Proteolytic venoms: using size-exclusion chromatography and parallel bioassays to study proteolytic activity of snake venom toxins

Axel de Monts de Savasse

Master of Pharmaceutical Sciences

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“Snakebite is the most important tropical disease you’ve never heard of.”

Kofi Annan, Secretary-general of the United Nations (1997 – 2006)

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Abbreviation	Meaning
BuMu	*Bungarus multicintus*, the many-banded krait
DaRu	*Daboia russelii*, the Russell's viper
DeAc	*Deinagkistrodon acutus*, the hundred-pace viper
DPBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
NTD	Neglected Tropical Disease
PLA_2	Phospholipase A_2
RFU	Relative fluorescent unit(s)
RP-HPLC	Reversed phase high-performance liquid chromatography
Rt	Retention time
SEC	Size exclusion chromatography
SVMPs	Snake venom metalloproteinases
SVSPs	Snake venom serine proteinases
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride, Trizma hydrochloride
UV/Vis	ultraviolet/visible light
VU	Vrije Universiteit Amsterdam
WHO	World Health Organization
3FTx	Three-finger toxin

1 Abstract

Snakebites are a major problem worldwide. Although it has always existed, the World Health Organisation (WHO) only recognised snakebites as a Neglected Tropical Disease (NTD) in 2017. By the same time, they acknowledged that they have been underestimating the mortality and morbidity caused by snakebites.

Snake Venom (SV) is known for many diverse types of activity as its compounds are complex and numerous. Different protein classes such as Phospholipase A_2 (PLA_2), Snake Venom Metalloproteinase (SVMPs) and Three-Finger Toxins (3-FTx) compose SV and each of them has complete different properties, targets and mechanisms of action. Some venoms are known for their high cytotoxicity, others for haemotoxicity or even neurotoxicity. Each family, sub-family and genus has its particularities, as evolution has selected the snakes according to their environment and their specific needs. That is why, through the years, various strategy of studying and curing SV have appeared.

In this Thesis, enhanced efforts have been done to study proteolytic venoms. A Size-Exclusion High-Performance Liquid Chromatography (SEC-HPLC) has been performed on different samples of venoms to separate them while doing parallel substrate degradation assays. The aim is to learn more about the proteolytic activity of SV toxins. Substrate degradation assays allows to study the degradation of Extracellular Matrix (ECM) by using *in vitro* fluorescent compounds like modified collagen and gelatine. Those assays can be done in parallel of the separation using an in-home modified *fractioner* that collects the eluent after the SEC-HPLC.

Impressive results have emerged, however further improvement can be done to improve the repeatability of the assays. Moreover, inhibitors of specific protein classes and further assays such as *egg-yolk micelles degradation assay*, showing the disruption of the cellular membrane, have yet to be performed.

2 Résumé

Les morsures de serpents constituent un problème mondial majeur. Bien qu'elles aient toujours existées, elle ne furent reconnues par l'Organisation Mondial de la Santé comme une Maladie Tropicale Négligée uniquement qu'en 2017. En même temps, ils ont reconnu avoir sous-estimé la mortalité et la morbidité causées par les morsures de serpents.

Le venin de serpent est connu pour beaucoup de différents types d'activités puisque ses composants sont très nombreux et complexes. Plusieurs classes de protéines telles que les Phopsholipase A_2 (PLA_2), les Snake Venom Metalloproteinase (SVMPs) et les Three-Finger Toxins (3-FTx) composent les venins de serpents et chacune d'entre elles ont des propriétés, cibles et mécanismes d'action différents. Certains venins sont connus pour leur haute cytotoxicité, d'autres pour leur hématotoxicité ou encore leur neurotoxicité. Chaque famille, sous-famille ou genre ont leurs particularités, puisque l'évolution a sélectionnés les serpents en fonction de leurs environnements et leurs besoins spécifiques. Tout ceci fait que, au fil du temps, plusieurs stratégies d'étude des venins et la façon de les soigner sont apparues.

Dans cette *Thèse,* des efforts accrus ont été fait pour étudier les venins protéolytiques. Une chromatographie d'exclusion stérique a été réalisée sur différents échantillons de venins pour les séparer tout en faisant des essais de dégradation de substrats en parallèle. Le but étant d'en apprendre plus sur l'activité protéolytique des toxines du venin de serpent. L'essai de dégradation de substrats permet d'étudier la dégradation de la matrice extracellulaire en utilisant, *in vitro*, des composés fluorescents tels que de la gélatine et du collagène modifiés. Ces essais peuvent se faire en parallèle de la séparation en utilisant un *fractioner*, modifié sur place, qui récolte l'éluent après la chromatographie.

De bons résultat sont ressortis, cependant des améliorations peuvent être faites pour améliorer la répétabilité des essais. De plus, des inhibiteurs spécifiques à certaines protéines et d'autres essais tel que l' « *essai de la dégradation des micelles de jaune d'œuf »*, qui montre une rupture de la membrane cellulaire, doivent être effectués.

3 Introduction

Snakes are ectothermic animals, which means that they do not produce (or in a very tiny amount) their heat. Their heat is not regulated by their metabolism, which means that snakes must rely on sunshine or on their warmth' environment to maintain their bodies' proper temperature. This is the main reason they are located mainly in hot and tropical countries. However, the superfamily Colubroidea which includes almost all the venomous species has a worldwide distribution, with only Antarctica being excluded as there is no venomous snake on this continent (1,2).

Because it does not concern northern Europe directly, snakebite envenoming has been underestimated for many years, even by the World Health Organisation (WHO)(3). Only in 2017, it has been considered a Neglected Tropical Disease (NTD)(4). Research then gets little consideration and lacks fundings. Furthermore, snakebite envenoming is considered as a disease of poverty as it is concerning particularly Africa and Asia(5). Indeed, ten percent of the 2 500 000 bites have sequels and the number of deaths per year is estimated at 85 000 of which 15 400 to 57 600 occurring in Asia and 3 500 to 32 100 in sub-Saharan Africa (5,6). The problem of snakebites "demands an integrated multifocal approach, targeting complex problems and involving many participants"(6).

Venoms have widely evolved through the animal world. They are present among many organisms such as spiders, jellyfish, and snakes and they can serve different purposes: venoms can be used for defence or predation(7).

Throughout time, venoms have been the result of *positive Darwinian selection* among the snakes(8). Snake venoms (SV) got more and more complex and new effects and mechanisms of action started to appear. It is a mixture made of different protein families with many toxins and toxin isoforms. 63 protein families have been identified within 132 different snakes from different families (*Elapididae* family and *Viperidae* family including *Viperinae* and *Crotalinae* subfamily)(9). It can give a preview of how complex a venom mixture can be and how hard it is to identify a single protein within the mixture.

Intraspecific variation of the composition of SV is known within some species like the Monocled Cobra (*Naja kaouthia*)(10). This is due to variation in the habitat, alimentation and age. Interspecific and intraspecific variation of the composition of SV make it harder to analyse and study. Developing a method to study independently each protein or protein family that can

be used and repeated even within poor countries, where snakebite envenoming touches the most people, and with enough efficiency and repeatability is tough. However even within the most developed countries it's not that simple.

The properties of SV are numerous: from huge cytotoxicity to enormous medical potential(1,11). The pharmacological application has only started to be studied in the recent years but is clear. The mechanisms of action behind the toxins of venoms (including SV) only begin to be understood and the scientists are hoping for therapeutical molecules to be extracted and used, whether in snakebite envenoming medication or in another therapeutical field such as cancer therapy(12).

The toxicity of the SV can be classified into three main effects: cytotoxicity (local tissue damage); neurotoxicity and haemotoxicity (see Figure 1). Of course, it cannot be reduced to those three effects, but it gives an idea of the diversity of the effects. Each of them is caused by specific toxin families and is distributed differently within species, genera and family (1,9). As the analysis methods have evolved and new technologies have emerged, it is important to note that some toxin families or toxins have been classified in a wrong way or with wrong effects. The nomenclature is a mess and can be erroneous. For instance, a new kind of toxin could have been classified as a "cardiotoxin" in the 1970's but is in fact non-specific to the heart. And similar structural newly-discovered toxins could be named after this first cardiotoxin(13).

