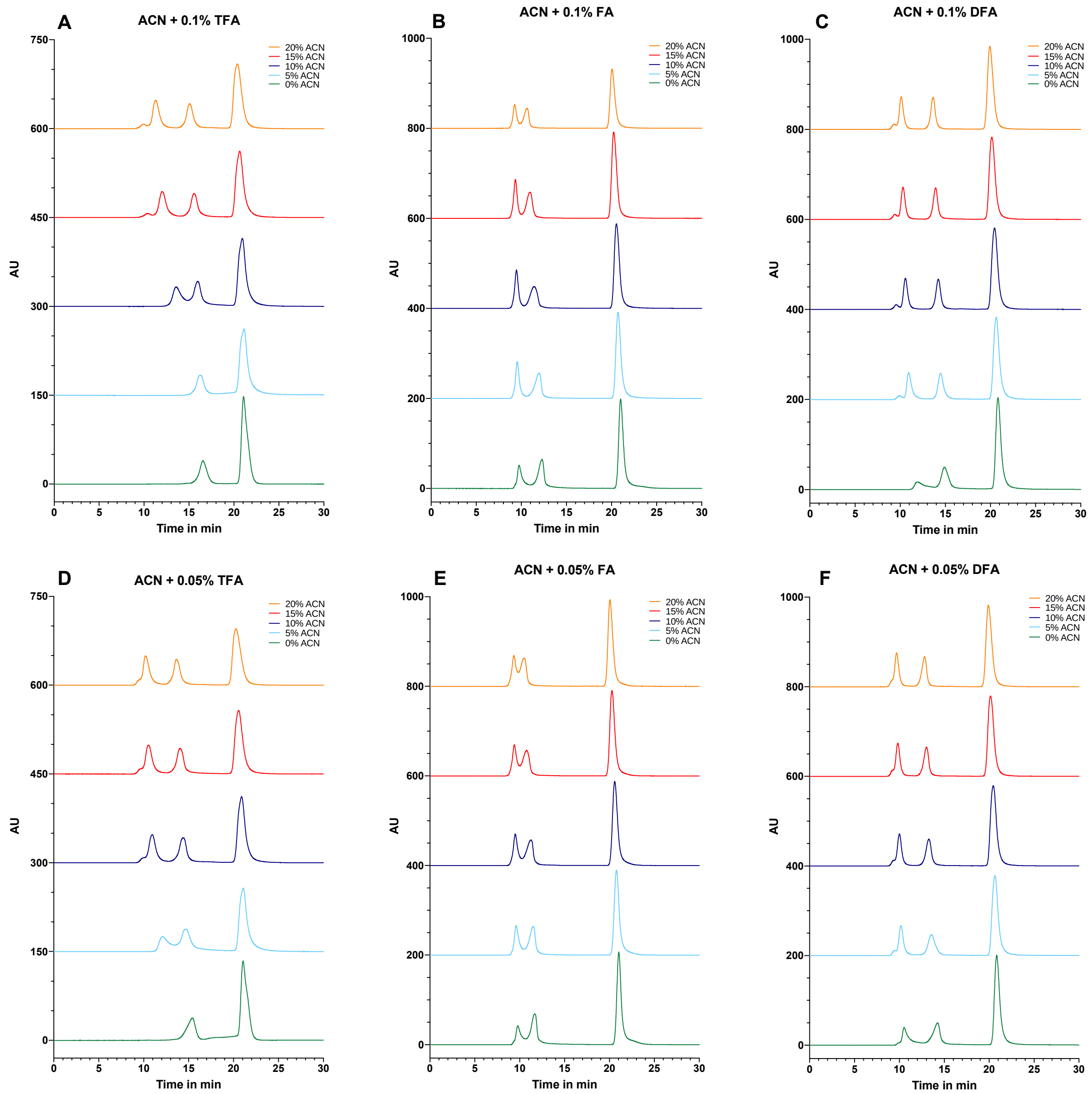


*Figure S6: Separation of the protein mix with eluents tested containing five concentrations of ACN with 0.1% TFA (**A**), 0.1% FA (**B**), 0.1% DFA (**C**), 0.05% TFA (**D**), 0.05% FA (**E**) or 0.05% DFA (**F**).*

Separation using different concentrations of IPA was tested next with acidifiers TFA and FA, see Figure S7. Again, addition of FA showed poor separation compared to TFA, where 0.1% FA had a better resolution between the eluting peaks in contrast to 0.05% FA. The 10% IPA concentration was sufficient for separation of the compounds in the protein mix with addition of TFA. IPA seemed to give a slightly better separation than ACN, however it did give unusual and broader peak shape of first two elution peaks. At higher concentrations of IPA, the last eluting peak even tended to split in two co-eluting peaks with bad peak shape. IPA significantly increases the viscosity of the mobile phase, which causes an elevation in the backpressure [8]. The SEC column used in this study has an optimum performance at a pressure below 140 bar, which was exceeded when using 20% IPA at flowrates of 0.3 ml/min or higher. Therefore, ACN was used further as organic solvent in the eluent.

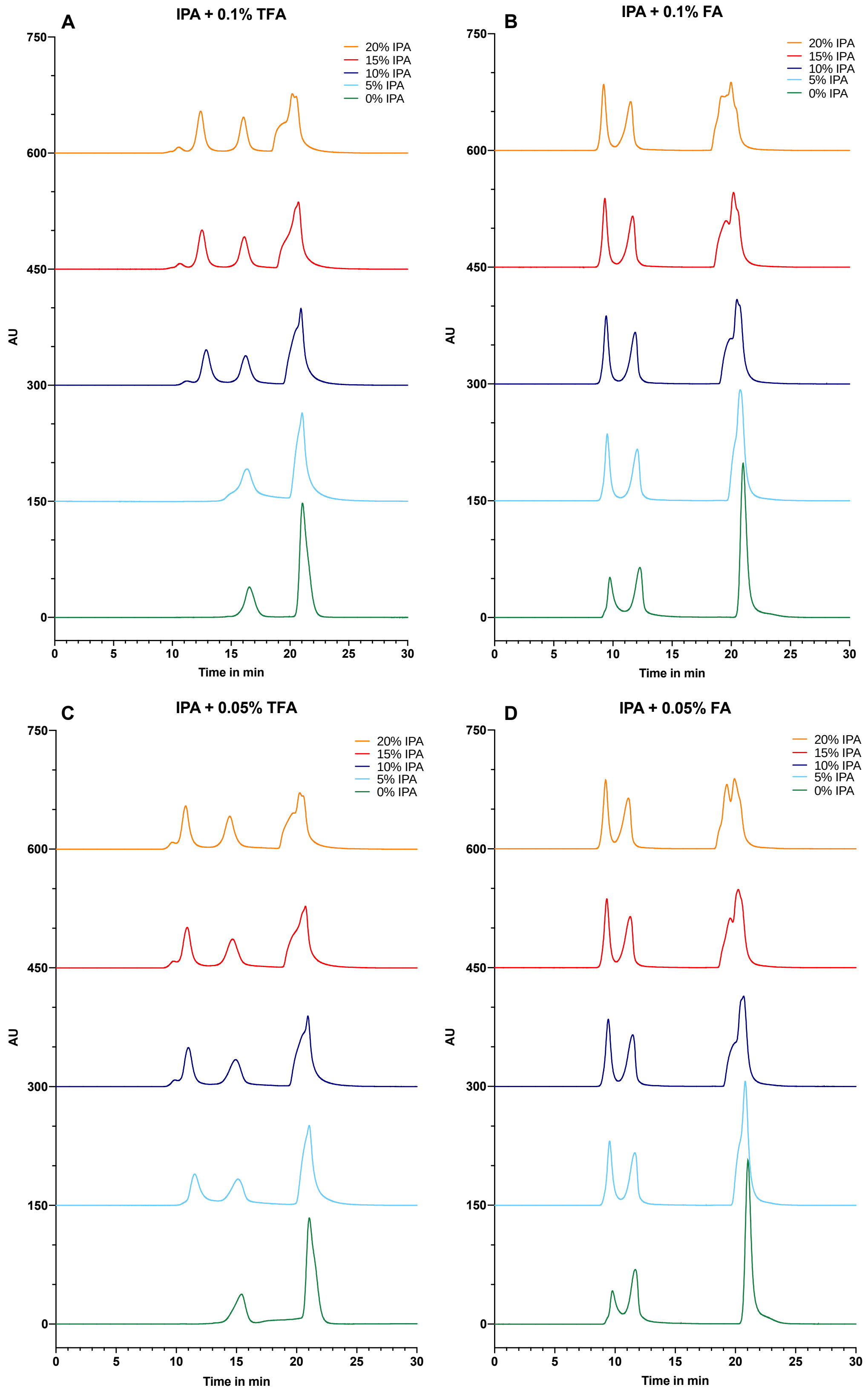


Figure S7: Separation of the protein mix with eluents tested containing 5 concentrations of IPA and 0.1% TFA (A), 0.1% FA (B), 0.05% TFA (C) or 0.05% FA (D)

S3.4. SEC optimisation with venom

Since the combination of organic solvent with TFA in Milli-Q showed best results with PM separations, evaluation of separations with venom were restricted to these conditions. Venoms of *B. multicinctus* and *D. russelii*, were chosen for their complexity and known effects on coagulation modulation, see SI section S4 for their venom compositions. The largest toxins (SVMPs and SVSPs) are expected to elute first, followed by PLA_2s and ending with 3FTxs.

Separation of the venom from *B. multicinctus* showed similar results when different mobile phases were used. In Figure S8A-D, all chromatograms resulting from a total of 16 different mobile phase compositions tested for both organics combined with TFA at different concentrations are presented. In all chromatograms there are three clear main peaks visible, with an additional shoulder peak at the beginning of the first peak. Between the organic solvents IPA and ACN, better separation was observed with ACN. This was concluded due to an additional visible shoulder peak at RT 18-19 min shown in Figure S8A and at RT 17-18 min in Figure S8B. Whether this shoulder peak occurs, depends on the concentration of ACN in combination with the concentration of TFA added. For 0.1% TFA, this shoulder peak was visible at the range of 5% to 20% ACN, whilst when 0.05% TFA was added the peak disappeared at 20% ACN. When IPA was used as organic solvent, separation with 20% IPA again showed the peak splitting effect.

Analytical separations of venom from *D. russelii* evaluated with different mobile phase compositions were difficult to compare by only SEC-UV data. This is due to the many dissimilarities in retention time profiles, as shown in Figure S8E-H. Some peaks showed shifts in retention time with increasing organic solvent concentration in the mobile phase, including a peak eluting after the column volume time (thus the compound(s) representing this peak had secondary binding interactions with the SEC column material). The retention time of this specific peak decreased with increasing concentrations of organic eluent (indicating the reduction of possible secondary interactions with the column material). It is well known that separation of proteins by SEC can cause interactions with the SEC surface material due to hydrophobicity of some proteins [9]. A higher concentration of ACN in the mobile phase will cause these proteins to be better soluble in the mobile phase and interact less with the SEC material.

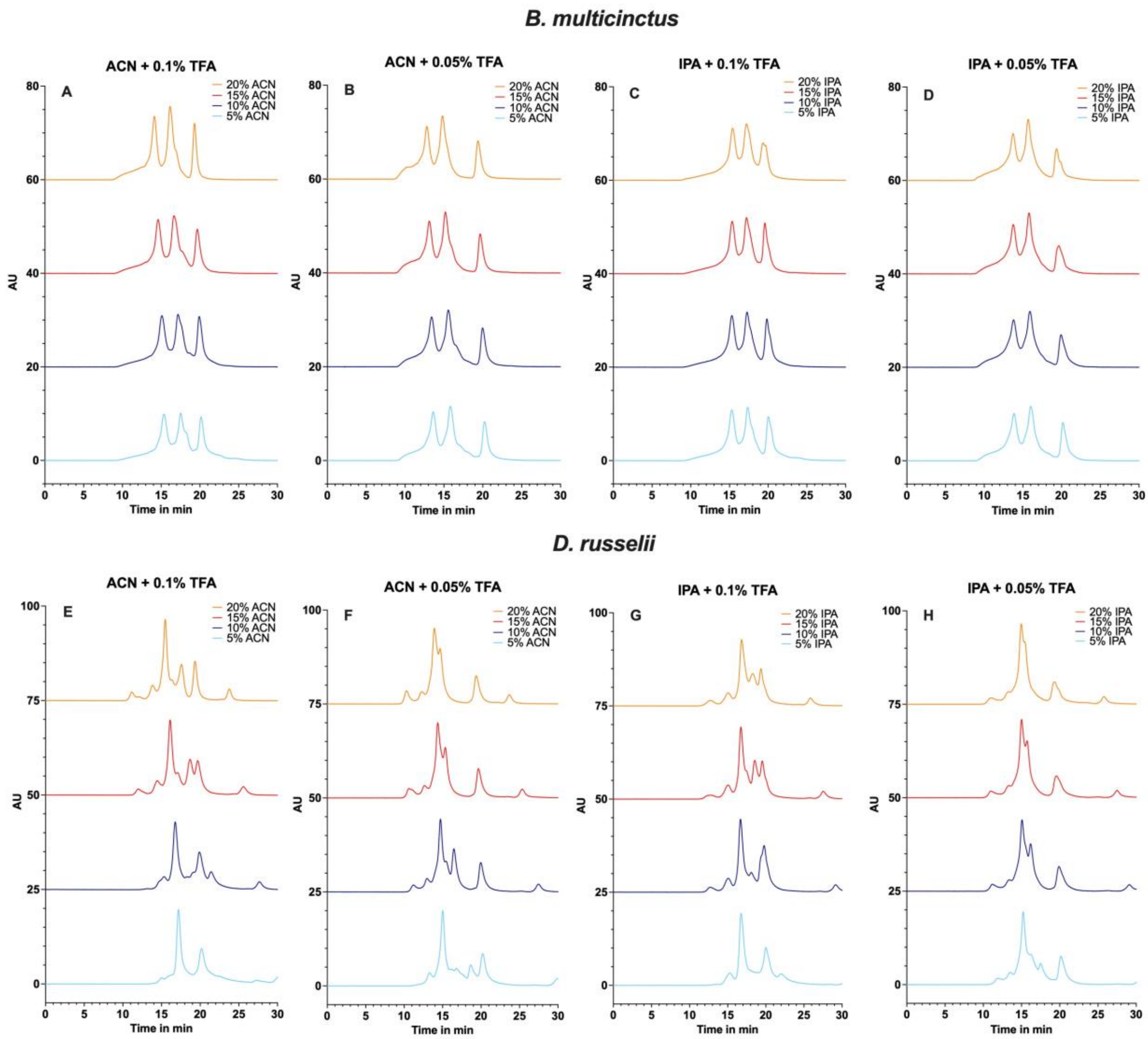


Figure S8: SEC separation of B. multicinctus venom by 5-20% ACN + 0.1% TFA (A), 5-20% ACN + 0.05% TFA (B), 5-20% IPA +0.1% TFA (C) and 5-20% IPA + 0.05% TFA (D). SEC separation of D. russelii venom by 5-20% ACN + 0.1% TFA ©, 5-20% ACN + 0.05% TFA (F), 5-20% IPA + 0.1% TFA (G) and 5-20% IPA + 0.05% TFA (H). See SI document Prism S2 for all raw data.

To also study the effect of DFA and FA for SEC separations of the two venoms, additional experiments were conducted. Results are given in Figure S9. Separations with 0.1% or 0.05% DFA gave similar results in combination with 10%, 15% and 20% ACN. Separations with FA as acidifier again showed less separation of which separation by 20% ACN + 0.05% FA gave the worst separation.

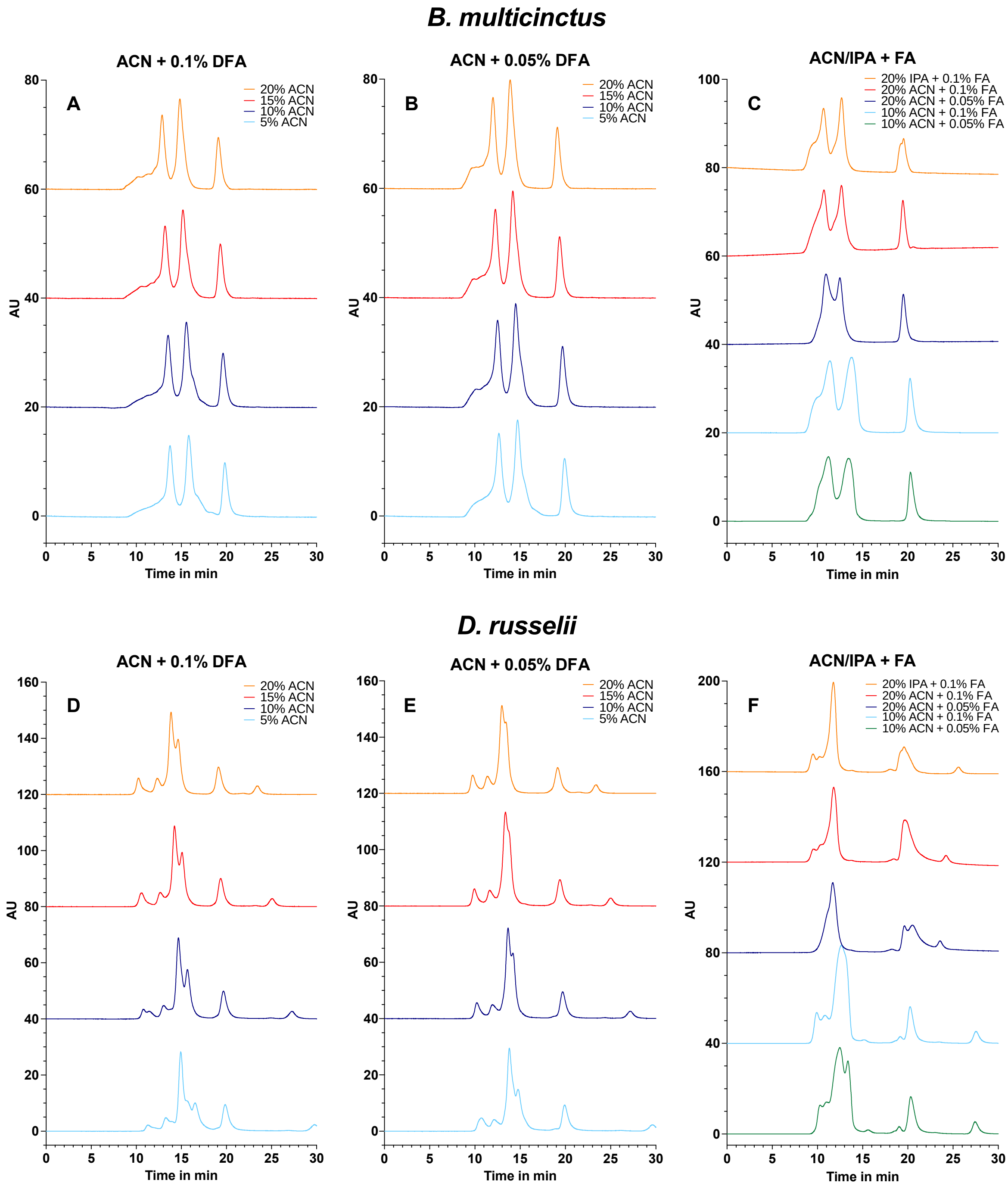


Figure S9: Separation of 1 mg/ml venom from B. multicinctus and D. russelii by mobile phases containing different concentrations of ACN or IPA in combination with different concentrations of DFA or FA. See SI document Prism S2 for all raw data.