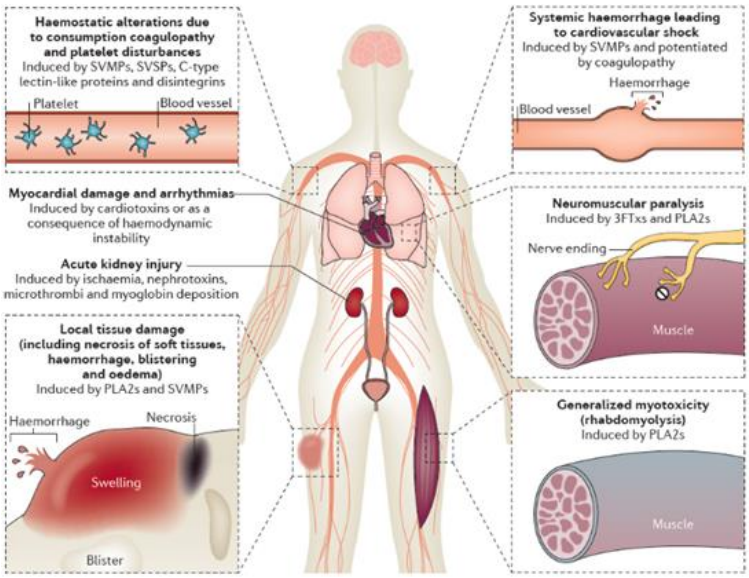


Figure 1|**Action of snake venom toxins on different body systems:** The toxins that compose SV have a wide range of activities. In this figure, the three main effects can be schematized: the cytotoxicity, mainly induced by PLA_2 and SVMPs; the neurotoxicity, mainly induced by the 3-FTx and PLA_2 that affect the neuromuscular junction and the haemotoxicity, induced by many different families and mechanisms. Taken from *Snake Envenoming*, Gutiérrez et al. 2007

Venomous snakes include many genera and families but in this *Thesis*, only two families will be discussed: the *Viperidae* (including *Viperinae* and *Crotalinae* subfamily) and the *Elapidae* families (phylogenetic classification by Pyron et al, 2010)(14).

The *Viperidae* family is historically and commonly known for its cyto- and haemotoxicity; the *Elapidae* family for its neurotoxicity(1,15). However, the

respective pathologies of each effect and the different toxins and toxin families can be found in every venomous snake, with different amount and intensity. The major toxin families are Phospholipase A_2 (PLA_2), Snake Venom Metalloproteinase (SVMPs), Snake Venom Serine Proteinase (SVSPs) and the Three-Finger Toxins (3-FTx). There are plenty of others such as Cysteine-rich Secretory Protein (CRISP), C-type Lectin-like protein (CTL), Dendrotoxin (DTx), disintegrins…

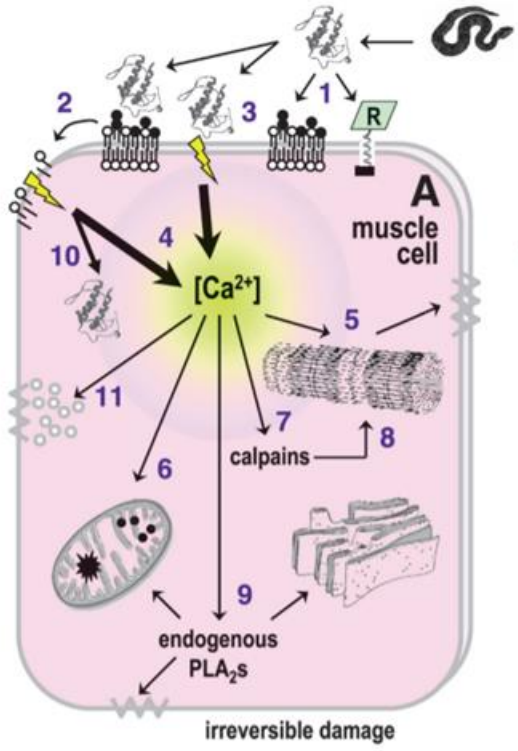


Figure 2|**Schematic representation of the action of PLA_2 on skeletal muscle cells:** *Step 1*: PLA_2 binds to receptors (R) or goes into the membrane. *Step 2*: Hydrolysis of the membrane. *Step 3*: Direct perturbations of the membrane. *Step 4*: Increase of cytosolic Calcium ions concentration. *Step 5*: Hypercontraction of myofilaments caused by Calcium influx. *Step 6*: Severe impairment of mitochondrial functions. *Step 7 and 8*: Activation of calpains (Ca^{2+}-dependent proteinases) causing degradation of the cytoskeletal, affecting the cell mechanical stability. *Step 9*: PLA_2 Ca^{2+}-dependent are activated which causes more hydrolysis and disruption. *Step 10*: PLA_2 can directly enter the cell and cause even more damage. *Step 11*: Vesicles fusion to the damaged cell membrane. Taken, simplified and adapted from *Cellular pathology induced by snake venom phospholipase A2 myotoxins and neurotoxins: common aspects of their mechanisms of action*, Montecucco et al. 2008

The cytotoxicity (local tissue damage) is mainly induced by PLA_2 and SVMPs. Indeed, the disintegration of the muscles (myonecrosis) is mostly due to the action of PLA_2. It's an enzyme that hydrolyse the phospholipids of the cell membrane and thus causes its disruption. Research has shown that PLA_2 can be divided into many groups, and some are tissue-selective and destroy specific tissues but let others unaffected(16,17). Furthermore, ions influx is disturbed due to the membrane disruption. Calcium penetrates into the cytosol and multiple events follow: "myofilament hypercontraction; mitochondrial dysfunction and other degenerative effects"(1). In addition, the hydrolyse of phospholipids frees fatty acids (among other things) that are precursors of eicosanoids (prostaglandins, leukotrienes…). "[They] play a significant role in the inflammatory pathway […]"(18). Finally, PLA_2 also have effects on the mitochondria by disrupting it, affecting its pores and causing shape changing (see Figure 2)(19). The blood vessel is also affected, mainly by SVMPs. They hydrolyse the basement membrane, composed of collagen (mainly type IV). This causes the capillary wall to be weakened and can lead to its disruption(see Figure 4)(20). SVMPs also hydrolyse Extracellular Matrix (ECM) and therefore affect the inflammatory

pathway. This affects tissues other than vessels(1). Obviously, damaging local tissues with the cytotoxicity of the SV can affect the muscles as well as the blood and the neurons, naming them only. A clear separation of the effects is useless as they are all connected, and a proper distinction of toxin families is complex.

The neurotoxicity is mainly inducted by α-neurotoxins and ß-neurotoxins. α-neurotoxins affect the postsynaptic cellule of the neuromuscular junction (21). 3-FTx belong to this protein family. 3-FTx have a common structure of three β-stranded loops (see Figure 3), which gave it their names(22). Their specific structure allows them to bind to the cholinergic receptor on the muscle fibre, which inhibits the binding of acetylcholine to its receptor and therefore provoking muscle paralysis. The binding is caused by amino acids residue in the long chain of the 3-FTx. They can also bind to other receptors such as β-adrenergic receptors(23).

Figure 3|**Representation of the 3-FTx and the nicotinic acetylcholine receptor:** The left half represents the 3-FTx. Their shapes are a common structure of three β-stranded loops. They can form covalent or non-covalent heterodimers or homodimers, and covalently linked dimers have been observed. The right half represents the nicotinic acetylcholine receptor, with some of its subtypes. This receptor is a hetero- or homo-pentameric transmembrane allosteric protein. The binding of the 3-FTx to the nicotinic acetylcholine receptor is caused by amino acids residue in the long chain of the 3-FTx. Taken, adapted and modified from *"**Graphical Abstract"**, Snake three-finger α-neurotoxins and nicotinic acetylcholine receptors: molecules, mechanisms and medicine*, Nirthanan, 2020.

As a proof of the importance of SV research, it is extremely important to note that "the discovery of [an α-neurotoxins (α-bungarotoxin)], almost six decades ago, exponentially expanded our knowledge of membrane receptors and ion channels This included the localisation, isolation and characterization of the [postsynaptic nicotinic acetylcholine receptors] and by extension, the pathophysiology and pharmacology of neuromuscular

transmission and associated pathologies[…]"(24). ß-neurotoxins have effect on the presynaptic cellule of the neuromuscular junction(25). PLA_2 that have presynaptic effects can therefore be considered as ß-neurotoxins. Indeed, if the cell membrane is disrupted, ions will enter the membrane and the polarity of the neurone membrane will be affected. This causes the neurone to lack its potential of action. This effect is only one example among many.

The haemotoxicity is mainly caused by SVMPs(26). Indeed, some SVMPs can target the microvasculature and affect the inflammatory pathway, as already explained above regarding the cytotoxicity, as they can degrade the ECM (see Figure 4). In the same way, it's the identic for PLA_2, also briefly explained above.

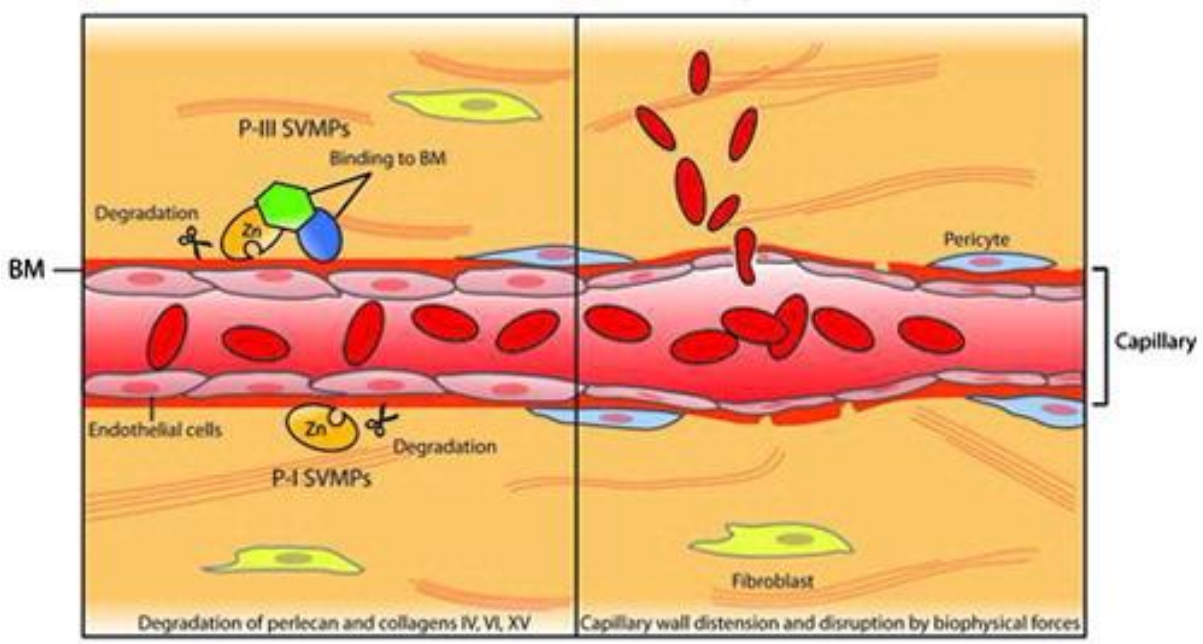


Figure 4|**Summarised mechanism of microvessel disruption by SVMPs:** Some SVMPs are degrading the Basement Membrane (BM), causing endothelial cells to be weakened, and disruption can happen. It directly affects the inflammatory pathway. Taken, adapted and modified from "***Graphical Abstract***", *Key events in microvascular damage induced by snake venom hemorrhagic metalloproteinases*, Gutiérrez et al. 2011.

The haemotoxicity caused by SVMPs depends on their ability to target and degrade the BM components (non-specifically) such as collagen types IV (27). The clotting process is directly linked to the prothrombin being cleaved as thrombin which, once activated, acts as a serine protease that turns a soluble fibrinogen into insoluble fibrin. This process is the last part of what is called the "coagulation cascade"(28). Many prothrombin activators are found in SV(29). Some are part of the SVMPs family, but others are part of the serine proteinases. Those events lead to an alternation of blood clotting and therefore some SV can induce systemic bleeding(30). Many other proteins and protein families are untold in this *Thesis* but, regarding the haemotoxicity, a lot of them have numerous other effects such as inhibiting the angiotensin-

converting enzyme(31). All those here undetailed effects change the haemodynamic and therefore can be responsible for cardiotoxicity and sometimes even cardiovascular shock(32).

In conclusion of these short analysis of the snakebite envenoming and its toxicity, naming all the effects and detailing all the mechanisms of action or targets hiding behind each protein families is not possible. However, an idea of how dangerous a SV can be is now possible (see Figure 5). The therapeutical and medical potential behind the research is only beginning, and tremendous progresses are expected in the antivenom field.

Figure 5|**Consequences of a snakebite by *Bothrops atrox*, more known as the *common lancehead*, on a 12-year-old boy in Peru:** The *Bothrops arthox* is a snake from the *Viperidae* family. The boy reached the hospital after 48 hours as the haemorrhage was still going, but it was too late for him to get an antivenom. His arm was amputated 13 days after the bite in a regional hospital. Taken and modified from *Snakebite envenoming from a global perspective: Towards an integrated approach*, Gutiérrez et al. 2009.

The most used SV in this Thesis is the SV *from Deinagkistrodon acutus* (DeAc), a snake frome the *Viperidae* family(33). A lot of proteolytic activity is therefore expected. The “Big Four” is a name given to the four most venomous species in India where, according to the WHO, there is the highest mortality due to snakebite in the World(3,34). It is composed of *Bungarus caeruleus* (the common krait), *Daboia russelii* (DaRu) (the Russell’s viper), *Echis carinatus* (the Indian saw-scaled viper) and *Naja naja* (the Indian cobra). The Russell’s viper consists in 43 % of the snakebites in India, a research has shown(35). This snake is also from the *Viperidae* family. Therefore, those two SV from DaRu and DeAc are mostly known for their cyto- and haemotoxicity(33).

The DaRu SV is mostly composed of PLA_2, but also SVMPs and SVSPs among many others. A research (*Proteomics, functional characterization and antivenom neutralization of the venom of Pakistani Russell's viper (Daboia russelii) from the wild*, Faisal et al. 2017) has shown that

DaRu SV is composed of 2,5 % of SVMPs, another (*Unraveling the Proteome Composition and Immuno-profiling of Western India Russell's Viper Venom for In-Depth Understanding of Its Pharmacological Properties, Clinical Manifestations, and Effective Antivenom Treatment*, B. Kalita et al. 2016) has shown more than 24 %(36,37). This confirms the intraspecific variation of the SV and the difficulty to properly analyse it. However, it is certain that SVMPs are present in the DaRu SV. As already discussed, some components of the ECM, such as the collagen type IV, are hydrolysed by SVMPs (also hyaluronidases and others).

This *Thesis* will focus on separating the venoms using Size-Exclusion High-Performance Liquid Chromatography system (SEC-HPLC) while doing bioassays in parallel.

Doing so allows to analyse the venom, in a non-quantitative approach, based on the size of the proteins of the SV, as it has already been done (*Theory and practice of size exclusion chromatography for the analysis of protein aggregates*, T-Y. Huang et al. 2018)(38). In recent years, huge progress has been made in protein analysis and "numerous techniques have been developed to monitor protein aggregation"(39). However, to create a method to separate the SV without losing the protein activities is though. Usually to get a proper separation, the solvents used such as Acetonitrile are denaturating the proteins, as they changes the helix forms at high concentration(40). To create a fast, reliable, repeatable and global method for all snake species and, at the same time, to keep the proteins active, is the first goal of this *Thesis*. Concerning the identification of the proteins, the method used is often proteomics, unfortunately this field will not be detailed in this *Thesis*(9) but leads will be given.

Once the eluent is detected after the SEC system, it will go through a "fractioner", which is more detailed 4.3.2 Fractionation. The separated SV will then be collected and tested to perform a substrate degradation assay on modified collagen type IV and gelatine. Those components are specially modified to be easily and precisely analysed. As stated above, SVMPs hydrolyse those. Effects with a part of the separated SV, which would then correspond to SVMPs, on the collagen and gelatine is then largely expected. This is the second goal of this *Thesis*.

Finally, the last objective is to make a parallel between the separation and the bioassays, using the chromatograms and the results obtained.

The objective of this *Thesis* is to create a global method of separation of SV and of bioassays to study more easily specific toxins that have been separated. Therefore, it allows in the future to:

- Test inhibitors on specific toxins or toxin families. Already existing or not-yet-discovered or -tested inhibitors could be tried;
- Perform more precise proteomics as the SV is properly separated, leading to a more detailed SV proteomic database;
- Study more accurately a specific toxin or toxin family, separated on the basis of their size, by performing already existing or not-yet-existing bioassays, helping to understand more precisely about them and their properties, targets and mechanisms of action;
- Possibly imagine a new antivenom or new medical and therapeutical application based on more known SV;
- Possibly improve this method, rendering it more accessible, faster and cheaper for every country in the world where the number of samples of SV and the technologies are limited.