A selection between the two organic solvents was made for follow-up research. Addition of IPA to the mobile phase increased the backpressure of the column significantly more than ACN. This phenomenon is likely the result of the high viscosity of IPA [8]. Castells *et al* 1997 [10] also suggested that the viscosity of IPA was responsible for broader an non-gaussian peaks. Due to the higher viscosity of using IPA in the mobile phase opposed to the lower viscosity of the injected samples, the peaks get instable and can therefore cause peak splitting. According to Goyon *et al* 2017 [8], IPA is supposed to be a better organic solvent to retain proteins from interacting with the SEC-column surface. However, in the specific case of venom from *D. russelii*, ACN assumingly better prevented proteins from interacting with the SEC material assumed by the shorter retention time of the last eluting peak. An explanation for the better performance of ACN is that this organic modifier enhances electrostatic interactions and is therefore able to perturb ionic exchange [9,11]. Proteins with a large portion of basic amino acids will be protonated within the acidic conditions of the mobile phase and therefore can form secondary interactions with the hydrophilic film bonded silica surface of the SEC column. High concentrations of both ACN and IPA dissolved in water have the potency to unfold proteins [12]. Taking this into account, the lowest concentration of organic modifier which gives acceptable SEC separations was selected to avoid venom toxin denaturation. Since 10% ACN gave sufficient separation under all conditions tested, this concentration was chosen for further post-column bioassaying and MS analysis.

No initial selection was done for the tested acidifiers since their presence can also affect ionisation within the MS analysis and bioactivity of the toxins tested by bioassays. In addition, the three acidifiers FA, TFA and DFA in both concentrations of 0.05% and 0.1% were taken along to the next steps in this research.

S4. Venom composition

Venoms of the species *D. russelii* and *B. multicinctus* were chosen because of their complexity and distinct differences in composition. In Figure S10, pie diagrams are given for the toxin composition of both venoms according to two studies from literature [13,14]. The venom of *B. multicinctus* contains mostly 3FTxs (around 65%) of which approximately 41% are classified as α-bungarotoxins and the others as κ-bungarotoxins and regular 3FTxs. Around 25% are ß-bungarotoxins which consist of an A- and B-chain, where the A-chain is classified as a PLA_2 and the B-chain as a kunitz type toxin. Other toxins include PLA_2s, C-type lectins and sometimes also SVMPs [13,15]. Venom of *D. russelii* is known for huge interspecies variation in venom composition. In general it mostly consists of a wide variation of PLA_2s, with a lower concentration of SVMPs, (Kunitz type) SVSPs, VEGF toxins and phosphodiesterases [14]. The relevant mass range of venom toxins in *D. russelii* venom is larger for than for *B. multicinctus* venom due to the higher abundance of SVMPs, which can have a mass up to 100 kDa. SVMPs are expected to elute first on SEC separations as they comprise the largest venom toxins in these venoms (i.e., a mass range of 20 to100 kDa), followed by or in combination with SVSPs with a mass range of 26 to 67 kDa [16,17]. ß-bungarotoxins have a mass range of approximately 20-21 kDa, PLA_2s are within the range of 13-15 kDa and the smallest toxin families are the 3FTxs and kunitz type toxins with masses around 7 kDa [18–21].

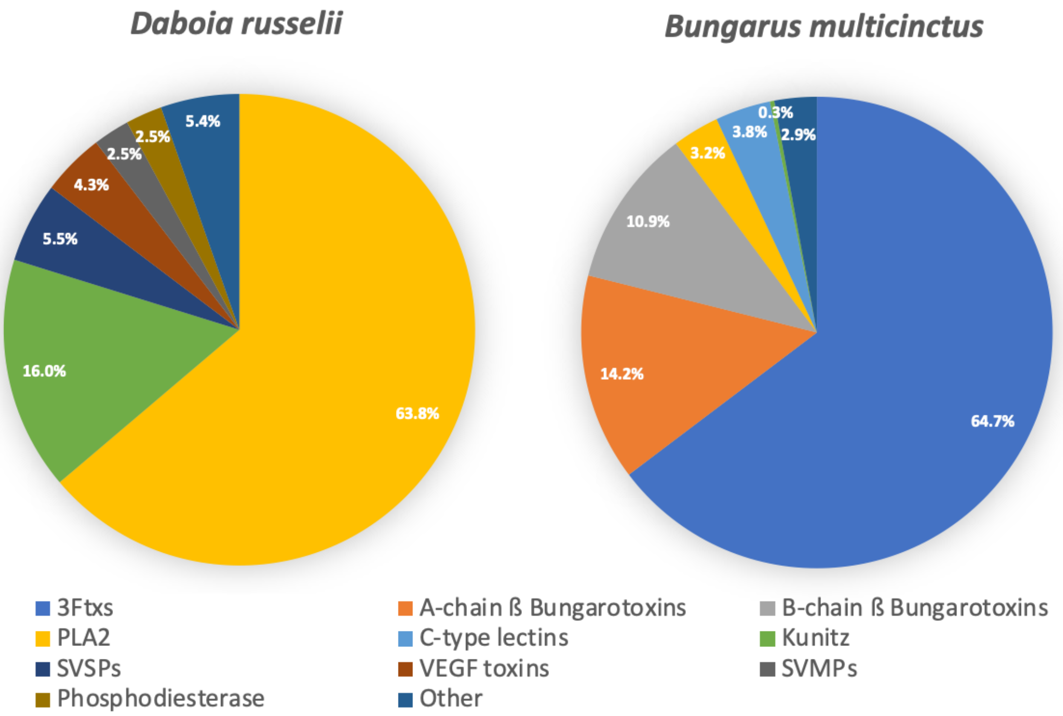


Figure S10: The toxin distribution in venoms of D. russelii and B. multicinctus. 3FTx, three-finger toxin; PLA_2, phospholipase A_2; SVSP, snake venom serine protease; VEGF, vascular endothelial growth factor; SVMP, snake venom metalloproteinase [13,14]

S5. Proteomics Results

Tables S1 and S2 include all venom toxins that were identified by proteomics analysis from venoms of the species *Bungarus multicinctus* and *Daboia russelii*. The tables include the protein codes from the MASCOT database, name of the toxins, toxin family they belong to, exact masses retrieved from MASCOT (excluding modifications such as PTMs), the number of modifications (mod.) like glycosylation sites or PTMs and the sum of the protein scores, for each of the 6 mobile phases tested for SEC separation, for both venoms. In case of *D. russelii* venom, also the exact species connected to the MASCOT code, *Daboia russelii russelii* (DABRR) or *Daboia russelii siamensis* (DABSI), was added to the table.

Table S1: Proteins identified for B. multicinctus venom separations. The proteins (i.e., venom toxins) were obtained through the MASCOT search engine from the Swissprot database and are all from one species, with code BUNMU. In the table the protein code, name, toxin family, exact mass derived from the peptide sequence and the amount of post translational modification (PTM) such as glycosylation's are displayed next to the sum of protein scores per separation. The Sum of protein scores is the sum of how many of the found peptides match with the sequence of the toxin. The more peptides match, the more likely it is that the matched protein is correct.

Bungarus multicinctus					*Sum of protein scores per separation*					
Code	***Name***	***Toxin Family***	***Exact mass***	***PTM***	***TFA 0.05***	***TFA 0.1***	***FA 0.05***	***FA 0.1***	***DFA 0.05***	***DFA 0.1***
3L21A	alpha-Bungarotoxin	3FTxs	7978.6		24094	38223	34417	28563	31188	36039
3L21V	alpha-Bungarotoxin Isoform V31	3FTxs	8003.63		25889	19475	28859	25780	23090	17378
3LK3	Kappa-3-bungarotoxin	3FTxs	7368.22		237	203	97	81	145	
3LK6	Kappa-6-bungarotoxin	3FTxs	7365.29		181	291	228	132	343	232
3LKB	Kappa-bungarotoxin	3FTxs	7260.22		276	133			259	
3NO41	Neurotoxin BM10-1-like	3FTxs	7233.21		36					
3NO4H	Long neurotoxin homolog	3FTxs	7352.27		798	1151	843	676	632	724
3NO52	Long neurotoxin homolog NTL2	3FTxs	7568.35		230	258	363	364	304	413
3NO5I	Gamma-bungarotoxin	3FTxs	7519.33			131				
3NOH	Toxin BMLCL	3FTxs	9012.78		5336	6306	625	1203	1924	3194
3NOH3	Cytotoxin-like protein TA-BMBGT3	3FTxs	9111.86		1496	1780	1211	945	1548	1928
3NOHE	Muscarinic toxin BM14	3FTxs	9068.89		6189	5978	3148	3146	2589	5677
3SO3	Short neurotoxin homolog NTL4	3FTxs	7201.62		2596	2786	1469	1986	2435	2984
3SO7	Short neurotoxin homolog	3FTxs	7154.46			194		222		136
3SO93	Neurotoxin-like protein pMD18-NTL3	3FTxs	7468.36		2114	2154	1930	1786	1612	1725
LECM2	C-type lectin BML-2	C-type lectin	15749.44	1		145			106	
OXLA	L-amino-acid oxidase	Oxidoreduct.	56836.01	2		52	30	2384	5296	7399
PA2A	Acidic PLA_2	PLA_2	12808.56		2873	1225	1380	1270	1019	821
PA2A4	Acidic PLA_2 beta-bungarotoxin A4 chain	PLA_2	13394.88		337	1714		2537	739	
PA2A6	Acidic PLA_2 beta-bungarotoxin A6 chain	PLA_2	13396.96		30	53	80	89	31	61
PA2B	Basic PLA_2 beta-bungarotoxin A-AL2 chain	PLA_2	13575.11		18165	23381	7811	18686	12209	14253
PA2B1	Basic PLA_2 beta-bungarotoxin A1 chain	PLA_2	13468.01		12301	1938	9001	2955	3129	1240
PA2B2	Basic PLA_2 beta-bungarotoxin A2 chain	PLA_2	13656.2		21525	10835	8566	6858	4033	
PA2B5	Basic PLA_2 beta-bungarotoxin A5 chain	PLA_2	13559.07		29	381		31		62
PA2B7	Basic PLA_2 beta-bungarotoxin A7 chain	PLA_2	13445.08		615	1771	1313	1382	405	651
PA2BA	Basic PLA_2 beta-bungarotoxin A-AL1 chain	PLA_2	13472.08		27116	39265	14669	28957	23492	31146
PA2BC	Basic PLA_2 beta-bungarotoxin A-AL3 chain	PLA_2	13966.33		35					
VKTH1	Kunitz-type serine protease inhibitor homolog beta-bungarotoxin B1 chain	Kunitz-type	7177.34		1312	148	403		438	174
VKTH2	Kunitz-type serine protease inhibitor homolog beta-bungarotoxin B2 chain	Kunitz-type	7186.37		718		331		650	93
VKTH3	Kunitz-type serine protease inhibitor homolog beta-bungarotoxin B3 chain	Kunitz-type	7205.28		2422				712	70
VM3	Zinc metalloproteinase-disintegrin-like BmMP	SVMP	47743.54	1	235	1103		889	1879	1872
VNP	Natriuretic peptide BM026	Natriuretic peptide	4943.54	2	37	949	39		35	39
		Total detected toxins per separation			***28***	***28***	***22***	***23***	***27***	***24***

Table S2: Proteins identified for D. russelii venom separations. The proteins (i.e., venom toxins) were obtained through the MASCOT search engine from the Swissprot database and are majorly from two species, the Daboia russelii russelii (DABRR) and the Daboia russelii siamensis (DABSI). In the table the protein code, name, toxin family, exact mass derived from the peptide sequence and the amount of post translational modification (PTM) such as glycosylation's are displayed next to the sum of protein scores per separation. The Sum of protein scores is the sum of how many of the found peptides match with the sequence of the toxin. The more peptides match, the more likely it is that the matched protein is correct.

Daboia russelii						*Sum of protein scores per separation*					
Species	***Code***	***Name***	***Toxin Family***	***Exact mass***	***PTM***	***TFA 0.05***	***TFA 0.1***	***FA 0.05***	***FA 0.1***	***DFA 0.05***	***DFA 0.1***
DABRR	NGFV	Venom nerve growth factor	NGF-beta	13,268.32	1					31	
DABRR	OXLA	L-amino-acid oxidase	Oxidoreduct.	54,826.05	2	4338		1083	12991	21070	25120
DABSI	OXLA	L-amino-acid oxidase	Oxidoreduct.	46,338.57	2	855		53			
DABSI	PA2A	Acidic PLA_2 Drs-PLA_2	PLA_2	2,012.07			25				
DABSI	PA2A7	Acidic PLA_2 RV-7	PLA_2	13,655.72		1082	1442	8290	7610	3778	3494
DABRR	PA2B	Basic PLA_2 RVV-VD	PLA_2	13,602.94			105				
DABRR	PA2B1	Basic PLA_2 Drk-b1	PLA_2	14,066.30							76
DABSI	PA2B1	Basic PLA_2 DsM-b1/DsM-b1'	PLA_2	14,045.35		83	89		370	23	336
DABRR	PA2B3	Basic PLA_2 3	PLA_2	13,663.18		68786	90425	40483	33435	40909	46041
DABSI	PA2B4	Basic PLA_2 RV-4	PLA_2	13,789.20		2721	1952		3284	89	2274
DABRR	PA2B5	Basic PLA_2 VRV-PL-V	PLA_2	13,563.13		62957	66232	51720	40805	47877	51458
DABRR	PA2B8	Basic PLA_2 VRV-PL-VIIIa	PLA_2	13,587.20		36376	39843	30315	22701	25927	32780
DABSI	PA2BS	Basic PLA_2 DsM-S1	PLA_2	13,615.21		413	6088			7653	1786
DABSI	SL3	Snaclec 3	Snaclec	14,481.62				1870	1847	1160	111
DABSI	SL4	Snaclec 4	Snaclec	14,368.69				1523	1286	667	374
DABSI	SL5	Snaclec 5	Snaclec	14,727.86				412	166	62	
DABSI	SL6	Snaclec 6	Snaclec	14,227.80		78		35		31	
DABSI	SL7	Snaclec 7	Snaclec	15,696.16		2975		773	715	6395	2779
DABSI	SLA	Snaclec dabocetin subunit alpha	Snaclec	15,150.20				29	64	155	
DABSI	SLLC1	Snaclec coagulation factor X-activating enzyme light chain 1	Snaclec	14,452.80	1	426	137			60	272
DABSI	SLLC2	Snaclec coagulation factor X-activating enzyme light chain 2	Snaclec	15,922.45	1	85	38	57	76	37	56
DABRR	TXVE	Snake venom vascular endothelial growth factor toxin VR-1	VEGF	12,539.92	1	3884	4951	2846	2603	5508	5041
DABSI	TXVE	Snake venom vascular endothelial growth factor toxin VR-1	VEGF	12,539.92	1	1297	327				212
DABRR	VKT4	Kunitz-type serine protease inhibitor 4	Kunitz	6,696.99	1				176		349
DABSI	VKTB1	Kunitz-type serine protease inhibitor B1	Kunitz	6,869.09		463	277	293	500	642	1147
DABSI	VKTC1	Kunitz-type serine protease inhibitor C1	Kunitz	6,866.13	1	39		34	76	82	170
DABSI	VKTC3	Kunitz-type serine protease inhibitor C3	Kunitz	6,994.14		80		134	66	518	876
DABSI	VM3CX	Coagulation factor X-activating enzyme heavy chain	SVMP	48,125.17	4	3959	4375	1323	1319	1630	2821
DABSI	VSPA	Factor V activator RVV-V alpha	SVSP	26,153.10	1	2956	872	1210	418	2578	3103
DABSI	VSPAF	Alpha-fibrinogenase-like	SVSP	25,782.81	1	1607	1439	842	863	1844	1711
DABSI	VSPB	Beta-fibrinogenase-like	SVSP	25,350.33	4	2721	3544	2098	1133	1865	2317
DABSI	VSPG	actor V activator RVV-V gamma	SVSP	26,138.03	1	829	1789		1209	562	
				Total detected toxins per separation		***23***	***19***	***21***	***23***	***27***	***24***

All proteins have a protein score chromatogram (PSC) available for each measurement. All EICs per fractionated plate are given in Figure S11 for *B. multicinctus* and Figure S12 for *D. russelii*. The data was plotted in Prism, see SI documents Prism S3-S8 for *B. multicinctus* and Prism S9-S14 for *D. russelii*.