4 Materials and Methods

4.1 Chemicals and Solvents

All the water used in the experiments was purified to Milli-Q water grade using an in-house Milli-Q® Reference Water Purification System (Millipore, Amsterdam, The Netherlands). Gibco™ Dulbecco's phosphate buffered saline (no calcium, no magnesium, pH 7.0-7.3; DPBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). 2-propanol HPLC grade was purchased from VWR International (Radnor, PE, USA).

4.2 Venoms

The venoms were sourced from the Faculty of Sciences, Bio Analytical Chemistry, Vrije Universiteit Amsterdam (VU). They have been provided by Prof. Kini R. Manjunatha (Department of Biological Sciences, National University of Singapore (NUS), Singapore), Prof. Dr. Freek Vonk (Faculty of Sciences, Chemistry and Pharmaceutical Sciences, VUA, The Netherlands and Naturalis Biodiversity Centre, Leiden) and the herpetarium of the Centre for Snakebite Research & Interventions at the Liverpool School of Tropical Medicine (LSTM, Liverpool, UK).

The venoms were housed in a -80°C freezer with a 5 mg/mL concentration after being flash-frozen in liquid nitrogen. When using 2.5 and 1 mg/mL concentrations, the venoms were diluted with Milli-Q water.

4.3 Liquid Chromatography and Fractionation

4.3.1 Size Exclusion Chromatography

Size Exclusion Chromatography (SEC) was performed on a Shimadzu (Kyoto, Kyoto prefecture, Japan) HPLC system., using Shimadzu Lab Solutions software. An autosampler Shimadzu SIL-20AC Prominence was used to inject the venom samples put in vials. All the injections in the following methods are 10 µL except for the protein mixture, in which 5 µL was injected (prepared with the manufacturer's instructions). The column is a Sepax (Newark, DE, USA) Zenix SEC-300 column (300 Å, 5 µm, 4.6 mm x 300 mm) and was housed in a Shimadzu CTO-10AC VP column oven and set to 27 °C for all the following assays. The pump is a Shimadzu LC-10Ai (Kyoto, Kyoto prefecture, Japan) and was set at a flow rate of 0.35 mL/min for all the following assays. The elution was then monitored with a Shimadzu SPD-

20A Prominence UV/Vis detector at 220nm. This wavelength was used for all the following methods. The method was settled for 20 minutes for all the following assays. The mobile phase was always isocratic and consisted of DPBS with different concentrations: from 100 % DPBS to 10/90 % DPBS/MilliQ.

A solvent loop was added to the pump system using 2-propanol and MilliQ 20/80% as DPBS is a saline solvent, it can precipitate and form crystals and therefore damaging the pump. This solvent loop is done to prevent this to happen as it flushes the solvent used.

4.3.2 Fractionation

The goal of the fractionation is to parallel the separation of SV with the substrate degradation assays results. This is the key to this Thesis and its experiments. Combining the data from the separation and the substrate degradation assays can result in finding active proteins on specific substrates. Furthermore, as said in the introduction, SV contain a lot of different protein families with multiple effects. If a set of wells filled with fractionated SV matches with an activity on a specific substrate, then the separation of proteins is efficient, and a protein family can be determined and separated.

After the column and the detector, 90 % of the eluent was settled to go the fractioner Gilson ASTED-XL (Middleton, WI, USA) which is controlled by the Ariadne software (in-home software, v1.08j, VU Amsterdam, Amsterdam, The Netherlands). The flow rate of the fractioner is then 0.315 mL/min (90% of 0.35 mL/min). The fractioner was settled with the software to drop the eluent in a 384-well flat-bottom plate (Greiner Bio-One, Kremsmünster, Austria) every 12 seconds in a different well, 6.00 minutes after the beginning of a SEC-HPLC analysis up to 16.00 minutes, resulting in 50 wells fill with 62 µL.

$$\text{Flow rate of the Splitter} = 0.315 \text{ mL/min}$$

$$0.315 \text{ mL/min} = 315 \text{ µL/min}$$

$$\frac{315}{60} = 5.25 \text{ µL/sec}$$

$$5.25 \text{ x } 12 = 62 \text{ µL/well}$$

This results in having a plate with 50 wells full of separated venoms. Having 62 µL in each wells allows a fractionated plate of venom to be used in multiple assays (see Figure 6), saving a lot of time and resources (venoms, solvent…). However, as the fractionation is done like this, it's important to note that its accuracy is not huge. In practical, the wells contain less than 60

µL. This can be improved by a better, but far more expensive splitter, such as a FractioMate (Spark-Holland and VU, Emmen and Amsterdam, The Netherlands).

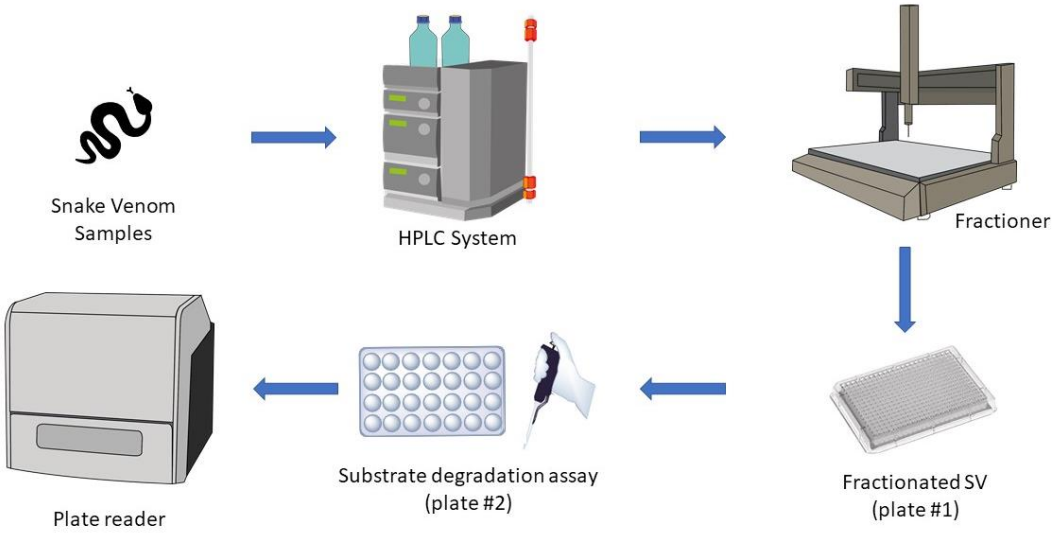


Figure 6| **Schematic representation of the SEC-HPLC system combined with the fractioner followed by substrate degradation assays**: The Snake Venom samples are put in vials in the auto-sampler at the desired concentration (5; 2.5 or 1 mg/mL). The SEC-HPLC system is composed of the auto-sampler (Shimadzu SIL-20AC Prominence), the column (Sepax Zenic SEC-300), the column oven (Shimadzu CTO-10AC VP), the pump (Shimadzu LC-10Ai) and the detector (Shimadzu SPD-20A Prominence UV/Vis). The eluent goes to the fractioner (Gilson ASTED-XL) after the detector and is dropped in a plate (Greiner 384-well flat-bottom plate) called in this diagram plate #1, containing fractionated SV. Fractionated SV can be taken manually with pipette to a new plate called here plate #2. In this diagram it is represented by a different plate to make it clearer however plate #2 is also a Greiner 384-well flat bottom plate. After 4hours incubation (Heracell 240 CO2 incubator, ThermoFischer), the plate is read by a plate reader (Varioskan LUX Multimode Microplate Reader 3020-444). The pictures "HPLC System"; "Fractioner"; "Plate #1" and "Plate reader" were taken from Arif Arrahman (PhD Student, VU Amsterdam, The Netherlands).

4.4 Protein mixtures

To condition the column and test its efficiency, solvent DPBS was used, as it would be used for the future assays. A protein mixture was prepared according to the manufacturer's instruction (Bovine Serum Albumin (BSA) 1.0 mg/mL (66 kD); Ribonuclease A 1.0 mg/mL (13.7 kD); Uracil 0.1 mg/mL (120 D); diluted in DPBS) to try the column and using Uracil as the smallest molecule to determine the dead time. The mixture was then put in a vial, in the autosampler. Five µL was then injected with a flow rate of 0.35 mL/min, eluted through the column and detected with the UV/Vis detector at 220 nm.

4.5 Optimizing the solvent for the assays

"Buffered Saline Solutions are isotonic saline solutions used to maintain pH and osmotic balance as well as provide cell with water and essential inorganic ions"(41). DPBS was chosen as a solvent to separate the venoms for many reasons. As a Buffered Saline Solution, it maintains pH and osmotic balance of the cells. It is then better to separate venom with DPBS to proceed with *In Vitro* bioassays for a next step as it has already been shown to be good for some assays(42).