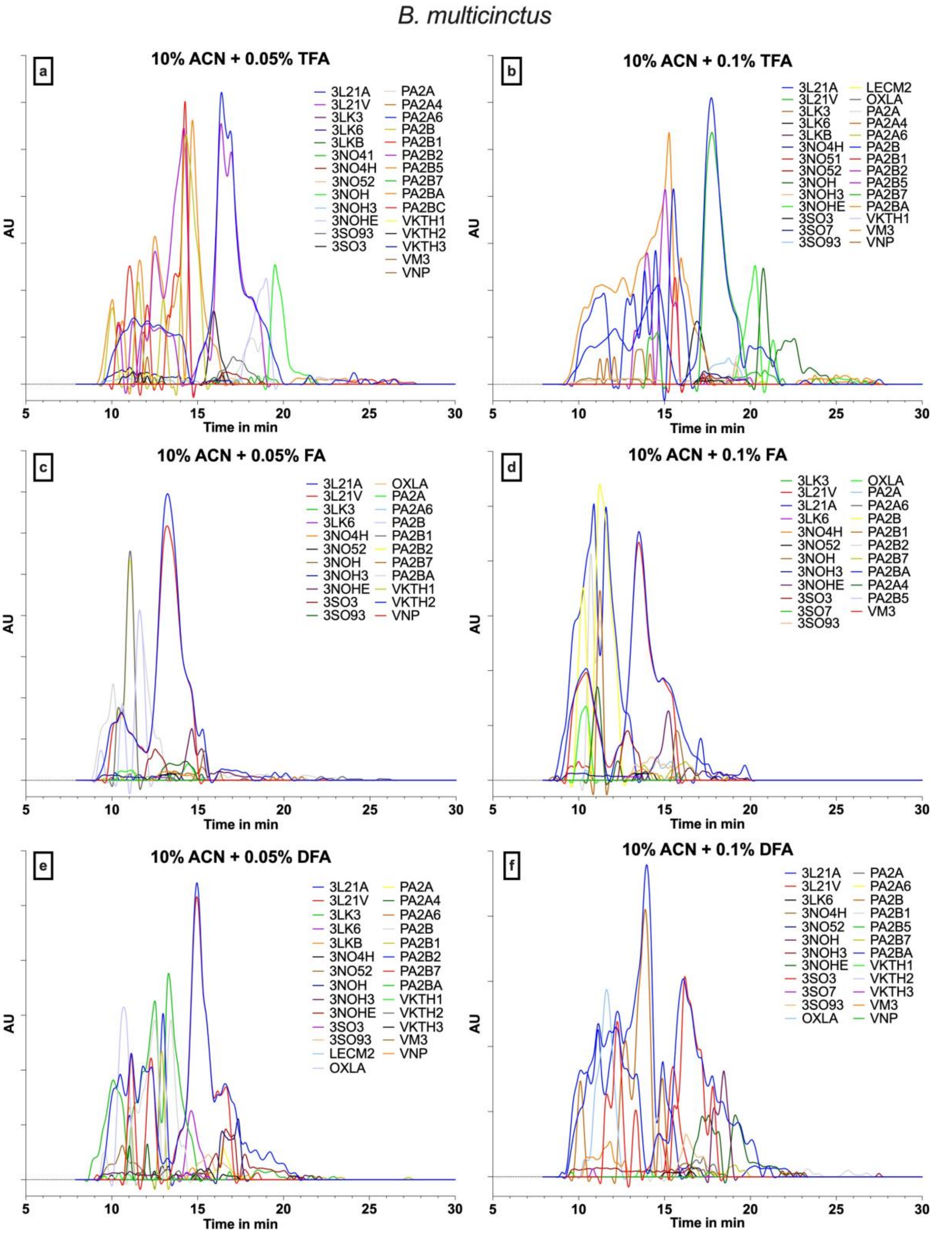


Figure S11: A visualization of the PSCs from the identified proteins in the proteomics analysis per sample for the B. multicinctus venom. Proteins identified in the fractionated plates from separation using 10% ACN + 0.05% TFA (a), 10% ACN + 0.1% TFA (b), 10% ACN + 0.05% FA (c), 10% ACN + 0.1% FA (d), 10% ACN + 0.05% DFA (e) and 10% ACN + 0.1% DFA (f).

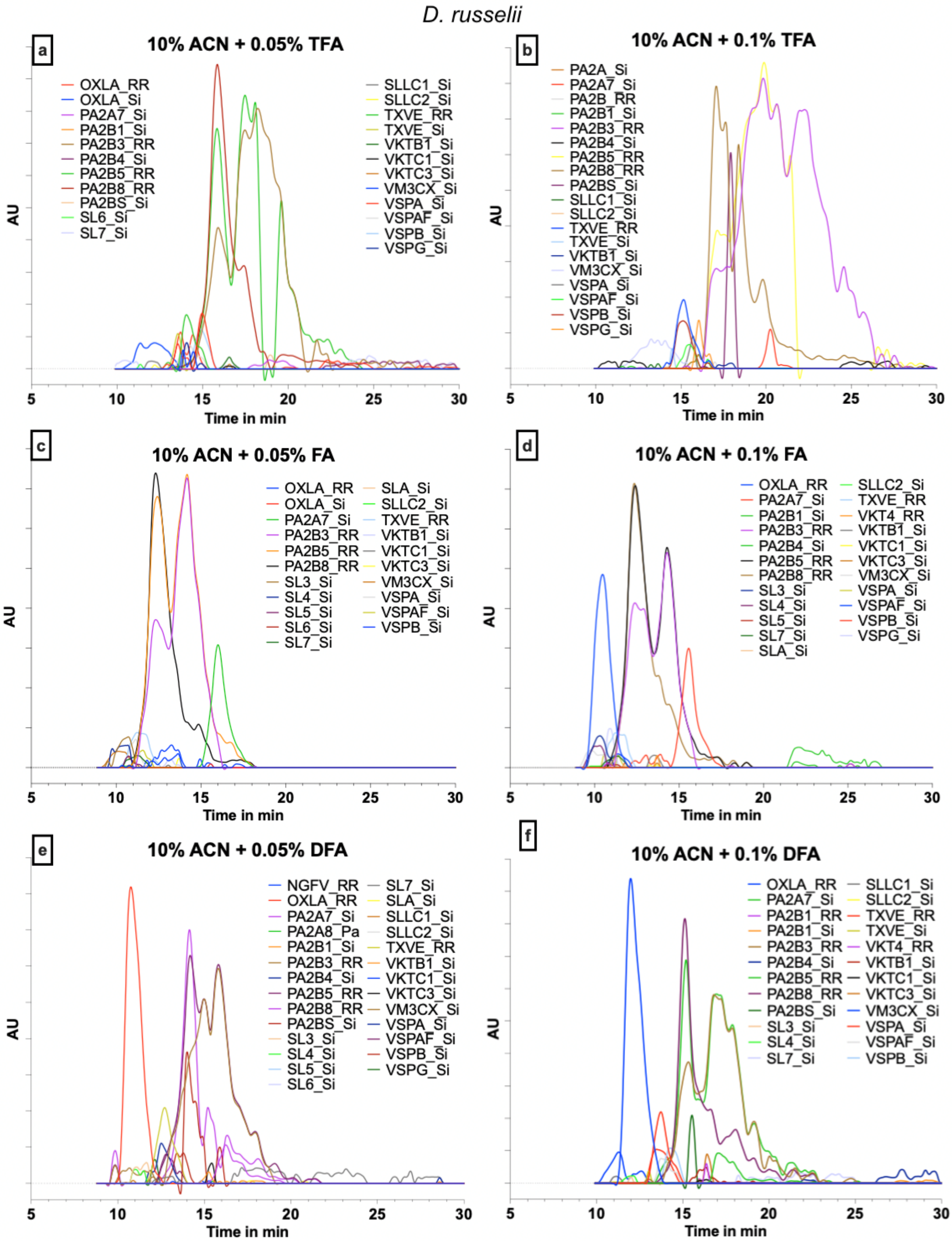


Figure S12: A visualization of the PSCs from the identified proteins in the proteomics analysis per sample for the D. russelii venom. Proteins identified in the fractionated plates from separation using 10% ACN + 0.05% TFA (a), 10% ACN + 0.1% TFA (b), 10% ACN + 0.05% FA (c), 10% ACN + 0.1% FA (d), 10% ACN + 0.05% DFA (e) and 10% ACN + 0.1% DFA (f).

S6. Plasma coagulation assay results

Plasma coagulation assay data for venom of *B. multicinctus*, separated with SEC using 6 different mobile phase compositions are shown in Figure S13. The UV data using 280 nm is shown in black with the absorption unit (AU) on the left y-axis. Anticoagulation is shown in blue with the absorption intensity on the right y-axis. The procoagulation is shown in red with the average rate on the right y-axis. The data was plotted in Prism, see SI documents Prism S3-S8 for the raw data.

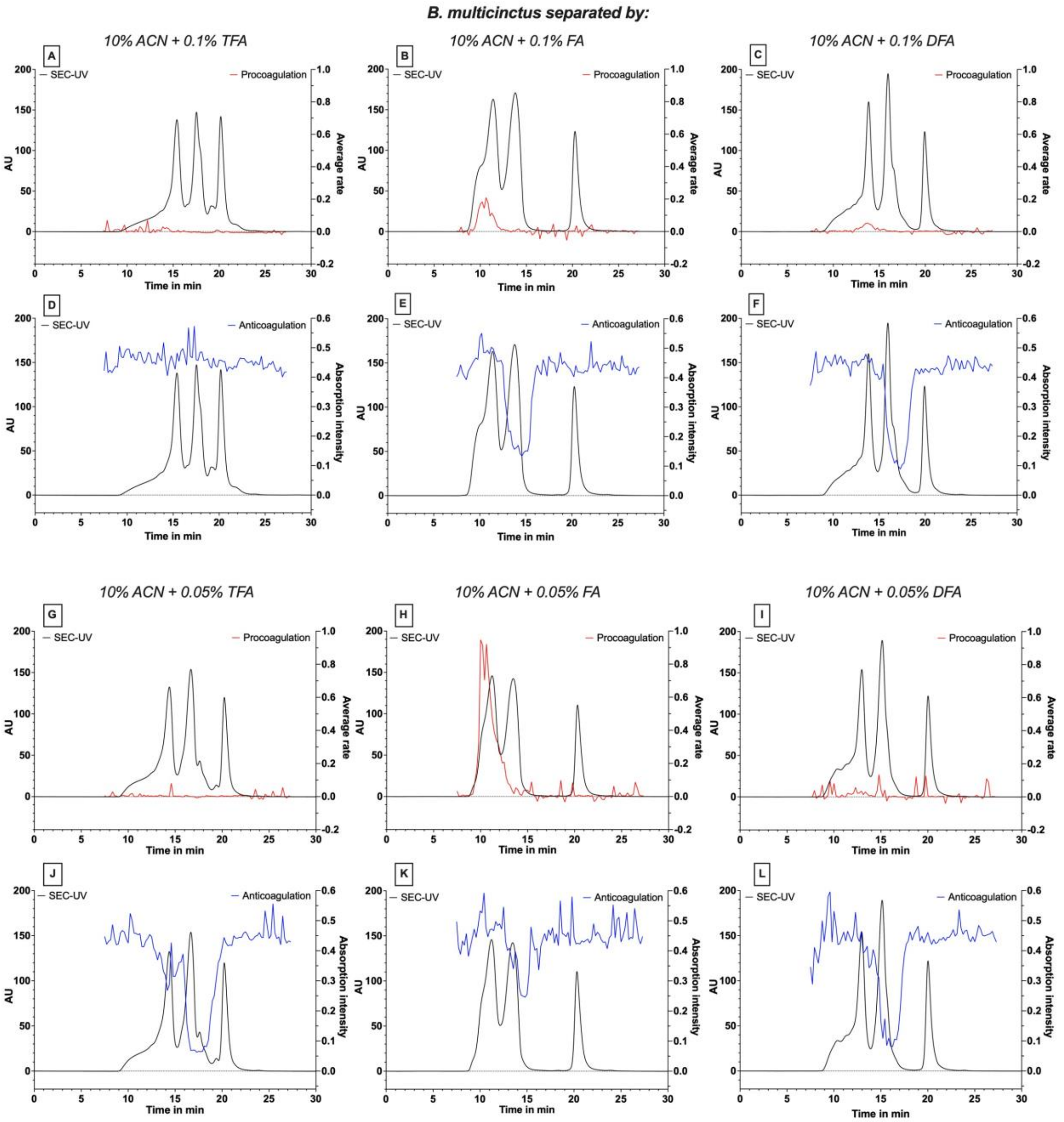


Figure S13: Plasma coagulation bioassay results of B. multicinctus venom after SEC separation, presented as bioassay chromatograms. Pro- and anticoagulation results for SEC separation using 10% ACN + 0.1% TFA (A+D), 10% ACN + 0.1% FA (B+E), 10% ACN + 0.1% DFA (C+F), 10% ACN + 0.05% TFA (G+J), 10% ACN + 0.05% FA (H+K) and 10% ACN + 0.05% DFA (I+L). as eluent.

Plasma coagulation assay data for venom of *D. russelii*, separated by SEC using 6 different mobile phase compositions are shown in Figure S14. The UV data using 280 nm is shown in black with the absorption unit (AU) on the left y-axis. Anticoagulation is shown in blue with the absorption intensity on the right y-axis. The procoagulation is shown in red with the average rate on the right y-axis. The data was plotted in Prism, see SI documents Prism S9-S14 for the raw data.

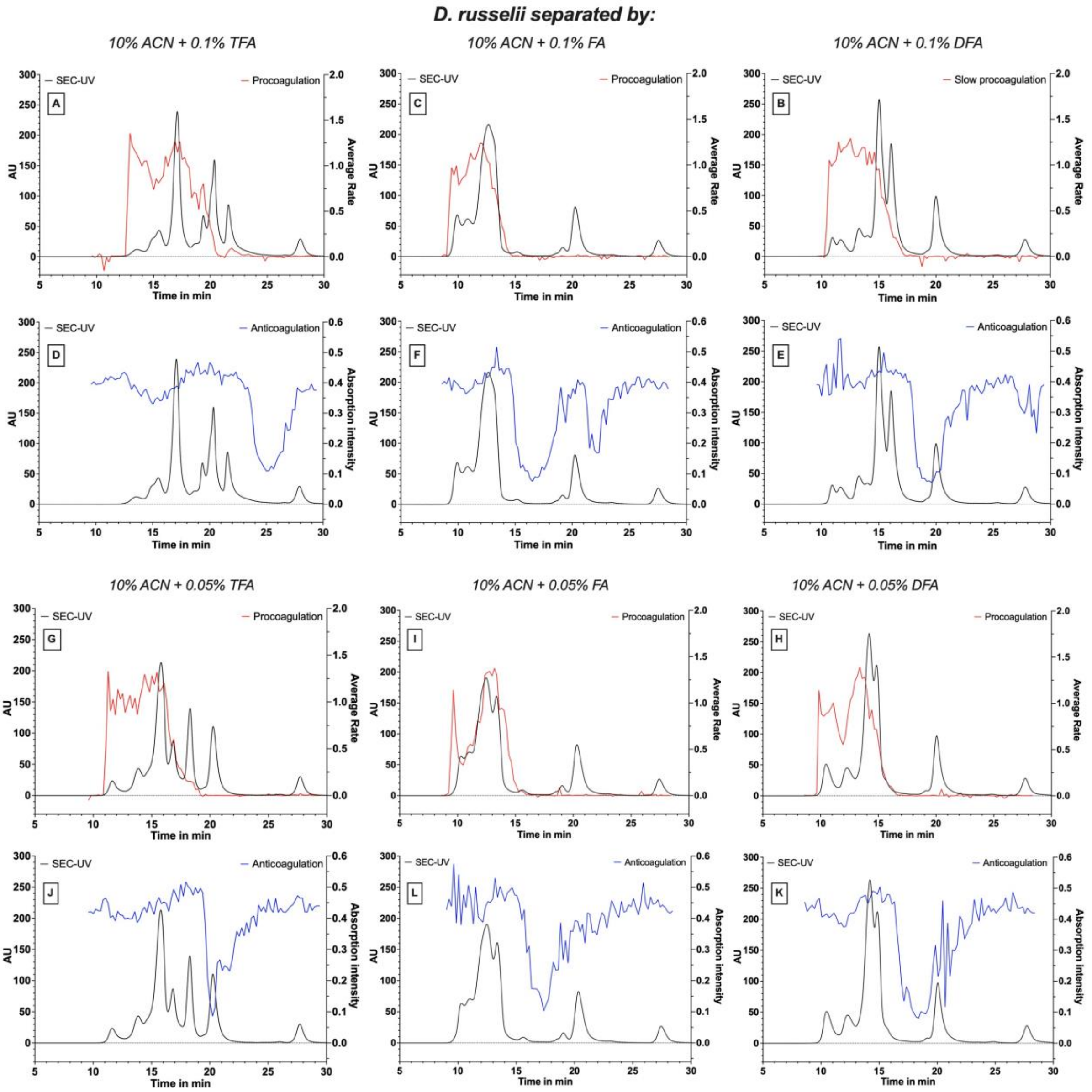


Figure S14: Plasma coagulation bioassay results of D. russelii *venom after SEC separation, presented as bioassay chromatograms. Pro- and anticoagulation results for SEC separation using 10% ACN + 0.1% TFA (A+D), 10% ACN + 0.1% FA (B+E), 10% ACN + 0.1% DFA (C+F), 10% ACN + 0.05% TFA (G+J), 10% ACN + 0.05% FA (H+K) and 10% ACN + 0.05% DFA (I+L) as eluent.*

An overlay was made of the bioassay chromatogram data (with and without marimastat added to the bioassay), UV data and proteomics data of *D. russelii* venom SEC-separated using mobile phase of 10% ACN and 0.05% DFA (See Figure S15). This overlay shows which toxins elute at the same time as that the anti- and procoagulation were detected.

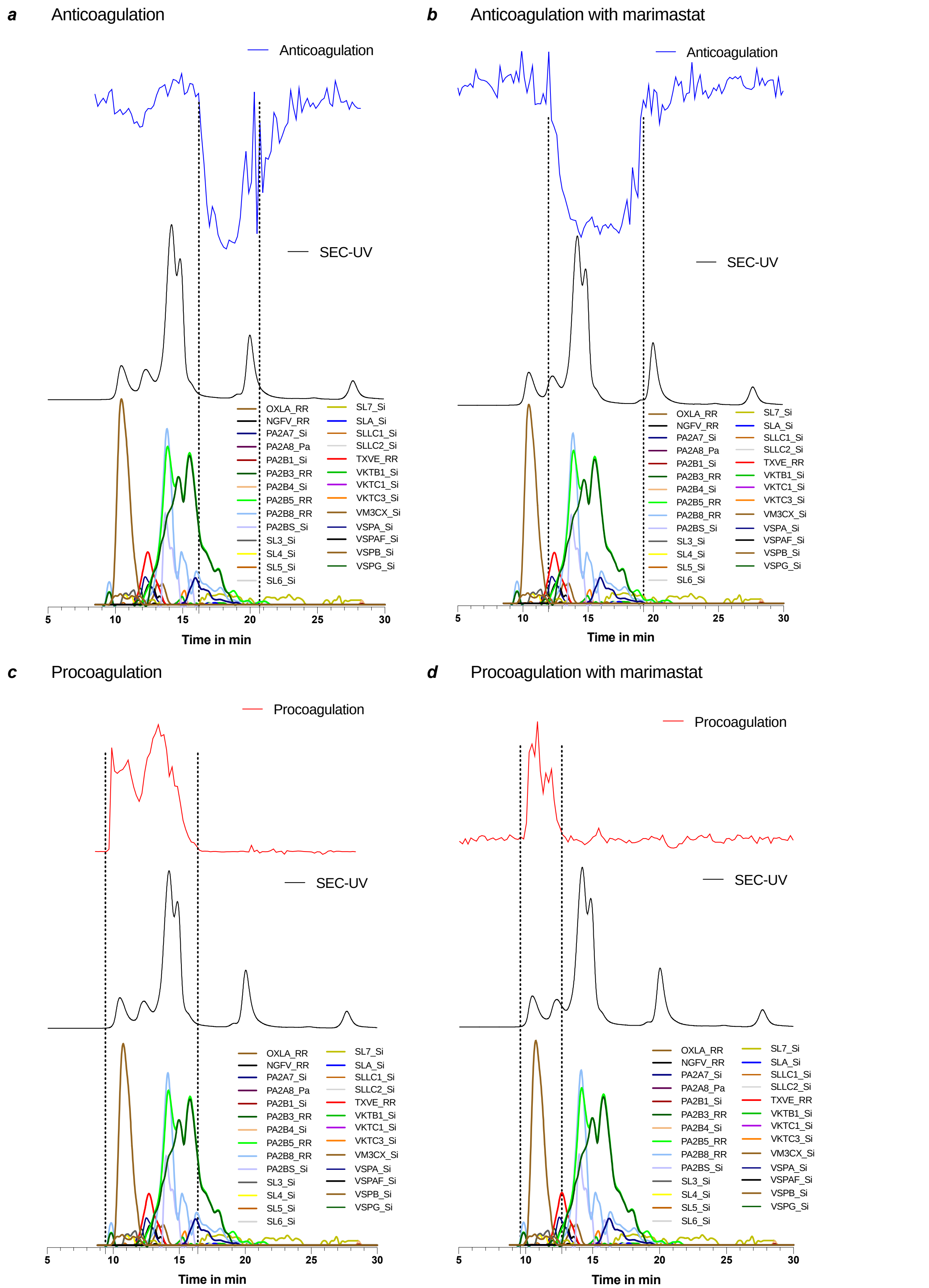


Figure S15: Overlay of the bioassay, UV and proteomics data of D. russelii venom. (a) anticoagulation with the regular assay, (b) anticoagulation with assay including marimastat, (c) procoagulation with the regular assay and (d) procoagulation with the assay including marimastat.

S7. SEC-MS raw data

Raw data including all deconvoluted masses, the TICs and EICs of the SEC-MS measurements for both venoms are represented in this SI. The TICs and EICs are visualised in PowerPoint documents, see documents PP S1 for *B. multicinctus* and PP S2 for *D. russelii*.

S7.1. Deconvolution Data

Each table contains deconvolution data for both venoms from the separation by:

- Table S3: 10% ACN + 0.1% TFA
- Table S4: 10% ACN + 0.05% TFA
- Table S5: 10% ACN + 0.1% FA
- Table S6: 10% ACN + 0.05% FA
- Table S7: 10% ACN + 0.1% DFA
- Table S8: 10% ACN + 0.05% DFA

Table S3: A list of all deconvoluted m/z values from the MS data from the SEC separation using 10% ACN + 0.1% TFA as the mobile phase. The delay of 1.2 min between the HPLC and MS systems is processed in the retention time (RT).

MS analysis of separation by 10% ACN + 0.1% TFA									
Bungarus multicinctus					*Daboia russelii*				
m/z	Charge	Mass	Intensity	RT	*m/z*	Charge	Mass	Intensity	RT
2299.5728	9	20692.54	10477	13.8 - 16.5	2276.0485	11	25029.02	2357	14.4-16.1
2306.7966	9	20744.04	10133	13.8 - 16.5	2471.3139	10	24708.68	2263	14.4-16.1
2309.6844	9	20766.03	8746	13.8 - 16.5	1943.3013	7	13598.03	290545	16.2-18.7
2301.2355	9	20702.05	6626	13.8 - 16.5	1675.3732	8	13397.81	37761	16.2-18.7
2293.5656	9	20639.05	3996	13.8 - 16.5	1938.0047	6	11624.26	35092	16.2-18.7
2327.9225	9	20934.13	2520	13.8 - 16.5	2269.8471	6	13613.49	28749	16.2-18.7
1597.706	5	7985.11	461366	16.6 - 19.4	1941.0105	7	13590.85	20373	16.2-18.7
1603.3099	5	8013.57	156205	16.6 - 19.4	1661.2399	8	13284.43	18801	16.2-18.7
1502.0522	5	7506.71	56762	16.6 - 19.4	3400.0219	8	27195.02	15173	16.2-18.7
1600.9033	5	8004.36	43791	16.6 - 19.4	2273.344	6	13637.68	9882	16.2-18.7
1328.5852	6	7967.80	31582	16.6 - 19.4	1645.1081	8	13155.45	9780	16.2-18.7
1472.2395	5	7358.38	30992	16.6 - 19.4	1921.829	6	11527.15	7242	16.2-18.7
1606.5072	5	8029.89	26188	16.6 - 19.4	1781.496	7	12465.59	6501	16.2-18.7
1475.6397	5	7374.11	25545	16.6 - 19.4	1797.6509	7	12578.68	6337	16.2-18.7
1505.6517	5	7524.79	24079	16.6 - 19.4	1950.2932	7	13646.74	23020	18.9-20.1
1817.2831	4	7267.67	18402	16.6 - 19.4	2278.6692	6	13669.99	7813	18.9-20.1
1609.7041	5	8045.34	13877	16.6 - 19.4	2283.6694	6	13700.09	6847	18.9-20.1
1591.3068	5	7954.30	10639	16.6 - 19.4	2286.1627	6	13714.97	5790	18.9-20.1
1509.2511	5	7542.92	8605	16.6 - 19.4	1887.2606	7	13204.48	5098	18.9-20.1
1461.2286	5	7302.74	8527	16.6 - 19.4	1921.7052	7	13446.94	4692	18.9-20.1
1324.2508	6	7942.81	8100	16.6 - 19.4	1865.964	7	13057.28	4180	18.9-20.1
2016.8623	4	8065.75	7156	16.6 - 19.4	1905.6975	7	13334.24	3888	18.9-20.1
1432.6773	5	7160.31	6948	16.6 - 19.4	1952.1508	7	13659.72	36240	20.2-21.3
1455.6283	5	7276.03	7584	16.6 - 19.4	1888.831	7	13217.60	6536	20.2-21.3
1586.098	5	7928.35	5882	16.6 - 19.4	1923.5616	7	13459.95	6356	20.2-21.3
1312.5748	6	7872.72	5197	16.6 - 19.4	1907.2644	7	13346.76	5352	20.2-21.3
1498.6474	5	7490.46	4953	16.6 - 19.4	1867.8196	7	13070.12	4987	20.2-21.3
1466.6364	5	7331.86	4856	16.6 - 19.4	1952.1486	7	13660.15	87564	21.3-24.2
1315.2439	6	7888.02	4624	16.6 - 19.4	1888.8278	7	13217.45	13223	21.3-24.2
1815.7536	5	9075.89	10292	19.7 - 21.5	1923.5587	7	13459.96	12670	21.3-24.2
1812.3538	5	9058.65	8887	19.7 - 21.5	2280.1671	6	13677.23	11527	21.3-24.2
1818.9556	5	9092.58	4905	19.7 - 21.5	1907.4051	7	13347.09	10412	21.3-24.2
1804.7292	5	9021.24	2002	19.7 - 21.5	1867.817	7	13070.48	10214	21.3-24.2
1807.7368	5	9037.18	1718	19.7 - 21.5	2283.8297	6	13699.64	7684	21.3-24.2
2280.1812	4	9119.06	1524	19.7 - 21.5	1950.0042	7	13654.44	7637	21.3-24.2
1804.5334	5	9019.90	5035	21.7 - 23.6					

1801.3314	5	9002.96	4218	21.7 - 23.6					
1807.531	5	9036.50	2069	21.7 - 23.6					

Table S4: A list of all deconvoluted m/z values from the MS data from the SEC separation using 10% ACN + 0.05% TFA as the mobile phase. The delay of 1.2 min between the HPLC and MS systems is processed in the retention time (RT).

MS analysis of separation by 10% ACN + 0.5% TFA									
Bungarus multicinctus					*Daboia russelii*				
m/z	Charge	Mass	Intensity	RT	*m/z*	Charge	Mass	Intensity	RT
2069.696	10	20692.26	17918	12.9 - 15.3	2471.2931	10	24708.63	6845	12.7-14.5
2075.0981	10	20746.75	17226	12.9 - 15.3	2275.9359	11	25028.79	4860	12.7-14.5
2078.795	10	20781.29	15857	12.9 - 15.3	2270.2137	11	24970.81	2651	12.7-14.5
2064.5893	10	20641.08	6515	12.9 - 15.3	2600.0398	11	28589.36	1634	12.7-14.5
2300.9931	9	20716.39	10591	12.9 - 15.3	2613.4133	11	28736.47	1570	12.7-14.5
2080.3946	10	20804.51	6390	12.9 - 15.3	1943.139	7	13597.59	337306	14.7-16.7
2088.3098	10	20878.03	4825	12.9 - 15.3	1675.3565	8	13397.66	38470	14.7-16.7
2095.0144	10	20939.10	4346	12.9 - 15.3	1945.5644	7	13612.91	30387	14.7-16.7
2093.7154	10	20927.08	4006	12.9 - 15.3	1660.979	8	13283.65	23977	14.7-16.7
1997.2383	4	7986.99	2934	12.9 - 15.3	1698.3678	8	13590.72	22882	14.7-16.7
1597.6897	5	7985.02	609885	15.4 - 17.6	1937.9851	6	11623.46	30881	14.7-16.7
1603.2942	5	8013.46	193664	15.4 - 17.6	3022.3211	9	27196.59	10527	14.7-16.7
1501.8375	5	7506.54	66192	15.4 - 17.6	1644.9664	8	13154.80	9048	14.7-16.7
1600.8867	5	8004.20	54591	15.4 - 17.6	2273.4896	6	13629.77	7567	14.7-16.7
1328.5712	6	7967.66	50668	15.4 - 17.6	1693.1145	8	13543.44	5311	14.7-16.7
1472.4244	5	7358.59	41829	15.4 - 17.6	1952.5632	7	13660.77	7218	14.7-16.7
1475.4238	5	7373.87	35817	15.4 - 17.6	1689.4908	8	13512.04	5833	14.7-16.7
1817.2656	4	7267.49	34063	15.4 - 17.6	1797.7735	7	12578.85	5636	14.7-16.7
1505.6364	5	7524.76	33304	15.4 - 17.6	1781.6207	7	12465.76	5354	14.7-16.7
1606.691	5	8030.35	30092	15.4 - 17.6	1673.4788	8	13386.00	4825	14.7-16.7
1609.6884	5	8045.40	15700	15.4 - 17.6	1687.1143	8	13493.66	4426	14.7-16.7
1326.5736	6	7956.61	15388	15.4 - 17.6	1907.3025	6	11439.93	3622	14.7-16.7
1461.2139	5	7302.79	15556	15.4 - 17.6	1950.127	7	13646.16	39312	16.8-17.8
1324.0709	6	7941.60	13963	15.4 - 17.6	1887.0921	7	13203.72	9419	16.8-17.8
1509.2359	5	7542.17	12930	15.4 - 17.6	1921.8271	7	13446.08	7551	16.8-17.8
1432.8647	5	7160.25	11286	15.4 - 17.6	1380.985	5	6900.93	7244	16.8-17.8
1455.6123	5	7275.37	12695	15.4 - 17.6	1865.942	7	13056.65	7041	16.8-17.8
1312.7281	6	7873.23	8077	15.4 - 17.6	1905.5281	7	13333.87	6365	16.8-17.8
1315.0646	6	7887.05	7627	15.4 - 17.6	1909.6624	7	13362.91	5946	16.8-17.8
1466.6217	5	7331.60	7330	15.4 - 17.6	1925.6809	7	13475.70	5681	16.8-17.8
1586.0826	5	7928.31	7213	15.4 - 17.6	1890.9469	7	13233.36	4933	16.8-17.8
1498.6337	5	7490.41	6443	15.4 - 17.6	1681.4727	8	13475.95	4175	16.8-17.8
1317.0659	6	7899.08	6135	15.4 - 17.6	1870.3649	7	13086.81	3428	16.8-17.8
1503.761	6	9020.06	3804	17.7 - 19.1	1947.9838	7	13635.14	4931	16.8-17.8
1506.591	6	9038.75	3090	17.7 - 19.1	1400.8043	5	7000.28	2957	16.8-17.8
1801.3167	5	9002.17	3144	19.4 - 21.5	1902.8106	7	13318.58	2925	16.8-17.8
					1385.5783	3	4156.02	2755	16.8-17.8
					1370.9155	3	4112.04	2650	16.8-17.8
					1839.2199	7	12868.31	2530	16.8-17.8
					1366.2435	3	4097.35	2498	16.8-17.8
					1804.0538	7	12624.86	2543	16.8-17.8
					1954.5558	7	13674.72	28866	16.8-17.8
					2735.573	5	13673.12	15745	16.8-17.8
					1952.1276	7	13659.57	210712	17.9-19.8
					1888.9499	7	13217.19	40447	17.9-19.8
					1923.3958	7	12459.42	37351	17.9-19.8
					1907.3839	7	13346.48	32220	17.9-19.8
					1867.9408	7	13070.10	31847	17.9-19.8
					1949.9831	6	11695.36	18389	17.9-19.8
					1806.2006	7	12637.61	10966	17.9-19.8
					2273.733	6	13638.28	9398	17.9-19.8
					1840.9326	7	12881.82	10768	17.9-19.8
					1904.8103	7	13332.96	10085	17.9-19.8
					2736.3724	5	13678.40	9423	17.9-19.8
					1822.069	7	12750.53	8293	17.9-19.8
					2739.9651	5	13698.74	7362	17.9-19.8
					1789.904	7	12534.39	6864	17.9-19.8
					1859.6518	7	13013.46	6635	17.9-19.8
					3035.9723	9	27321.78	5390	17.9-19.8
					2743.367	5	13714.86	4678	17.9-19.8
					1697.2291	8	13574.03	3661	17.9-19.8
					1771.6151	7	12395.07	2398	17.9-19.8

Supporting Information document for the study: Analytical Size Exclusion Chromatography Coupled with Mass Spectrometry in Parallel with High-Throughput Venomics and Bioassaying for Venom Profiling

Table S5: A list of all deconvoluted m/z values from the MS data from the SEC separation using 10% ACN + 0.1% FA as the mobile phase. The delay of 1.2 min between the HPLC and MS systems is processed in the retention time (RT).