Different concentrations of DPBS with venoms from DeAc (*Deinagkistrodon acutus*) snake were tried. This snake specie was chosen as it's a snake from the *Viperidae* family, and therefore contains a big portion of SVMPs that have proteolytic activity(43). It is indeed a perfect venom to proceed a substrate degradation assay with gelatine and collagen. It is also possible to compare the results with venoms containing less SVMPs such as BuMu (*Bungarus multicintus*) which is known for its neurotoxicity (naming it only) and who doesn't directly degrade the cellular membrane(42).

100 µL of DeAc 1 mg/mL SV was put in a vial in the auto-sampler. 6 different solvents were made and consist in DPBS/MilliQ (100/0; 80/20; 60/40; 40/60; 20/80; 10/90). Ten µL was injected from the vial of DeAc SV in the auto-sampler and the pump was set at 0.350 mL/min. The elution was detected through the UV/Vis detector at 220 nm.

4.6 Separation of venoms from different species

Venoms from eleven different species were tested to the SEC-column: *Bungarus multicintus* (BuMu); *Calloselasma thodostoma* (CaRh); *Daboia russeli* (DaRu); *Deinagkistrodon acutus* (DeAc); *Dendroapsis angusticeps* (DeAn); *Dendroapsis jamesoni* (DeJa); *Dendroapsis jamesoni kaimosae* (DeJaKa); *Dendroapsis polylepis* (DePo); *Dendroapsis viridis* (DeVi); *Echis ocellatus* (EcOc) and *Naja mossambica* (NaMo).

The genus *Bungarus*, *Dendroapsis* and *Naja* are part of the *Elapidae* family. The genus *Calloselasma*, *Daboia*, *Deinagkistrodon* and *Echis* are part of the *Viperidae* family(33). Therefore, their venom composition are completely different(9).

Testing the same method of SEC-HPLC to different snakes, genus and even families to separate the venoms and make it a good separation would be the perfect way to optimize a global method for the future and to proceed in bioassays with the venoms. It is indeed a new path to find new

proteins, explore and improve the knowledge on venom protein families, and therefore leading to a better understanding of the venoms. This includes but not limits to new drug development by discovering protein structure while doing proteomics. It has already been proved that [MS (Mass Spectrometry) can be used for quantitative, non-invasive venom profiling](44).

The venom samples were stored at the concentration of five mg/mL. They were all diluted with MilliQ to one mg/mL. 30 µL of each SV were put in a vial, in the auto-sampler. 10 µL was injected. The solvent was 100 % DPBS and the pump had a flow rate of 0.350 mL/min. The detector UV/Vis was set to detected at 220 and 280 nm. The method was set to 20 minutes.

The same method has been done again with BuMu; DeAc and DaRu SV but with a concentration of 2.5 mg/mL, diluted with MilliQ. This was to try the method with higher concentration venoms, which can possibly lead to different separation. Those venoms with 2.5 mg/mL concentration were also used to perform *4.7 Substrate degradation assays by Snake Venoms*.

4.7 Substrate degradation assays by Snake Venoms

The following assays consist of measuring the degradation of gelatine and collagen caused by SV. This can be measured with fluorescence. Indeed, the gelatine and collagen are specially modified to contain fluorescents moiety. “The fluorescence signal is quenched until [an enzyme digests the substrate into] fluorescent fragments”(45).

SV from the *Viperidae* family are expected to have high haemotoxicity but also cytotoxicity(43). Furthermore, if any degradation of the substrate is observed, that would mean that those SVMPs also act on gelatine and collage, as gelatinase and collagenase would do, meaning that they can degrade the Extra Cellular Matrix (ECM), because one of the main fibrous protein of the ECM is collagen(46). This could explain some of the effects on the coagulation, as degrading the ECM would mean to free coagulation messenger in the local damaged tissue. Indeed, it has already been proven that SVMPs have many different activities(47). Therefore, it is expected for some SV, especially those from the *Viparidea* family, to degrade gelatine and collagen substrate.

The three SV used in these substrate degradation assays were the *Bungarus multicintus* (BuMu, *Elapidae* family); the *Daboia russelii* (DaRu, *Viperidae* family) and the *Deinagkistrodon acutus* (DeAc, *Viperidae* family)

As the substrate are light sensitive, the following methods are done while having the lowest light as possible in every step.

A special buffer from the EnzCheck™ gelatinase/collagenase assay kit was used (ThermoFischer™, Waltham, MA, USA) (10x buffer: 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6) and stored in a freezer at -18°C. Before use the buffer was defrosted and diluted 10 times to its final concentration (1x buffer: 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM sodium azide, pH 7.6) using sterile-filtered MilliQ filtered with syringe filter as recommended by the manufacturer. This buffer is used in the following methods.

The FG and FC were prepared by dissolving the vials from the EnzCheck™ gelatinase/collagenase assay kit in sterile-filtered MilliQ to a concentration of 1 mg/mL under sterile conditions and using a laminar flow and placed in an ultrasonic bath with warm water and agitated for five minutes as recommended by the manufacturer. They were placed in a fridge at 6°C and covered in tinfoil to prevent any reaction from the light. Those FC and FG are used in the following substrate degradation assays.

Positive and negative control were used. Positive control is collagenase from the EnzCheck™ gelatinase/collagenase assay kit for both the gelatine and collagen assay. It was dissolved with sterile-filtered MilliQ as recommended by the manufacturer and was diluted from 1000 U/mL to 1 U/mL with the special buffer. This positive control is the same for both FG and FC substrate degradation assay. The negative control is FG or FC and special buffer.

The snake venom use in the following methods were separated such as the method described in *4.3.2 Fractionation*.

The plates were always placed in an incubator after the special buffer, the SV and the FG/FC were put in the wells. The incubator (Heracell 240 CO_2 incubator, ThermoFischer™, Waltham, MA, USA) was kept at 37°C and 5 % CO_2 for four hours. The positive and negative controls were put in the exact same conditions and were also incubated for four hours. After this the plate was measured with a plate reader (Varioskan LUX Multimode Microplate Reader 3020-444) using the excitation wavelength of 490 nm and the emission wavelength of 525 nm. The plate reader was kept at 37°C and controlled by SkanIt RE 4.1 Software (SkanIt™, ThermoFischer™). The data was exported to Microsoft Excel using this Software. The Relative Fluorescence Unit (RFU) is measured with this plate reader, and an activity can be seen if any fluorescence light is emitted due to the degradation of the substrate.

4.7.1 Degradation of Fluorogelatine by Snake Venom

Three concentrations of SV were separated: 1, 2.5 and 5 mg/mL SV. The samples being stored in the freeze at – 80 °C at five mg/mL after freeze-dried, they were diluted when needed with MilliQ. For each of the three concentrations of SV, the assay was done with different amounts of fractionated SV: 2; 5 and 10 µL of fractionated SV in each well.

To begin, the 1x FG (1 mg/mL) was diluted ten times to obtain a 0.1x FG (0.1 mg/mL) in the 1x buffer prepared in 4.*7 Substrate degradation assays by Snake Venoms*. Then the solution of FG/buffer was put in 50 wells from a 384-well flat-bottom plate (Greiner Bio-One), the amount changes depending on how much of separated SV was put (Table 1) as the aim was to obtain wells of 50 µL and with the same substrate concentration. The fractionated SV was then added as the final step, as it would start the reaction of degradation. This results in a final volume of 50 µL, 0.5 µg of FG and 2, 5 or 10 µL of fractionated SV per well.

Venom from the fractionated plate (µL)	**0.1x FG (µL)**	**1x Buffer (µL)**	**Final amount per well (µL)**
2	5	43	50
5	5	40	50
10	5	35	50

Table 1| **Repartition of venom, FG and buffer in the three different assays**

4.7.2 Degradation of Fluorocollagen assay with Snake venom

To begin, the 1x FC (1 mg/mL) was diluted ten times to obtain a 0.1x FC (0.1 mg/mL) in the 1x buffer prepared in 4.*7 Substrate degradation assays by Snake Venoms*. Then the solution of FC/buffer was put in 50 wells from a 384-well flat-bottom plate (Greiner Bio-One) as the aim was to obtain wells of 50 µL (see Table 2). The fractionated SV was then added as the final step, as it would start the reaction of degradation. This results in a final volume of 50 µL, 2 µg of FC and 10 µL of fractionated SV per well.