MS analysis of separation by 10% ACN + 0.1% FA									
Bungarus multicinctus					*Daboia russelii*				
m/z	Charge	Mass	Intensity	RT	*m/z*	Charge	Mass	Intensity	RT
1724.9243	12	20692.89	44946	10.6 - 12.4	3616.0636	25	90359.88	1069	8.8-11.2
1890.0054	11	20778.12	31727	10.6 - 12.4	2200.4308	13	28592.51	2937	8.8-11.2
1886.6445	11	20739.94	36262	10.6 - 12.4	2211.6683	13	28738.59	2691	8.8-11.2
1876.9987	11	20641.38	16321	10.6 - 12.4	2267.0054	6	13597.75	242421	11.4-14.6
1726.176	12	20701.98	29614	10.6 - 12.4	1937.8281	6	11623.37	148514	11.4-14.6
1720.6681	12	20635.93	20467	10.6 - 12.4	1867.8105	7	13069.9	89962	11.4-14.6
1730.4241	12	20753.00	41991	10.6 - 12.4	1476.7549	9	13283.96	9596	11.4-14.6
1745.9342	12	20939.12	9310	10.6 - 12.4	1888.9642	7	13217.81	79290	11.4-14.6
1926.1181	7	13477.62	7099	10.6 - 12.4	1822.2255	7	12750.77	64814	11.4-14.6
1820.3219	4	7278.01	4796	10.6 - 12.4	1906.9725	7	13345.46	57179	11.4-14.6
1748.1003	12	20969.91	7445	10.6 - 12.4	1644.9761	8	13154.76	53266	11.4-14.6
1597.6921	5	7985.20	552058	12.6 - 16.9	1947.8313	6	11688.66	47754	11.4-14.6
1603.2969	5	8013.53	331287	12.6 - 16.9	1671.7141	7	11697.34	99270	11.4-14.6
1877.0481	4	7506.61	142368	12.6 - 16.9	1909.3096	6	11451.44	40424	11.4-14.6
1831.9035	7	12819.56	105834	12.6 - 16.9	1934.1548	6	11608.25	34084	11.4-14.6
1815.7433	5	9076.14	57326	12.6 - 16.9	1683.1076	8	13459.78	36644	11.4-14.6
1505.6379	5	7524.74	49234	12.6 - 16.9	1804.0717	7	12630.49	36898	11.4-14.6
1472.6258	5	7358.46	41910	12.6 - 16.9	1797.7833	7	12579.22	35370	11.4-14.6
1600.6827	5	8000.34	40285	12.6 - 16.9	1781.3435	7	12465.41	34300	11.4-14.6
1454.0115	5	7267.31	37139	12.6 - 16.9	1921.8232	6	11527.05	33600	11.4-14.6
1812.7417	5	9058.70	36682	12.6 - 16.9	2279.827	6	13674.56	30907	11.4-14.6
1834.3302	7	12834.26	33583	12.6 - 16.9	1934.1548	6	11600.65	34084	11.4-14.6
1799.4566	7	12591.65	32700	12.6 - 16.9	1474.758	9	13267.29	6984	11.4-14.6
1328.5711	6	7967.56	30352	12.6 - 16.9	1771.4818	7	12395.34	26785	11.4-14.6
1606.6904	5	8030.27	30112	12.6 - 16.9	1787.7729	7	12512.57	26853	11.4-14.6
1609.6881	5	8045.69	24347	12.6 - 16.9	1904.9689	7	13331.35	23262	11.4-14.6
1819.3381	5	9092.45	22277	12.6 - 16.9	1487.4342	9	13388.60	5687	11.4-14.6
1804.7227	5	9019.86	21688	12.6 - 16.9	1675.4914	8	13397.78	187178	11.4-14.6
1508.6358	5	7539.39	19723	12.6 - 16.9	1968.677	6	11806.23	20734	11.4-14.6
2250.8969	4	9002.24	7401	12.6 - 16.9	1978.3442	6	11866.06	19978	11.4-14.6
1461.6123	5	7305.08	18214	12.6 - 16.9	1882.1326	6	11290.30	32331	11.4-14.6
1326.5723	6	7955.35	15317	12.6 - 16.9	1997.1928	6	11979.34	18808	11.4-14.6
1498.6301	5	7490.40	15306	12.6 - 16.9	1762.9083	7	12336.14	17815	11.4-14.6
1475.6121	5	7376.84	15242	12.6 - 16.9	1820.0836	7	12741.02	20416	11.4-14.6
					1689.4974	8	13510.89	16963	11.4-14.6
					1838.9462	7	12872.08	21344	11.4-14.6
					1987.5243	6	11920.52	15473	11.4-14.6
					1886.9642	7	13207.28	21836	11.4-14.6
					2283.3266	6	13696.69	11155	11.4-14.6
					1931.4869	6	11584.95	14385	11.4-14.6
					1634.8392	7	11441.06	14123	11.4-14.6
					1865.9566	7	13058.45	31419	11.4-14.6
					1509.6674	9	13588.78	13739	11.4-14.6
					1813.9362	7	12692.86	13677	11.4-14.6
					1832.7988	7	12824.33	13214	11.4-14.6
					1753.0476	7	12265.77	12624	11.4-14.6
					1681.2322	8	13447.08	13960	11.4-14.6
					1769.1957	7	12383.31	10809	11.4-14.6
					1744.6156	7	12206.86	10789	11.4-14.6
					2028.2127	6	12164.73	10039	11.4-14.6
					1949.0735	7	13637.83	38551	14.8-16.2
					1916.4848	7	13411.10	12864	14.8-16.2
					1877.3241	7	13135.64	9422	14.8-16.2
					1895.6175	7	13263.71	6157	14.8-16.2
					1656.538	8	13246.01	4896	14.8-16.2
					1856.1689	7	12988.34	4565	14.8-16.2
					1543.3413	9	13883.42	19743	21.1-23.4
					1672.4221	7	11703.31	2184	21.1-23.4
					1698.8611	7	11887.53	1806	21.1-23.4
					1689.4857	8	13508.40	1264	21.1-23.4
					1531.7797	9	13778.90	1240	21.1-23.4

Supporting Information document for the study: Analytical Size Exclusion Chromatography Coupled with Mass Spectrometry in Parallel with High-Throughput Venomics and Bioassaying for Venom Profiling

Table 6: A list of all deconvoluted m/z values from the MS data from the SEC separation using 10% ACN + 0.05% FA as the mobile phase. The delay of 1.2 min between the HPLC and MS systems is processed in the retention time (RT).

MS analysis of separation by 10% ACN + 0.05% FA									
Bungarus multicinctus					*Daboia russelii*				
m/z	Charge	Mass	Intensity	RT	*m/z*	Charge	Mass	Intensity	RT
1544.2289	9	13882.09	5103	1.0 - 8.4	2383.4758	13	30972.09	3884	9.3-11.3
1699.0003	7	11886.95	913	1.0 - 8.4	2359.3129	13	30657.96	2294	9.3-11.3
1707.6169	8	13652.88	2023	1.0 - 8.4	1926.0457	13	25019.41	5068	9.3-11.3
1724.9206	12	20686.96	32912	8.6 - 12.2	1784.2534	14	24949.51	3243	9.3-11.3
1729.3396	12	20726.90	31875	8.6 - 12.2	3477.0974	26	90378.34	2029	9.3-11.3
1592.2346	12	19082.67	22717	8.6 - 12.2	2266.8402	6	13585.95	248619	11.3-13
1732.4201	12	20776.95	27566	8.6 - 12.2	1675.4924	8	13386.84	180504	11.3-13
1740.5956	12	20875.06	10856	8.6 - 12.2	1937.8294	6	11612.90	176089	11.3-13
1720.582	12	20634.90	14787	8.6 - 12.2	1644.9787	6	13144.74	56234	11.3-13
1456.4546	5	7277.24	6715	8.6 - 12.2	1658.8541	8	13260.74	42097	11.3-13
1726.0032	12	20696.87	21123	8.6 - 12.2	1921.6538	6	11515.84	39255	11.3-13
1597.6872	5	7978.38	482007	12.4 - 15.9	1781.4879	7	12456.34	36925	11.3-13
1603.292	5	8005.39	264255	12.4 - 15.9	1797.6428	7	12569.42	35263	11.3-13
1877.2931	4	7499.12	131493	12.4 - 15.9	1934.9907	6	11598.88	26962	11.3-13
1832.0409	7	12808.20	95572	12.4 - 15.9	1968.6775	6	11799.99	21403	11.3-13
1815.7375	5	9066.61	52441	12.4 - 15.9	1907.319	6	11431.84	20113	11.3-13
1505.633	5	7517.11	40901	12.4 - 15.9	1762.91	7	12328.29	18073	11.3-13
1472.6201	5	1352.05	35018	12.4 - 15.9	1673.1374	8	13368.80	17918	11.3-13
1812.3335	5	9049.60	34012	12.4 - 15.9	1987.5278	6	11912.08	17225	11.3-13
1600.6774	5	7992.36	32324	12.4 - 15.9	1626.8444	8	12997.67	17053	11.3-13
1799.4518	7	12581.08	29672	12.4 - 15.9	1879.9718	6	11269.77	34503	11.3-13
1328.5665	6	7959.34	24706	12.4 - 15.9	1689.5003	8	13500.91	14225	11.3-13
1606.6853	5	8022.37	24080	12.4 - 15.9	2269.6692	6	13603.94	15362	11.3-13
1834.1824	7	12826.20	28376	12.4 - 15.9	1509.7811	9	13568.91	15387	11.3-13
1818.7361	5	9083.62	22179	12.4 - 15.9	1832.6586	7	12815.54	12486	11.3-13
1609.6836	5	8037.36	21395	12.4 - 15.9	1744.6172	7	12199.25	12145	11.3-13
1804.716	5	9012.52	19695	12.4 - 15.9	1642.8479	8	13125.69	12031	11.3-13
1454.1993	5	7259.92	18345	12.4 - 15.9	3022.2265	9	27187.92	11940	11.3-13
1461.6061	5	7304.45	15318	12.4 - 15.9	1865.4659	6	11180.73	11259	11.3-13
1498.6257	5	7489.37	13846	12.4 - 15.9	1918.814	6	11498.82	10113	11.3-13
1822.7507	4	7287.04	13513	12.4 - 15.9	2277.3278	6	13648.87	271355	13.1-15.1
1807.7162	5	9035.88	12415	12.4 - 15.9	1949.9954	6	11687.90	227528	13.1-15.1
1476.6116	5	7377.41	11526	12.4 - 15.9	1806.0702	7	12628.41	166219	13.1-15.1
1326.5671	6	7954.37	11518	12.4 - 15.9	1867.9539	7	13059.57	126591	13.1-15.1
					1888.8186	7	13206.64	110394	13.1-15.1
					1789.9156	7	12514.32	106364	13.1-15.1
					1822.2238	7	12741.48	94585	13.1-15.1
					1840.9438	7	12872.52	93268	13.1-15.1
					1909.3092	6	11444.78	65099	13.1-15.1
					1907.2523	7	13337.69	59208	13.1-15.1
					1933.987	6	11589.85	52979	13.1-15.1
					1683.1056	8	13450.77	51544	13.1-15.1
					1882.132	6	11278.71	48848	13.1-15.1
					1771.4796	7	12385.28	42055	13.1-15.1
					1859.8049	7	13003.54	32577	13.1-15.1
					1978.5096	6	11857.98	30611	13.1-15.1
					1946.9892	6	11667.85	29751	13.1-15.1
					1997.3572	6	11971.07	28346	13.1-15.1
					1787.3402	7	12496.29	24461	13.1-15.1
					1904.6789	7	13318.62	22184	13.1-15.1
					1966.5041	6	11786.95	20531	13.1-15.1
					1752.9009	7	12257.24	18522	13.1-15.1
					1803.35	7	12608.37	17531	13.1-15.1
					2028.209	6	12157.19	14871	13.1-15.1
					1930.9763	6	11571.80	10894	13.1-15.1
					1768.7608	7	12366.24	10862	13.1-15.1
					1948.9279	7	13627.41	60796	15.2-16.6
					1916.6246	7	13400.28	32763	15.2-16.6
					1877.1779	7	13124.16	24573	15.2-16.6
					1895.4718	7	13252.21	16466	15.2-16.6
					1856.1688	7	12977.09	13483	15.2-16.6
					1656.4099	8	13236.19	12904	15.2-16.6
					1703.4331	8	13609.37	7282	15.2-16.6

Table S7: A list of all deconvoluted m/z values from the MS data from the SEC separation using 10% ACN + 0.1% DFA as the mobile phase. The delay of 1.2 min between the HPLC and MS systems is processed in the retention time (RT).

MS analysis of separation by 10% ACN + 0.1% DFA									
Bungarus multicinctus					*Daboia russelii*				
m/z	Charge	Mass	Intensity	RT	*m/z*	Charge	Mass	Intensity	RT
2076.1062	10	20754.35	31737	13.1 - 14.9	2246.8255	11	24707.53	12286	12.8-14.2
2069.7038	10	20692.41	31687	13.1 - 14.9	2086.2859	12	25028.50	7696	12.8-14.2
2078.8038	10	20781.59	25006	13.1 - 14.9	2081.3689	12	24971.60	5007	12.8-14.2
2073.8006	10	20736.72	18832	13.1 - 14.9	2383.467	12	28595.25	4791	12.8-14.2
2064.3973	10	20639.60	12394	13.1 - 14.9	2395.8855	12	28742.11	4708	12.8-14.2
1898.6496	11	20878.93	7814	13.1 - 14.9	1700.5077	8	13597.65	608257	14.3-15.9
1904.6531	11	20933.37	7724	13.1 - 14.9	1675.3674	6	13397.60	107574	14.3-15.9
1906.9291	11	20962.19	6300	13.1 - 14.9	1937.9971	6	11623.59	95271	14.3-15.9
1775.2185	9	15972.18	6023	13.1 - 14.9	1476.7566	9	13284.17	7456	14.3-15.9
1778.2206	9	15998.96	5808	13.1 - 14.9	1698.504	8	13591.07	35761	14.3-15.9
1331.5787	6	7985.08	661916	15.1 - 19.0	1702.3811	8	13612.71	33649	14.3-15.9
1336.2486	6	8013.45	297208	15.1 - 19.0	1644.9761	8	13154.79	28960	14.3-15.9
1328.5747	6	7967.57	92845	15.1 - 19.0	1934.9944	6	11605.54	16782	14.3-15.9
1502.04	5	7506.73	67672	15.1 - 19.0	1879.9718	6	11275.98	22571	14.3-15.9
1334.2428	6	8004.70	60782	15.1 - 19.0	1921.6519	6	11525.91	19557	14.3-15.9
1831.9018	7	12819.86	59691	15.1 - 19.0	1781.488	7	12465.39	18580	14.3-15.9
1338.9125	6	8030.06	35147	15.1 - 19.0	1797.5427	7	12578.61	18480	14.3-15.9
1513.4491	6	9076.08	32849	15.1 - 19.0	1707.2487	8	13650.10	14803	14.3-15.9
1461.4169	5	7304.38	31181	15.1 - 19.0	1673.2392	8	13387.22	12538	14.3-15.9
1472.427	5	7358.86	30190	15.1 - 19.0	1689.6264	8	13511.86	11359	14.3-15.9
1505.6386	6	7524.89	30455	15.1 - 19.0	1763.0537	7	12335.88	10082	14.3-15.9
1817.2681	4	7267.69	29655	15.1 - 19.0	1907.3175	6	11439.21	9673	14.3-15.9
1326.5766	6	7957.04	29360	15.1 - 19.0	1987.529	6	11920.75	9634	14.3-15.9
1510.4478	6	9058.15	25042	15.1 - 19.0	1952.1352	7	13659.66	212225	15.9-17.7
1341.5753	6	8045.26	24831	15.1 - 19.0	1888.9586	7	13217.30	93556	15.9-17.7
1834.3295	7	12834.79	24495	15.1 - 19.0	1867.8056	7	13070.06	78397	15.9-17.7
1475.6254	5	7374.87	23844	15.1 - 19.0	1907.2486	7	13346.52	71325	15.9-17.7
1324.0736	6	7941.49	23702	15.1 - 19.0	1923.4028	7	13459.52	70786	15.9-17.7
1455.6152	5	7276.08	24232	15.1 - 19.0	1949.8228	6	11695.96	39561	15.9-17.7
1312.5637	6	7872.25	16537	15.1 - 19.0	1806.0657	7	12637.59	33803	15.9-17.7
1516.1177	6	9092.61	16359	15.1 - 19.0	1841.082	7	12882.07	27846	15.9-17.7
1466.6217	5	7331.18	14858	15.1 - 19.0	1822.2192	7	12750.80	23856	15.9-17.7
1315.067	6	78867.00	14535	15.1 - 19.0	1904.6741	7	13327.37	21600	15.9-17.7
1321.5708	6	7926.60	14101	15.1 - 19.0	1790.0525	7	12524.59	20106	15.9-17.7
1458.6125	5	7290.88	12372	15.1 - 19.0	1859.8013	7	13013.97	15478	15.9-17.7
1317.5667	6	7901.60	12301	15.1 - 19.0	1380.7899	5	6901.10	10013	15.9-17.7
1509.2398	5	7541.91	12373	15.1 - 19.0					
1310.0637	6	7856.86	11877	15.1 - 19.0					
1319.5696	6	7614.60	11740	15.1 - 19.0					
1767.7402	4	7069.46	9246	15.1 - 19.0					

Table S8: A list of all deconvoluted m/z values from the MS data from the SEC separation using 10% ACN + 0.05% DFA as the mobile phase. The delay of 1.2 min between the HPLC and MS systems is processed in the retention time (RT).

MS analysis of separation by 10% ACN + 0.05% DFA									
Bungarus multicinctus					*Daboia russelii*				
m/z	Charge	Mass	Intensity	RT	*m/z*	Charge	Mass	Intensity	RT
2076.1957	10	20742.95	32771	12.2 - 14.0	2246.7402	11	24707.61	19572	11.6-13.1
1889.9029	11	20771.36	26195	12.2 - 14.0	2086.3749	12	25028.51	9479	11.6-13.1
2073.7902	10	20731.17	20644	12.2 - 14.0	2395.7254	12	28741.21	6644	11.6-13.1
1775.2079	9	15972.30	9832	12.2 - 14.0	2081.2889	12	24968.83	5352	11.6-13.1
1778.4337	9	15999.66	9285	12.2 - 14.0	1700.5064	8	13597.64	500958	13.2-17.1
1881.631	11	20678.79	34658	12.2 - 14.0	1952.1393	7	13660.12	175840	13.2-17.1
2071.0899	10	20699.51	22965	12.2 - 14.0	1675.4915	8	13397.71	88561	13.2-17.1
2064.4867	10	20630.37	14394	12.2 - 14.0	1888.8194	7	13217.29	80415	13.2-17.1
1331.5703	6	7985.01	778230	14.1 - 17.5	1867.8097	7	13070.09	66804	13.2-17.1
1336.2402	6	8013.36	333137	14.1 - 17.5	1907.8965	7	13347.26	62611	13.2-17.1
1328.5662	6	7967.54	108420	14.1 - 17.5	1923.5511	7	13460.06	61103	13.2-17.1
1501.8299	5	7506.54	71957	14.1 - 17.5	1937.9947	6	11623.83	59269	13.2-17.1
1832.033	7	12820.15	68372	14.1 - 17.5	1660.9881	7	13284.34	51333	13.2-17.1
1334.2348	6	8004.30	66483	14.1 - 17.5	1954.4234	7	13675.37	35276	13.2-17.1
1817.5076	4	7268.18	43257	14.1 - 17.5	1671.7132	7	11696.93	21945	13.2-17.1
1513.4401	6	9076.04	40321	14.1 - 17.5	1698.376	8	13590.71	29872	13.2-17.1
1461.4076	5	7304.15	36285	14.1 - 17.5	1702.5029	8	13613.52	25194	13.2-17.1
1338.9041	6	8029.64	36028	14.1 - 17.5	1645.2247	8	13155.52	20725	13.2-17.1
1326.5685	6	7956.94	32951	14.1 - 17.5	1789.9148	7	12524.46	18784	13.2-17.1
1505.6286	5	7524.81	32427	14.1 - 17.5	1859.6641	7	13012.34	15576	13.2-17.1
1510.4384	6	9058.21	31460	14.1 - 17.5	1891.1043	7	13233.13	14491	13.2-17.1
1472.6171	5	7359.27	27850	14.1 - 17.5	1797.6405	7	12578.77	13958	13.2-17.1
1324.0655	6	7941.41	27089	14.1 - 17.5	1658.8521	8	13269.48	13747	13.2-17.1
1341.5674	6	8045.40	27085	14.1 - 17.5	1781.4861	7	12465.59	13234	13.2-17.1
1834.3175	7	12834.45	26184	14.1 - 17.5	1956.9981	7	13695.02	12083	13.2-17.1
1475.614	5	7375.20	20280	14.1 - 17.5	1934.9906	7	13542.30	11626	13.2-17.1
1516.1084	6	9092.37	19015	14.1 - 17.5	1886.9637	7	13206.40	22833	13.2-17.1
1312.5555	6	7872.19	18656	14.1 - 17.5	1698.376	8	13576.21	29872	13.2-17.1
1248.8552	6	7489.84	16878	14.1 - 17.5	1685.2335	8	13475.73	10861	13.2-17.1
1315.2253	6	7887.50	16451	14.1 - 17.5	1804.0695	7	12629.33	10686	13.2-17.1
1321.7324	6	7927.27	15793	14.1 - 17.5	1870.0967	7	13085.40	11350	13.2-17.1
1317.5594	6	7901.46	14302	14.1 - 17.5	1806.0698	7	12639.61	32572	13.2-17.1
1458.6034	5	7290.97	13910	14.1 - 17.5	1689.4988	8	13511.50	9911	13.2-17.1
1319.5615	6	7914.61	13382	14.1 - 17.5	2176.9441	6	13055.74	4591	13.2-17.1
					1838.9457	7	12874.62	8248	13.2-17.1
					1959.6976	7	13713.97	8122	13.2-17.1
					1881.9648	6	11287.91	7911	13.2-17.1
					1694.8729	8	13556.13	7841	13.2-17.1
					1902.8227	7	13320.35	7490	13.2-17.1
					1820.3683	7	12743.59	6971	13.2-17.1
					1771.4798	7	12884.33	6711	13.2-17.1
					1987.5267	6	11920.65	6693	13.2-17.1
					1787.7729	7	12512.23	7056	13.2-17.1

S7.2. TICs and EICs

Each figure contains the TIC and EICs for one specific venom separation. The upper figure shows the TIC while the figure below shows the corresponding EICs. The delay of 1.2 min between the HPLC system and the MS is not converted in this data. The EICs m/z values are listed below the figure and divided into the RT section that was taken for deconvolution per peak. Figures S16-S21 show data from the *B. multicinctus* venom, while Figures S22-S27 show data for the *D. russelii* venom.

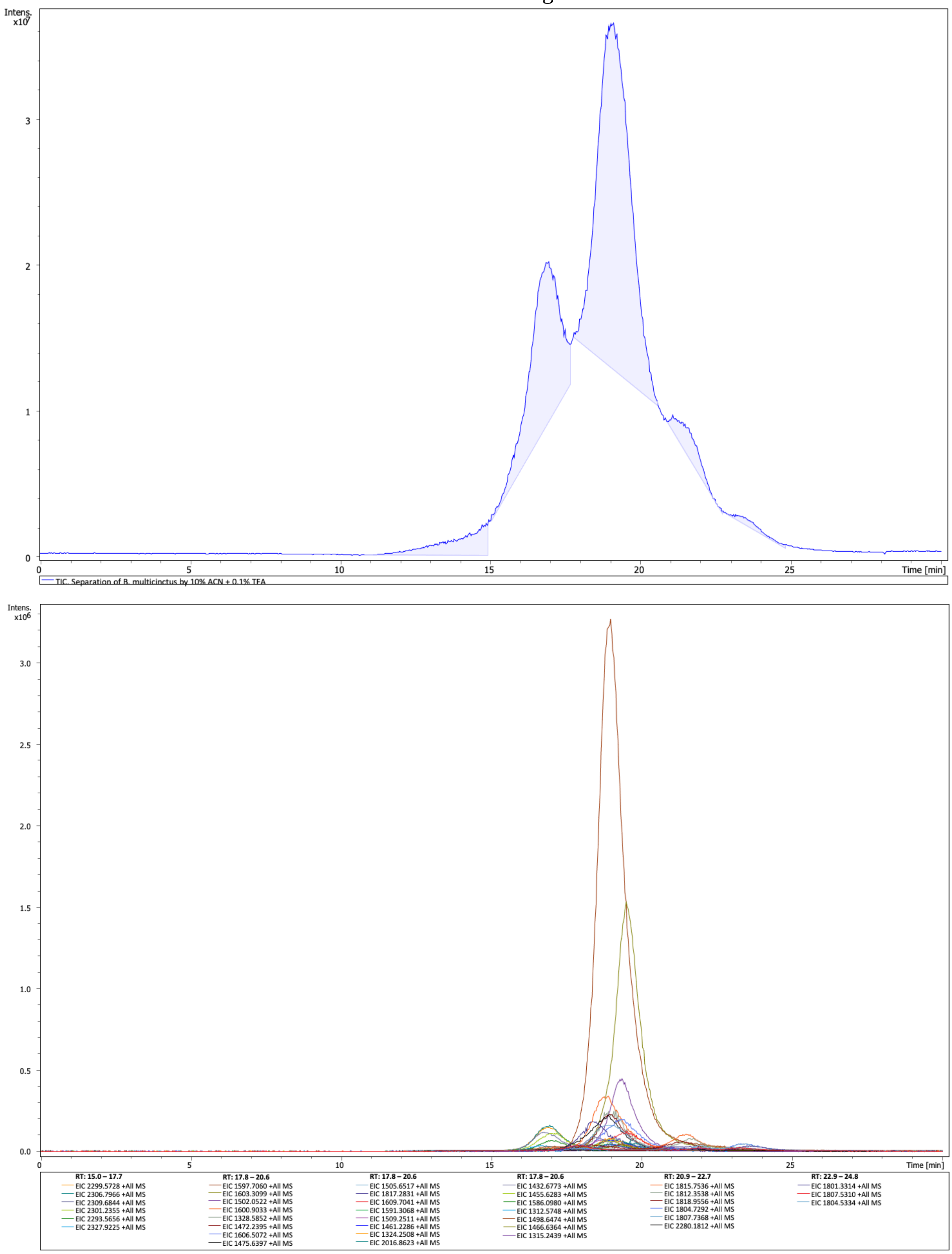


Figure S16: TIC and EICs of SEC-MS measurement of B. multicinctus venom separated using 10% ACN + 0.1% TFA.

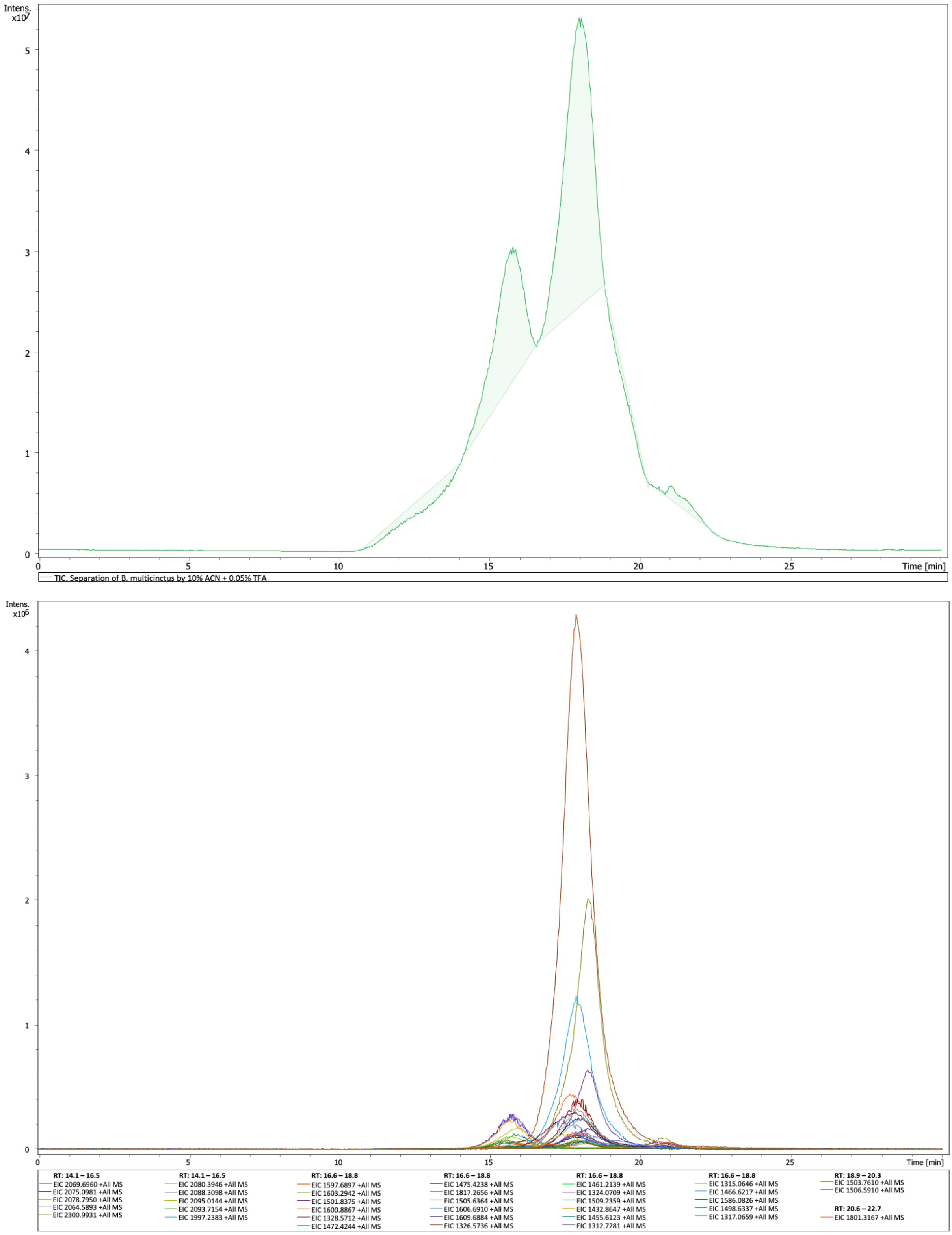


Figure 17: TIC and EICs of SEC-MS measurement of B. multicinctus venom separated using 10% ACN + 0.05% TFA.

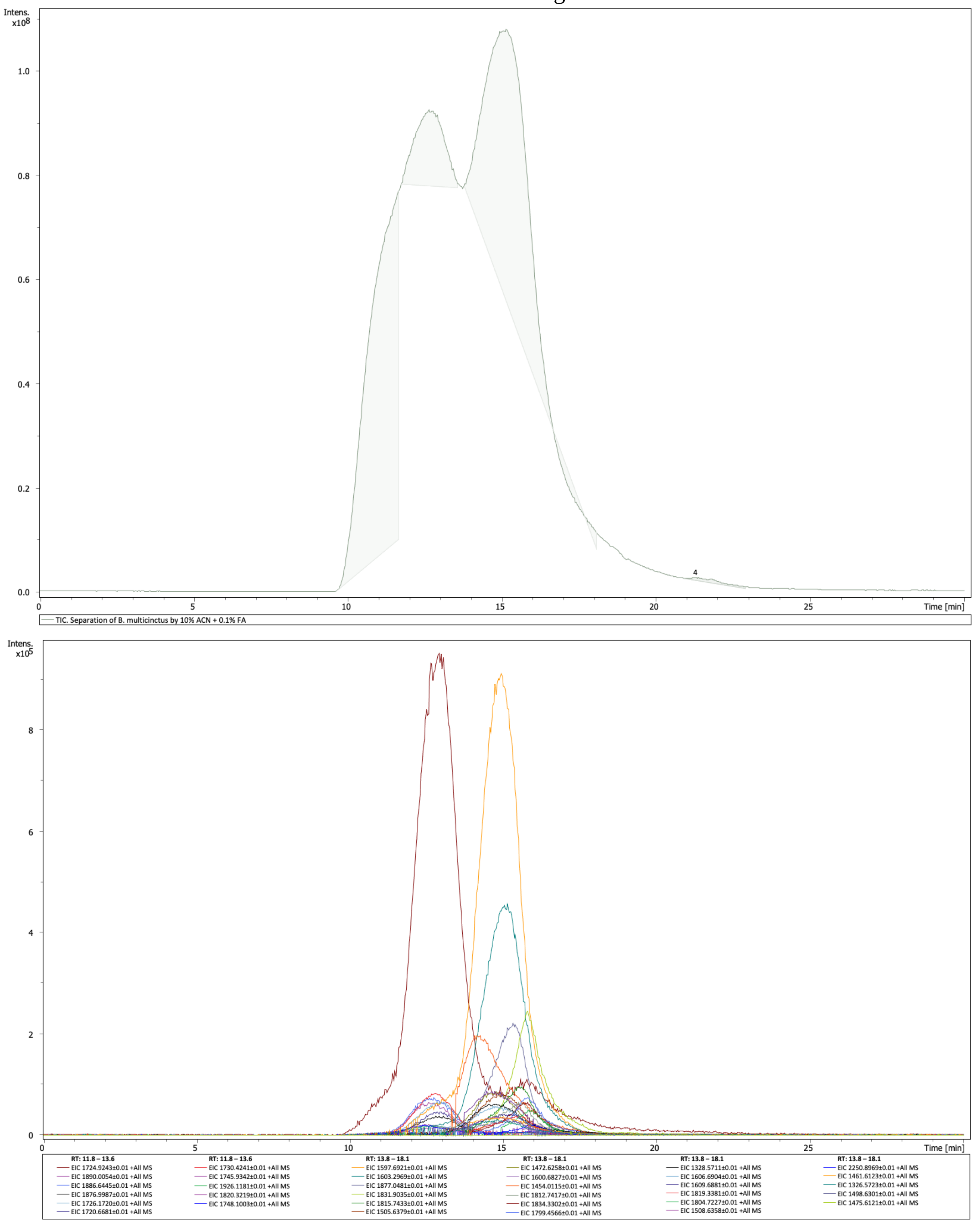
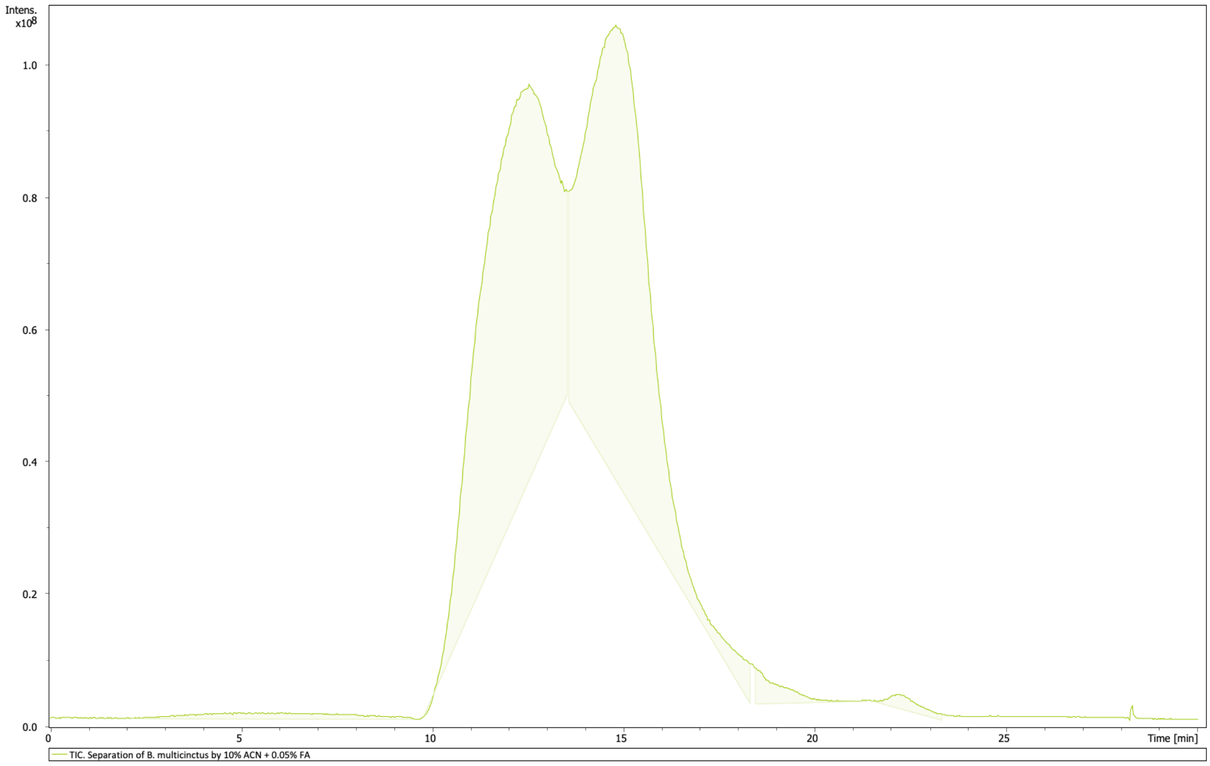


Figure S18: TIC and EICs of SEC-MS measurement of B. multicinctus venom separated using 10% ACN + 0.1% FA.