This substrate degradation assay was done with the BuMu, DaRu and DeAc fractionated SV using only one concentration and one amount of venom: 2.5 mg/mL SV and 10 µL of fractionated SV/well.

Venom from the fractionated plate (µL)	0.1x FC (µL)	1x Buffer (µL)	Final amount per well (µL)
10	20	20	50

Table 2| **Repartition of venom, FC and buffer in the assay**

5 Results and discussions

All data that follow unless specified were evaluated and treated with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad PRISM 8 (GraphPad Software Inc., San Diego, CA, USA).

5.1 Protein mixture

The chromatogram bellow (Figure 1) shows the absorbance of the protein mixtures made in *4.4 Conditioning the column* at 220 nm. As expected, a part of the BSA forms a dimer and even a trimer resulting in pics.(48) As the SEC column keep the small molecule such as Uracil (120 D) longer in the column because they get trapped in the stationary phase, it is then possible to determine the dead time. The chromatogram can be compared to the manufacturer's chromatogram (see Supplementary Data Figure 19).

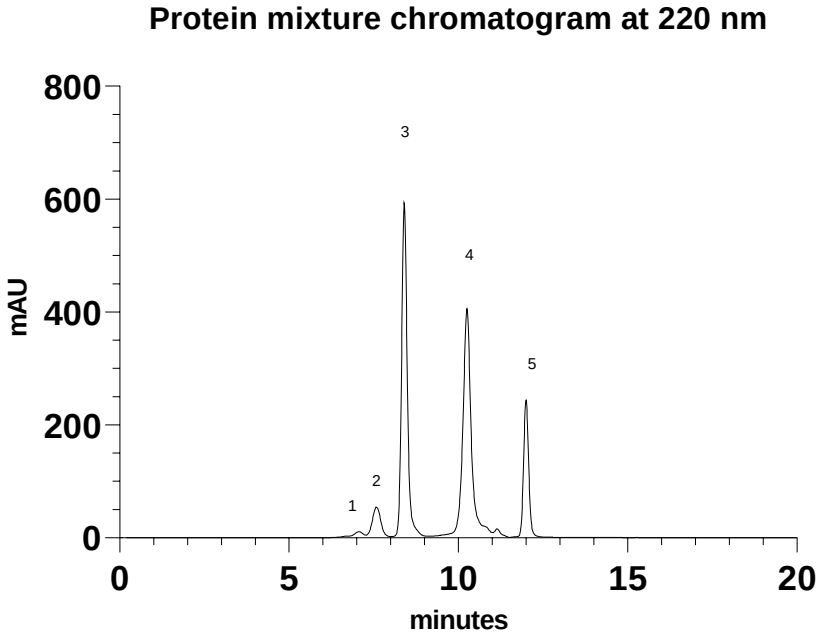


Figure 7| **Protein mixtures chromatogram at 220 nm:** The pic **1** corresponds to BSA trimer and the pic **2** to BSA dimer, both formed in vitro. The pic **3** corresponds to the BSA (66 kD), with a Rt (Retention time) of 8.3 min. The pic **4** corresponds to the Ribonuclease A (13.7 kD), with a Rt of 10.2 min. The pic **5** corresponds to Uracil (120 D) with a Rt of 12.1 min.

The column gives a proper separation and can therefore be used for the next following assays.

5.2 Optimizing the solvent for the assays

The chromatograms bellow (Figure 8) shows the absorbance of the different solvents tried that were made in *4.5 Optimizing the solvent for the assays*. The first pic appears at seven min in the six different concentrations except for the 10 % DPBS which appears at six minutes. The third and fourth pics of the 100 % DPBS are not well separated, however they consist of two different protein families as their retention time and the intensity are different. On the 80 % DPBS they are fusing even more, as only a small shoulder can be seen. Going with fewer DPBS makes these two pics completely fusing and not distinguishable.

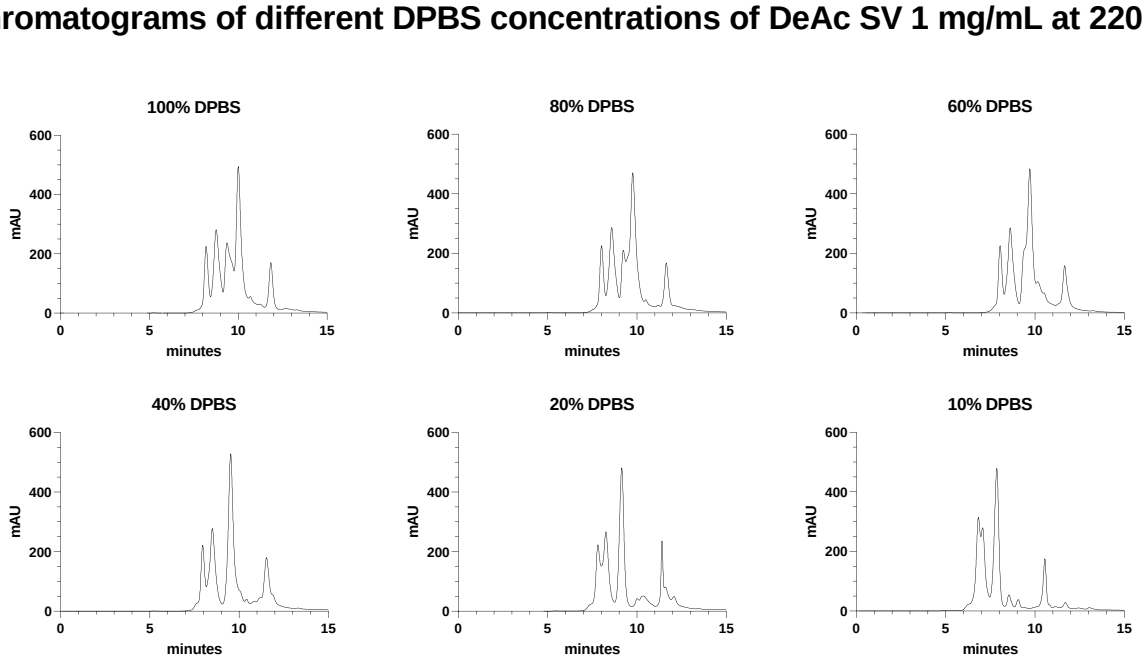


Figure 8|**Chromatograms of different DPBS concentrations of DeAc SV 1 mg/mL at 220 nm**: DPBS is mixed with MilliQ only, expect for first graph *100% DPBS* which is only DPBS.

In conclusion, the 100 % DPBS concentrations was chosen to do the following substrate degradation assay. However, the 20 % DPBS concentration was also tried to perform a substrate degradation assay using FG and the DeAc separated SV (see Supplementary Data Figure 20).

However, it is important to note that the separation is not perfect. Indeed, the separation of SV using DBPS leads to some problems. The separation must occur before the dead time. In this analytical method, the goal was to make it quick but if other venoms might be analysed or studied, a longer time could be needed. A better separation would also be great to perform more accurate needs like proteomics.

5.3 Separation of venom from different species

While testing different venom, it is important to note that there are inter- and intraspecific venom component variability(49). The intraspecific differences are caused by differences in habitat, age alimentation etc(10). This causes the separation to have completely different chromatograms (see Supplementary Data Figure 21), both for the absorbance than for the peaks, as expected. The three most relevant chromatograms (*Deinagkistrodon acutus* (DeAc), *Daboia russelii* (DaRu) and *Bungarus multicintus* (BuMu)) are shown in Figure 3 below. Those three SV will be used later in the substrate degradation assays.

The venoms contain proteins that have different properties, some being more hydrophobic than others, some bigger than others with higher molecular weight(50,51). As the solvent used is DPBS, which is an aqueous solvent, it's then completely expected that some compounds have various retention time. A lot of them have peaks that come far after 12.1 minutes, that corresponds to the Rt of the Uracil. The separation is therefore not suitable for those species using this method.