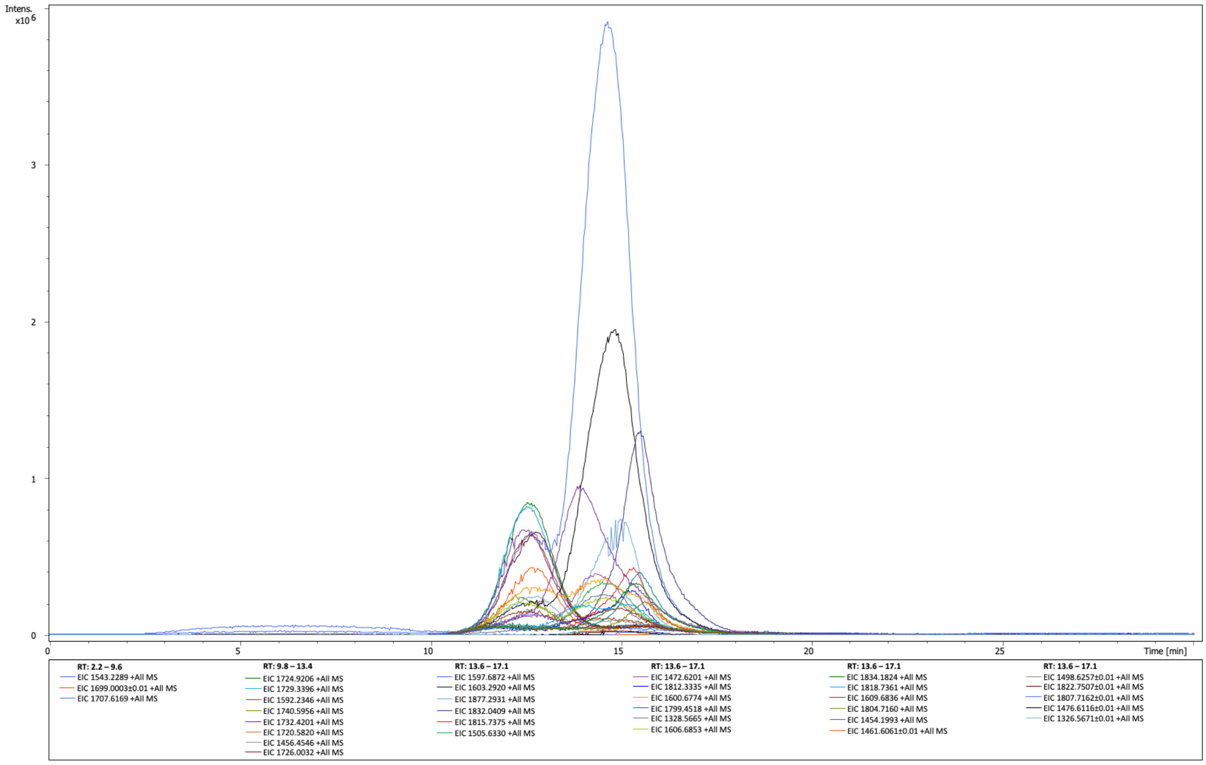


Figure S19: TIC and EICs of SEC-MS measurement of B. multicinctus venom separated using 10% ACN + 0.05% FA.

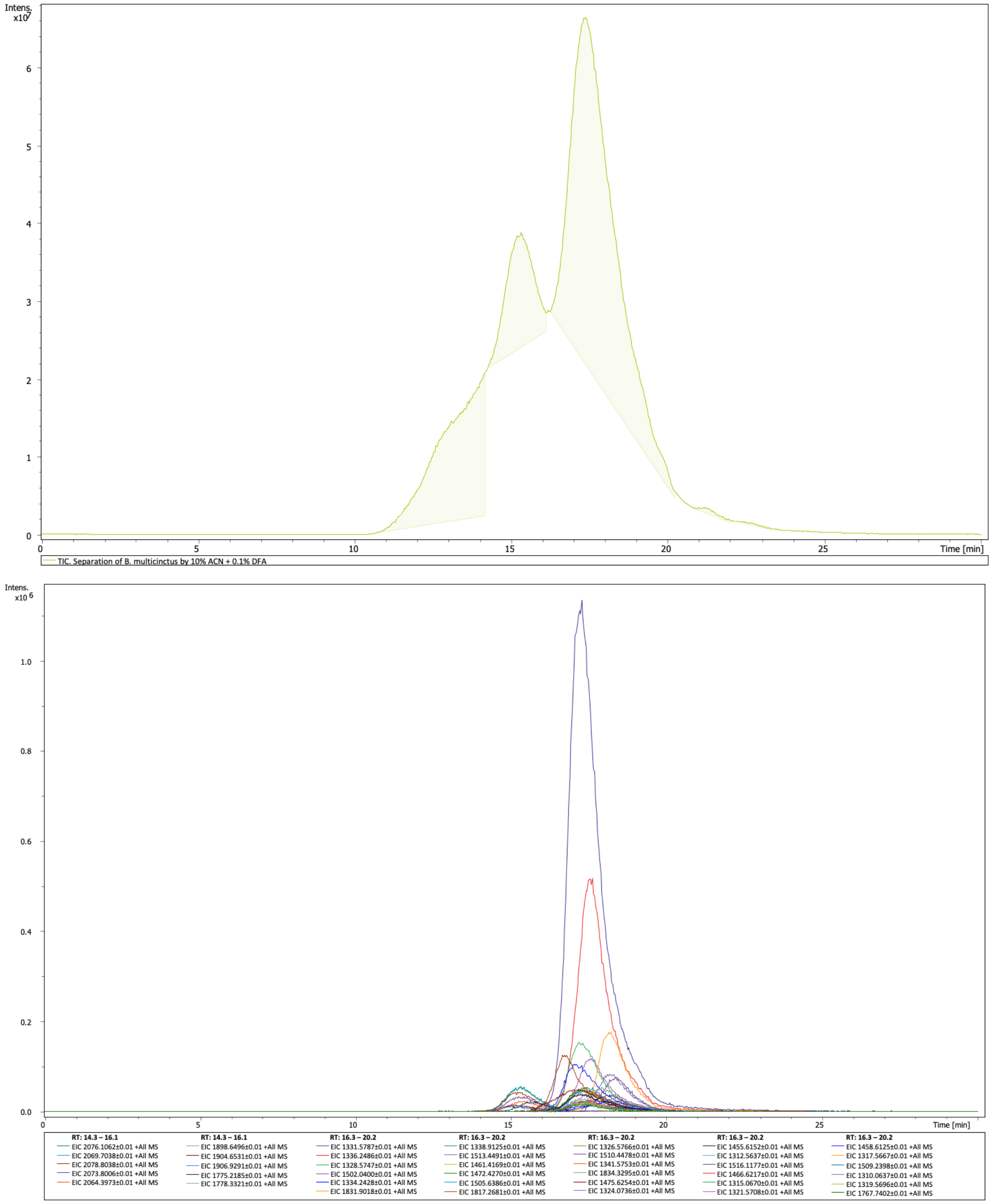


Figure S20: TIC and EICs of SEC-MS measurement of B. multicinctus venom separated using 10% ACN + 0.1% DFA.

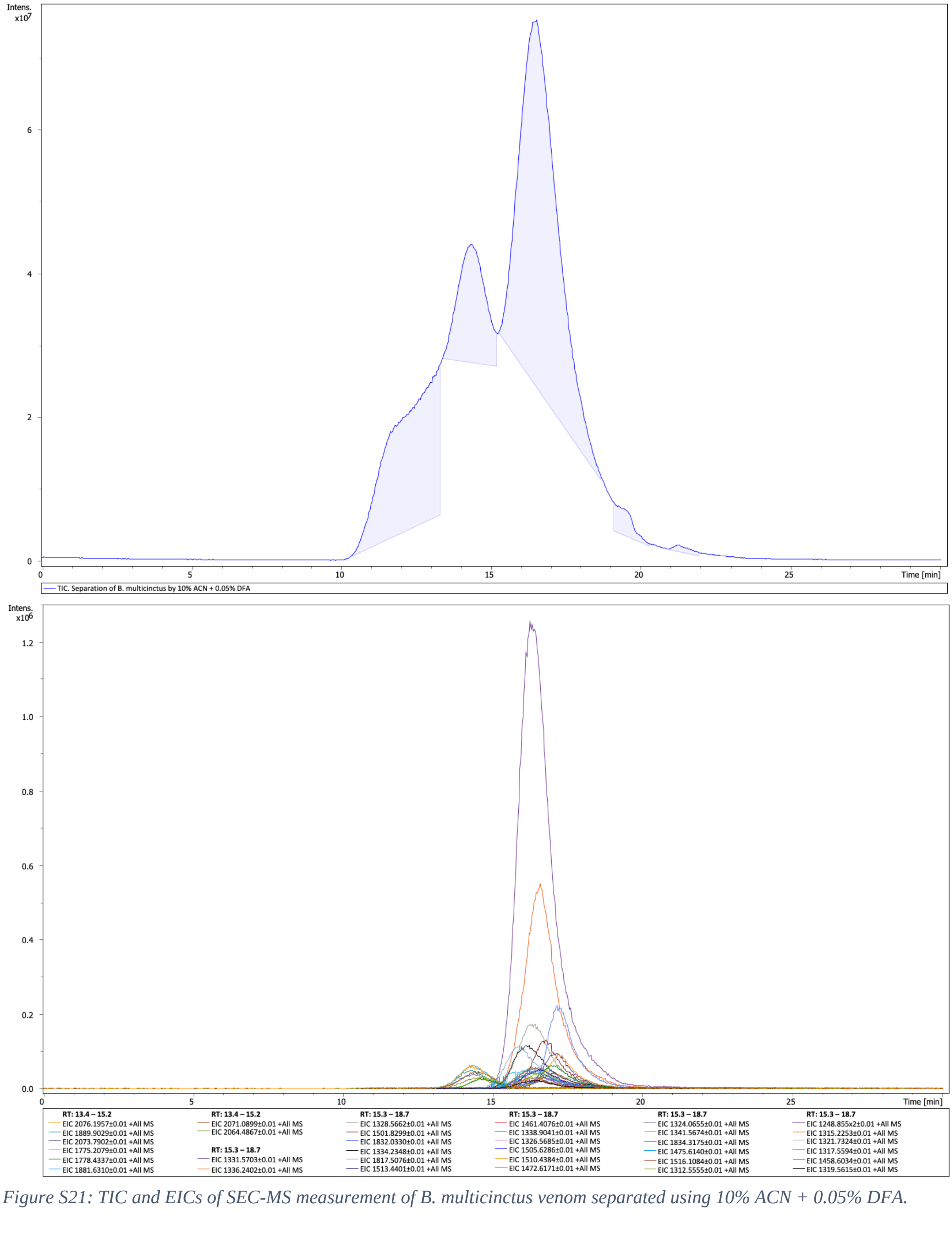


Figure S21: TIC and EICs of SEC-MS measurement of B. multicinctus venom separated using 10% ACN + 0.05% DFA.

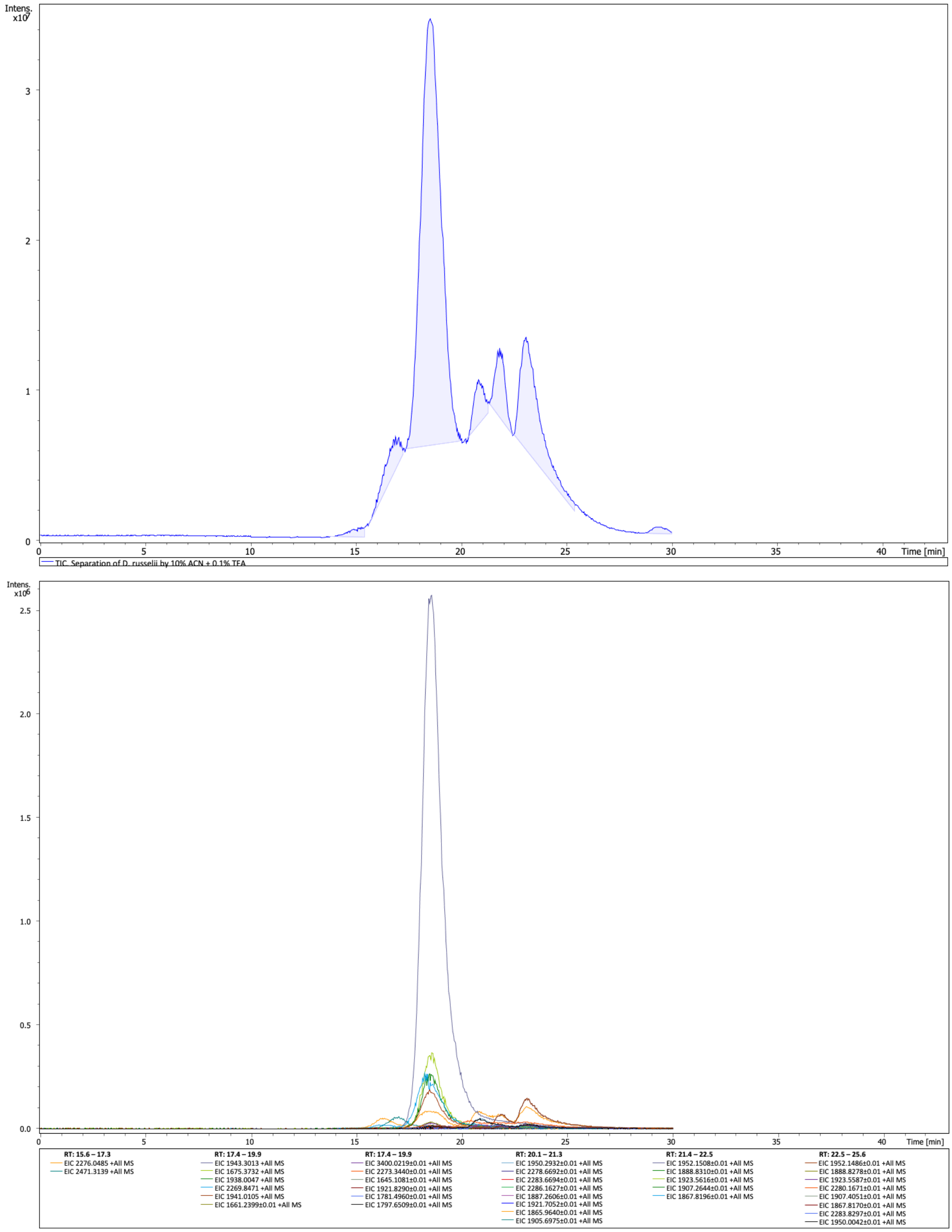


Figure S22: TIC and EICs of SEC-MS measurement of D. russelii venom separated using 10% ACN + 0.1% TFA.

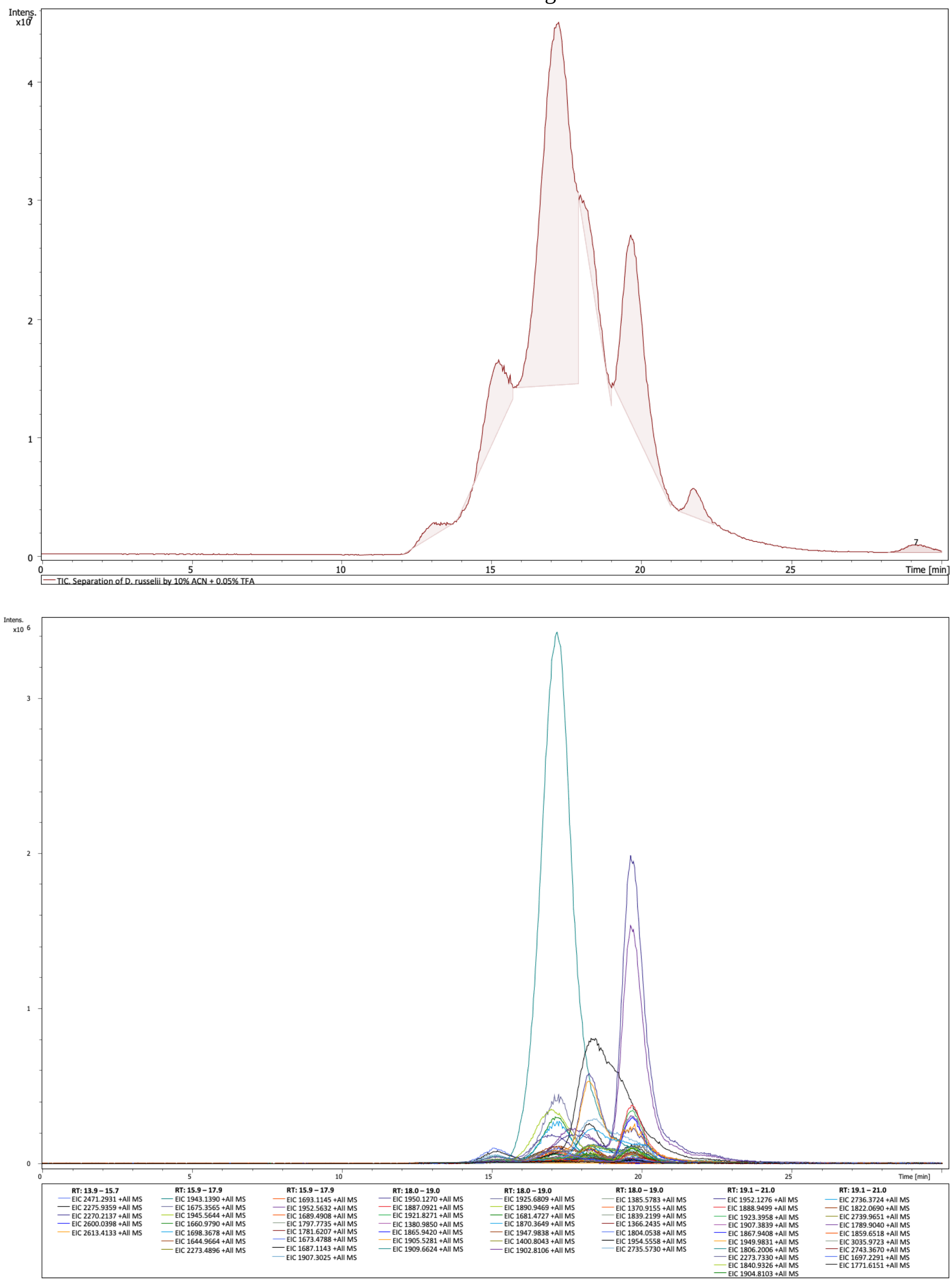


Figure 23: TIC and EICs of SEC-MS measurement of D. russelii venom separated using 10% ACN + 0.05% TFA.