Chromatograms at 220 nm of the *Deinagkistrodon acutus, Daboia russelii and Bungarus multicintus 1 mg/mL SV using 100 % DPBS*

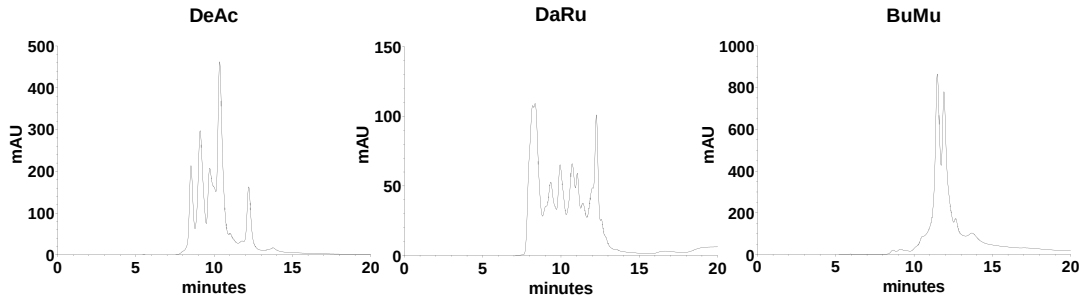


Figure 9| **Chromatograms at 220 nm of the Deinagkistrodon acutus, Daboia russelii and Bungarus multicintus 1 mg/mL SV using 100 % DPBS**: They have very different shaped chromatograms, as expected. Some compounds are not properly analysed and shown with the DaRu as it can been seen in Supplementary Data Figure 21.

In conclusion, some venoms such as DeAn, NaMo or DaRu (see Supplementary Data Figure 21), cannot be fully separated using this method. This is probably caused by the method, which consists of using DPBS as solvent but also using it in an isocratic manner. However, it's still possible to try substrate degradation assays with some of them acknowledging that the first peaks have activities. The Daboia genus is part of the *Viperidae* family known for its high enzymatic activities such as proteases and coagulant enzymes(52). That is also why the DaRu has been kept for the further assays.

Having the venom separated by DPBS solvent using SEC-HPLC allows to perform multiple assays in parallel of the separation, even if they are not fully separated. DPBS doesn't degrade the proteins and is perfect for bioassays as it will keep the isotonicity. This separation can be improved but is already a positive impact for future research.

5.4 Substrate degradation assays by Snake Venoms

In all the following substrate degradation assay graphs (FG and FC graphs), the y-axis is the RFU, and the x-axis corresponds to the t-time of the separation of SV. The fractioner was programmed to collect the eluent from six to sixteen minutes. The fractioner collects every 12 seconds (0.2 minute) and this makes an assay of 50 wells, that goes from 6.0 to 15.8 minutes included. However, the first and last two minutes were excluded only in the DeAc SV substrate degradation assays after the first few assays were made (see Figure 10).

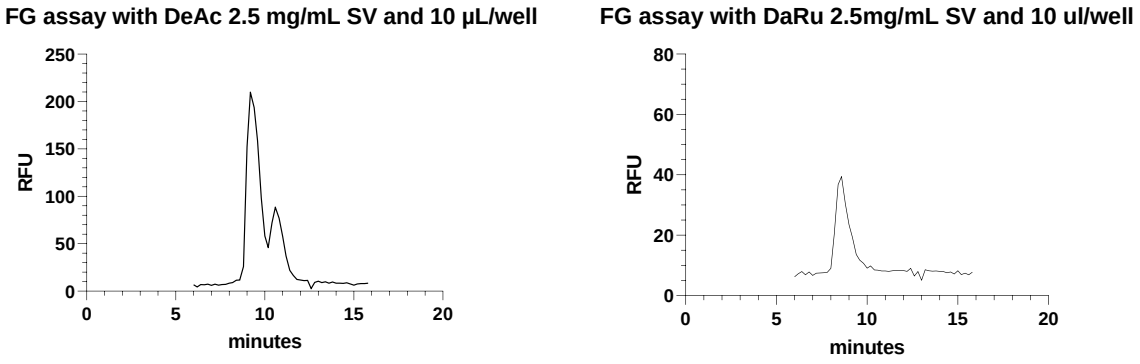


Figure 10|**Example of a FG assay with a set of 50 wells:** They both show the FG assay with the same SV concentration (2.5 mg/mL SV) and the same amount of SV per well (10 µL/well) but the left graph is with DeAc SV and the right graph is with DaRu SV. This example is shown to understand why the first and last two minutes were later cut in the further assays, reducing the amount of substrate needed for the assay (30 wells instead of 50) and the time needed to prepare the assay.

The set of data is then later shortened to 30 detected wells for the DeAc SV. This is a save of time in the preparation of the assay and a save of FG/FC substrate (saving 20 wells) and therefore of money.

Therefore, there are 30 wells and that corresponds to the separation of SV from 8.0 to 13.8 minutes included.

The positive and negative controls were made to confirm the data obtained later (See Figure 11). About the positive controls, they show that activity is highly expected when the degradation of the substrate occurs. Therefore, if any activity is detected on the assay with a separated SV,

this would mean that: this SV has indeed a proteolytic activity and the separated part responsible for the activity is a toxin or family toxin able to degrade the substrate.

The standard deviation (SD) of the FC positive control is quite huge (see Table 3 and Figure 11). To get a more accurate result of the collagenase activity on the FC assay with the exact same conditions, performing a new FC positive control can only have a positive impact on the global results. Also, more positive controls can be tested. Here, it was only threefold. However, those four graphs give a good idea of the results the separated SV would get if there were or not a sign of substrate degradation. Indeed, a proteolytic toxin that would degrade the gelatine is expected to get a RFU at around 265, and at around 1900 for the collagen.

	Mean (RFU)	SD (RFU)
FG positive control	265.1	44.3
FC positive control	1953.0	631.7
FG negative control	5.6	1.3
FC negative control	472.2	31.8

Table 3|**Values of the mean and Standard Deviation of the Positive and Negative controls of the FG and FC assays:** Those values were calculated on Excel based on the set of data obtained.

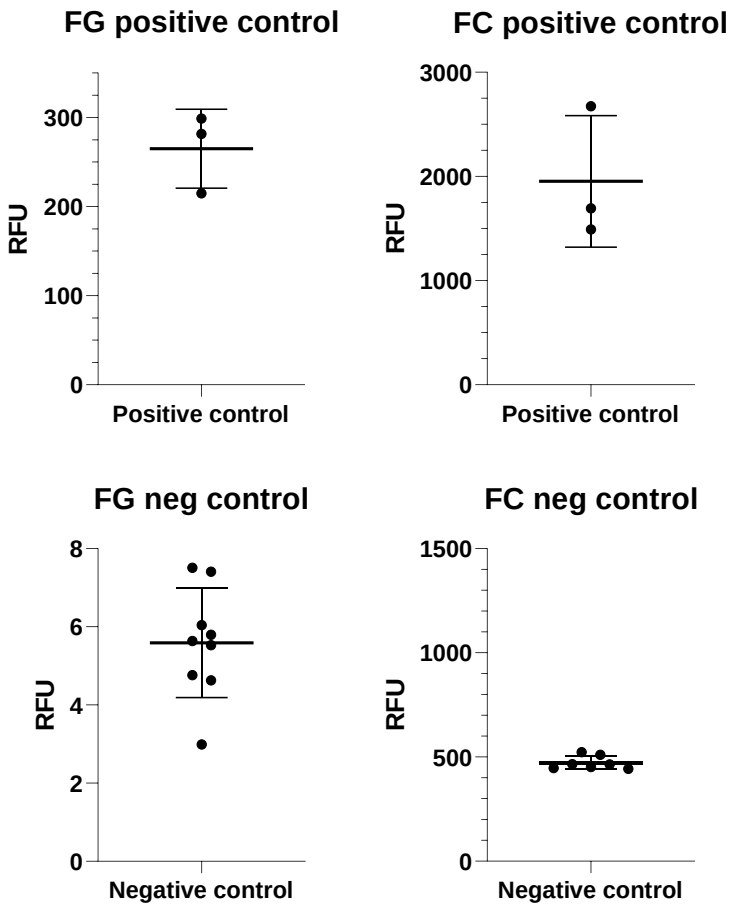


Figure 11|**Positive and negative control of the FG and FC assay:** The Positive controls were both made for FG and FC assay with collagenase 1U/mL. The negative controls were made with FG (for the FG assay) and FC (for the FC assay) normal concentration and special buffer. The graphs show the mean and the Standard Deviation SD

5.4.1 Degradation of Fluorogelatine by Snake Venom

The first graph (see Figure 12) shows the FG assay using the DeAc separated SV with 100% DPBS. The modified gelatine would emit light after an excited light (wavelength excitation is 495 nm and wavelength emission is 425 nm) only if it was degraded by SV. As already stated, the DeAc is a snake from the *Viperidae* family. It has proteolytic activity and is known for its cyto- and haemotoxicity. Therefore, high activity was expected.