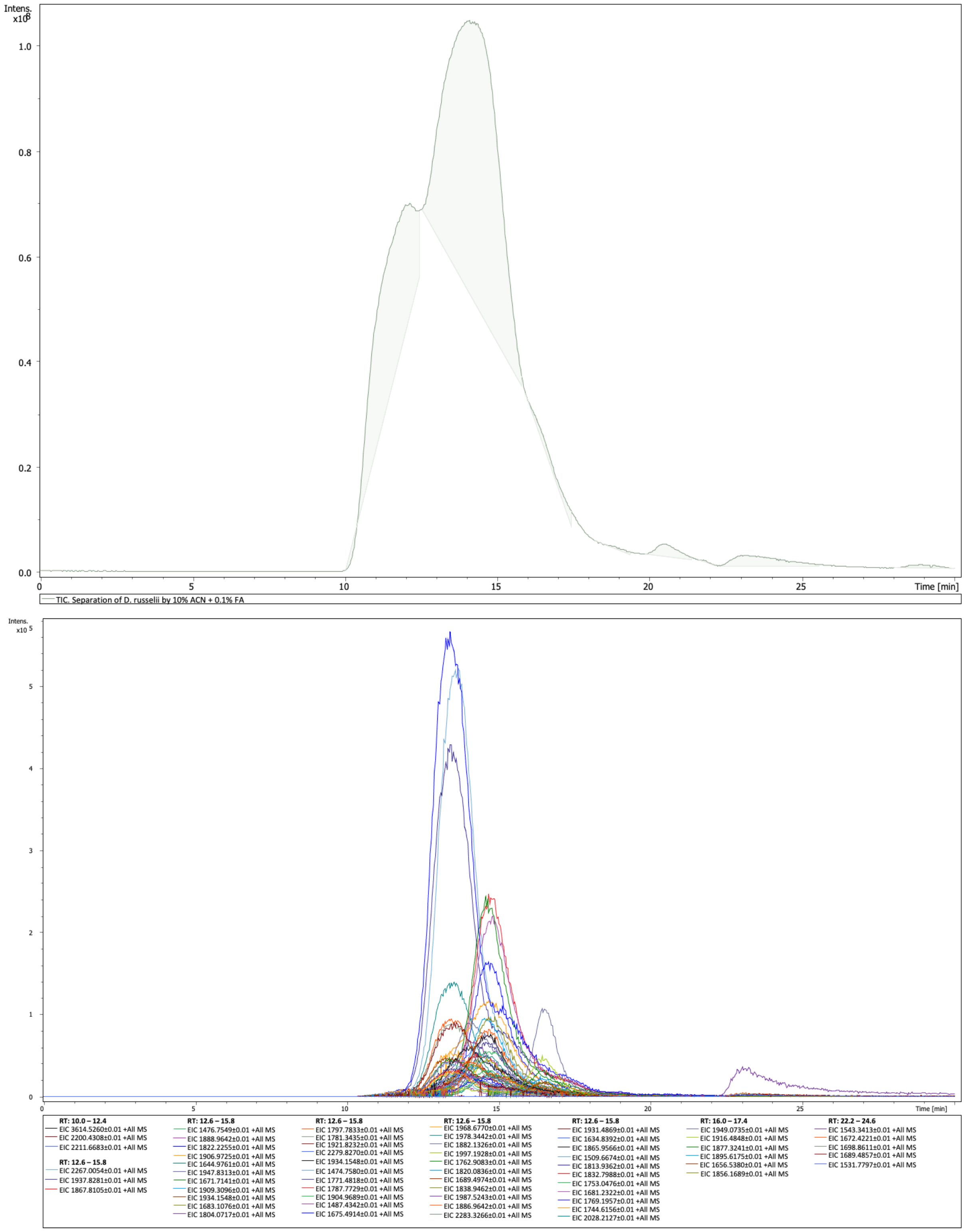
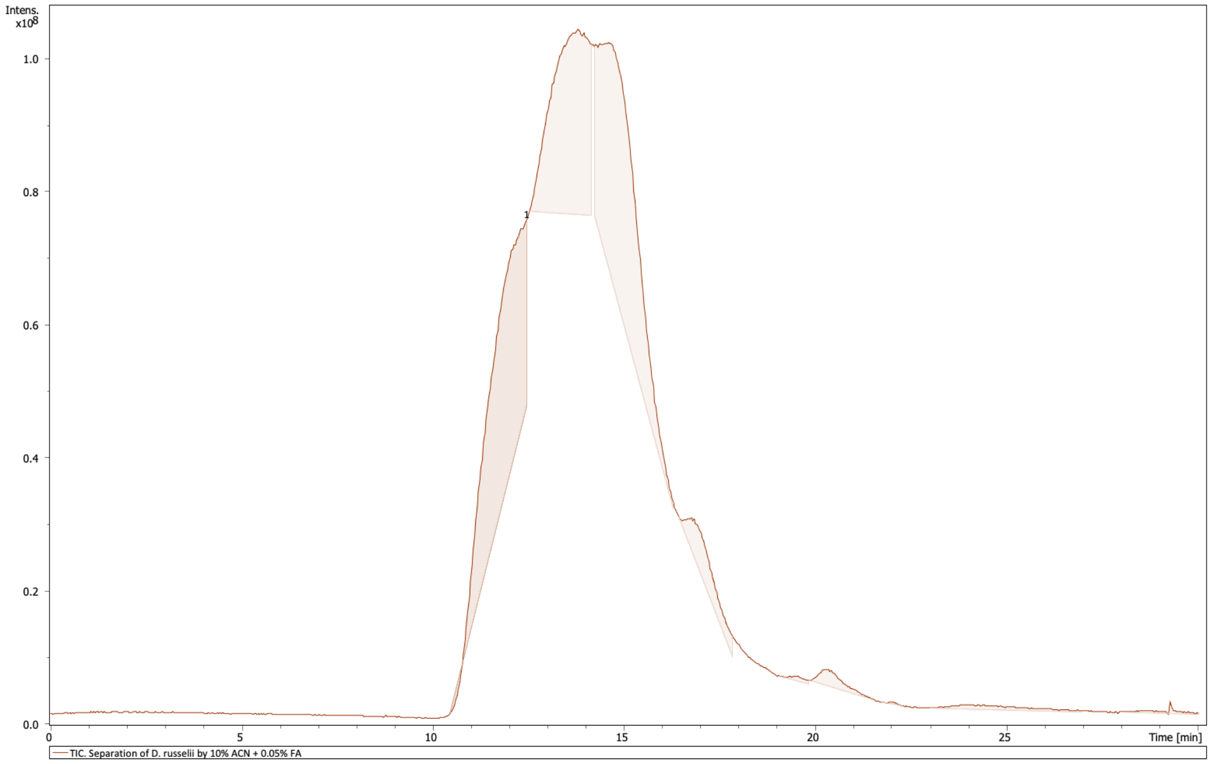


Figure S24: TIC and EICs of SEC-MS measurement of D. russelii venom separated using 10% ACN + 0.1% FA.



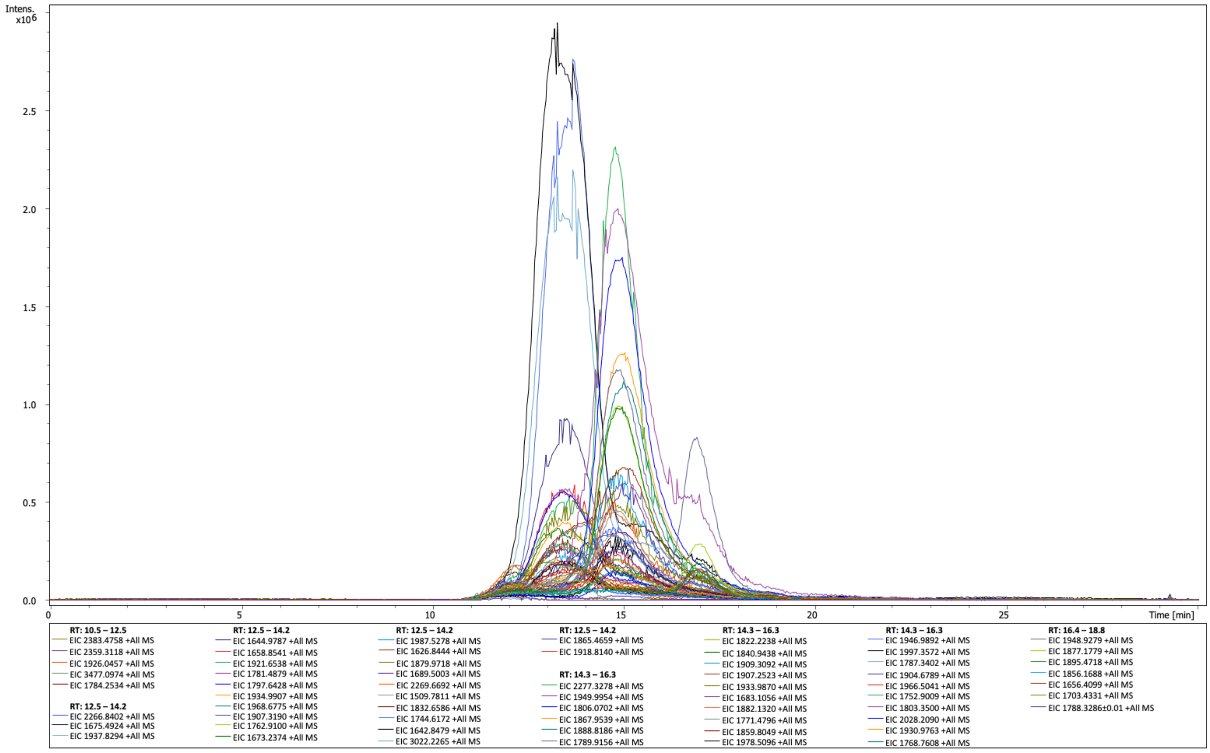


Figure S25: TIC and EICs of SEC-MS measurement of D. russelii venom separated using 10% ACN + 0.05% FA.

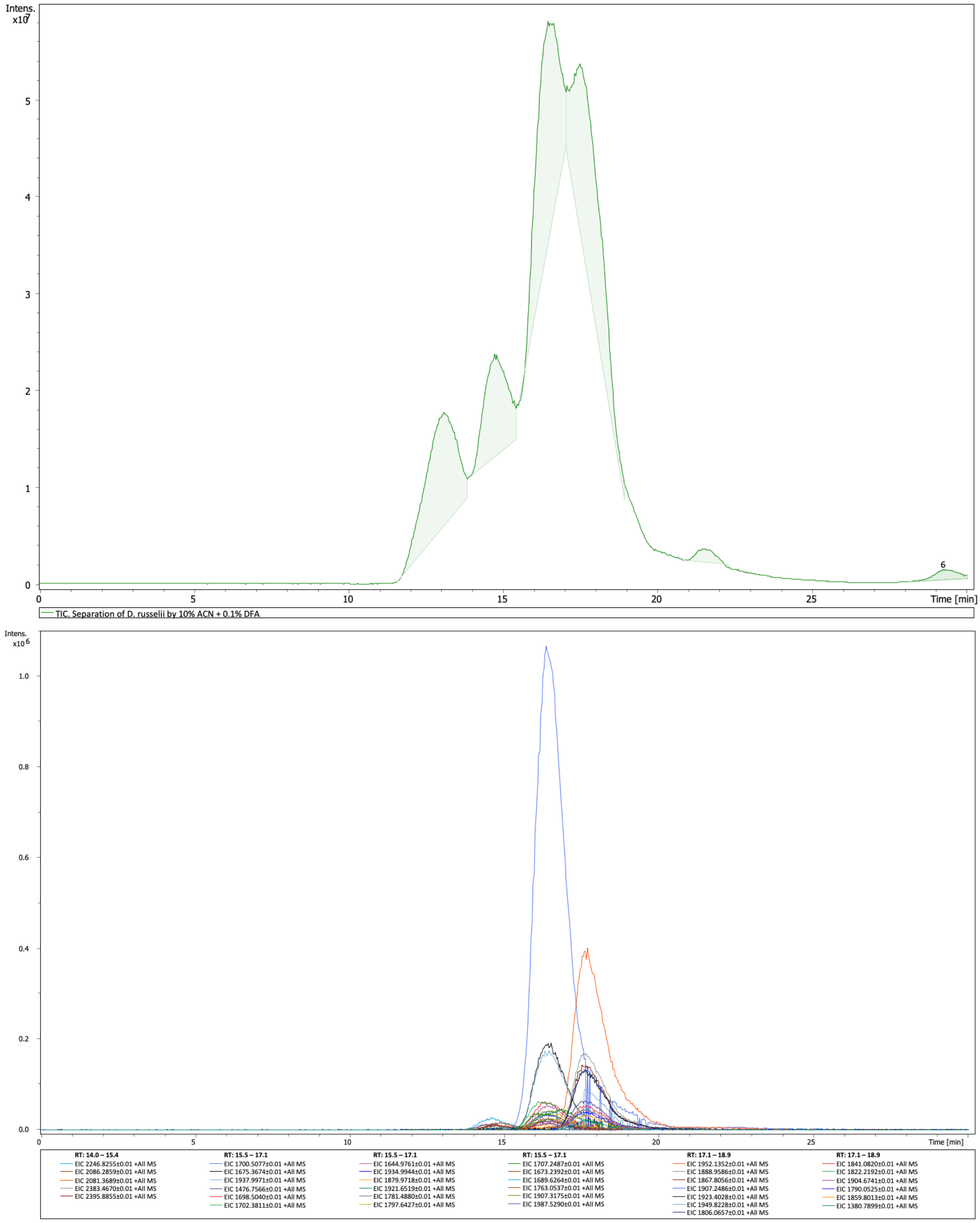


Figure S26: TIC and EICs of SEC-MS measurement of D. russelii venom separated using 10% ACN + 0.1% DFA

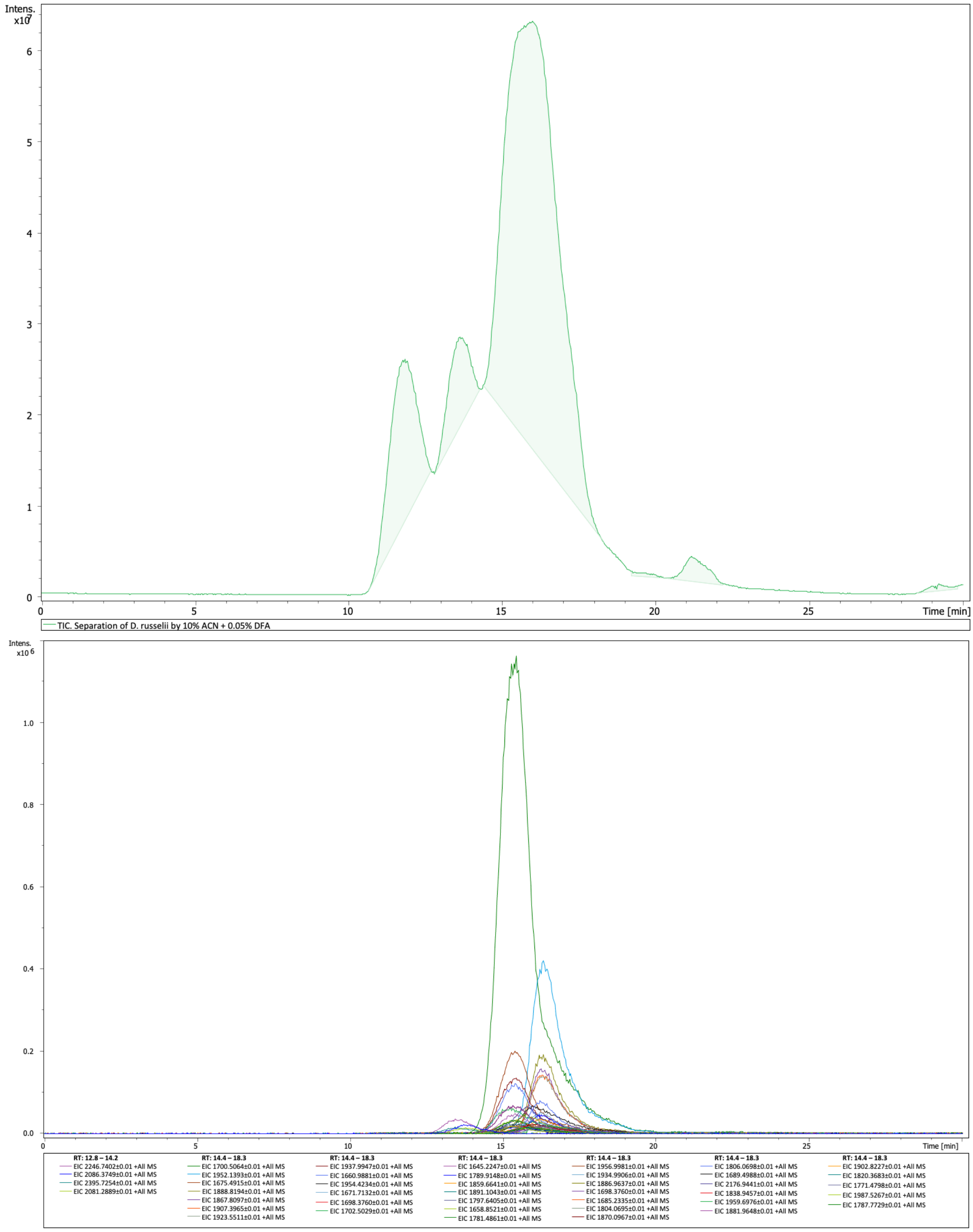


Figure S27: TIC and EICs of SEC-MS measurement of D. russelii venom separated using 10% ACN + 0.05% DFA.

S8. Reference material

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