The three concentrations and amounts of separated SV in each well were tried to know which one would fit the most for the assay in the future. The positive control on this FG was a mean of 265.1 RFU (SD: 44.3) and the negative control was a mean of 5.6 (SD: 1.3). The proteolytic activity of the DeAc SV on the FG is therefore here clearly shown, with two peaks at 9.2 and 10.6 minutes on all the graphs. This activity corresponds to a toxin or toxin family that degrade

the substrate. They correspond to SVMPs, that degrade the FG. The non-activity of all the other wells shows that there is nothing present in the separated SV that can degrade the FG.

However, the RFU difference between the 5 mg/mL SV 10µL/well, the 5 mg/mL 5 µL/well and the 2.5 mg/mL SV 10µL/well is not relevant and high activity is already demonstrated. The other graphs didn't show enough activity, compared to the positive control. Therefore, to save venoms, the future assays will usually consist only of the 2.5 mg/mL SV and 10 µL/well.

In conclusion, the SV separation was a success. Indeed, the fractioner collected the eluent where some wells had proteolytic activity of degrading the substrate (FG) and some didn't. SVMPs were there collected in the eluent from 8.8 minutes to 11.6 minutes. The 15 collected eluent at 8.8; 9.0; 9.2; 9.4; 9.6; 9.8; 10.0; 10.2; 10.4; 10.6; 10.8; 11.0; 11.2; 11.4 and 11.6 minutes all have SVMPs but at different concentration or with different efficiency of degrading the FG. The FG substrate degradation assay was also a success. Almost the same times are obtained with every graph, and it reinforces the repeatability of the assay.

The FG assay was performed again three times with the DeAc SV, with different samples and at different moment with the same conditions: 2.5 mg/mL SV and 10 µL/well (see Figure 13). This was made to confirm the repeatability and efficiency of this substrate degradation assay.

The FG assay was also performed with multiple parameters with the DeAc SV using 20 % DPBS as solvent (see Supplementary Data Figure 20). The results were high, but the two peaks are not properly separated. Indeed, a good parallel between the separation and the substrate degradation assay cannot be done. However, for future research and method development, in can be tried with different snakes, if the separation is properly done.

FG assays with DeAc SV

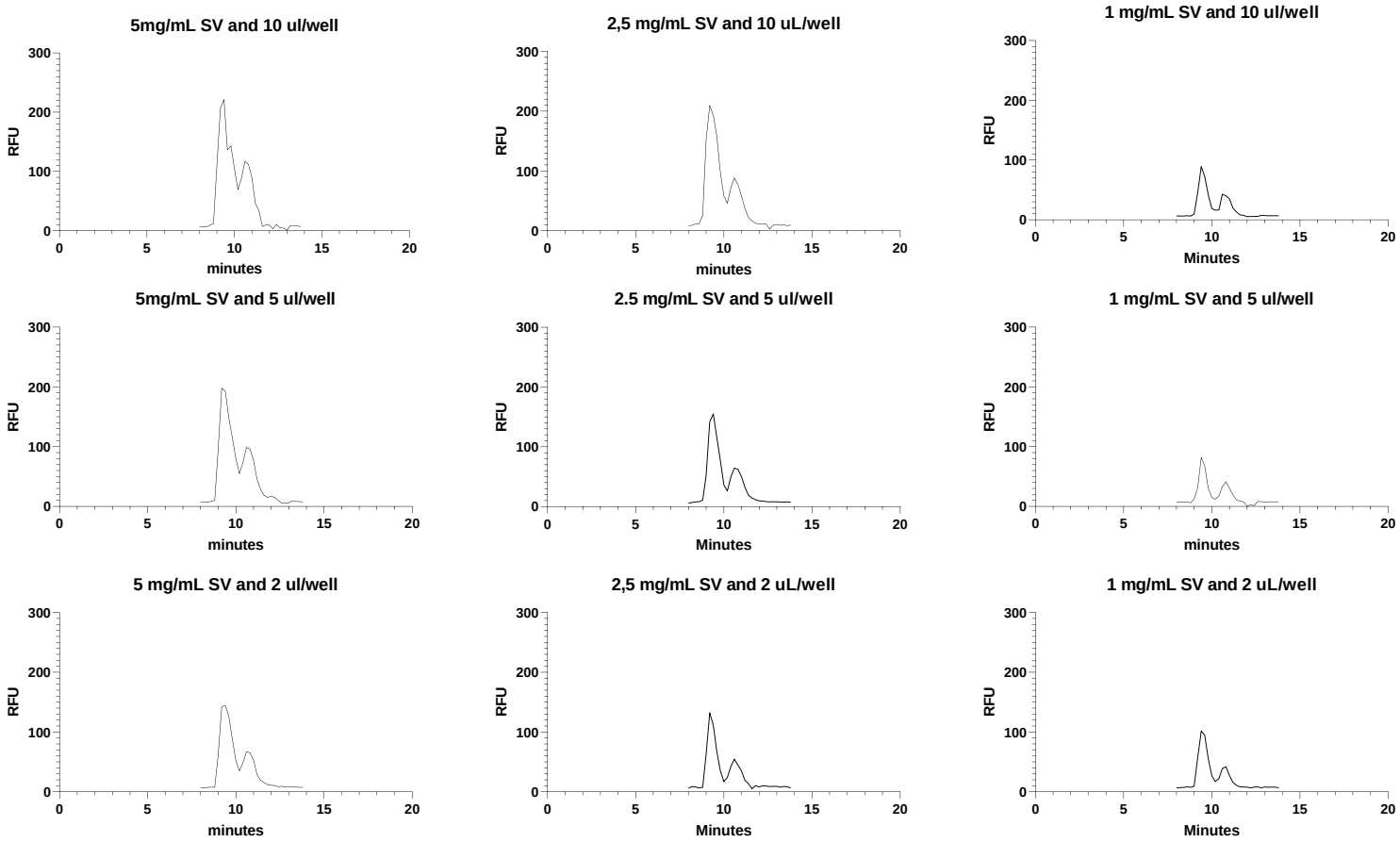


Figure 12|**FG assays with the DeAc SV:** The DeAc SV was separated with 100% DPBS and used for the FG substrate degradation assay. Each graph corresponds to a SV concentration and a different amount of SV in each well: 5 mg/mL SV concentration for the three left graphs; 2.5 mg/mL SV concentration for the three middle graphs and 1 mg/mL SV concentration for the three right graphs; 10 µL of SV/well in the three top graphs; 5 µL of SV/well in the three horizontal middle graphs and 2 µL of SV/well in the three bottom graphs.

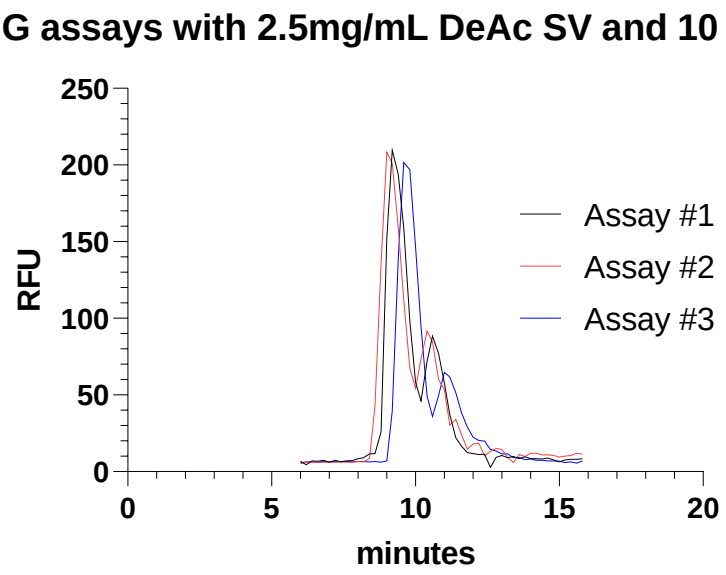


Figure 13|**FG assay using DeAc SV repeated at three different times with different samples but the same condition:** The three assays was performed using a 100% DPBS separation solvent with the DeAc SV and with a concentration of 2.5 mg/mL and 10 µL of fractionated SV/well.

Although there are a few translations of the values on the assays, they confirm the data previously obtained. The 2.5 mg/mL SV and 10 µL of fractionated SV/well are indeed the good and repeatable parameters.

The FG substrate degradation assay was performed using the same parameters (2.5 mg/mL separated SV; 10 µL of fractionated SV/well and 100 % DPBS separation solvent) with the DaRu and BuMu SV (see Figure 14). The DaRu is a snake from the *Viperidae*, like the DeAc. The BuMu is a snake from the *Elapidae* family.

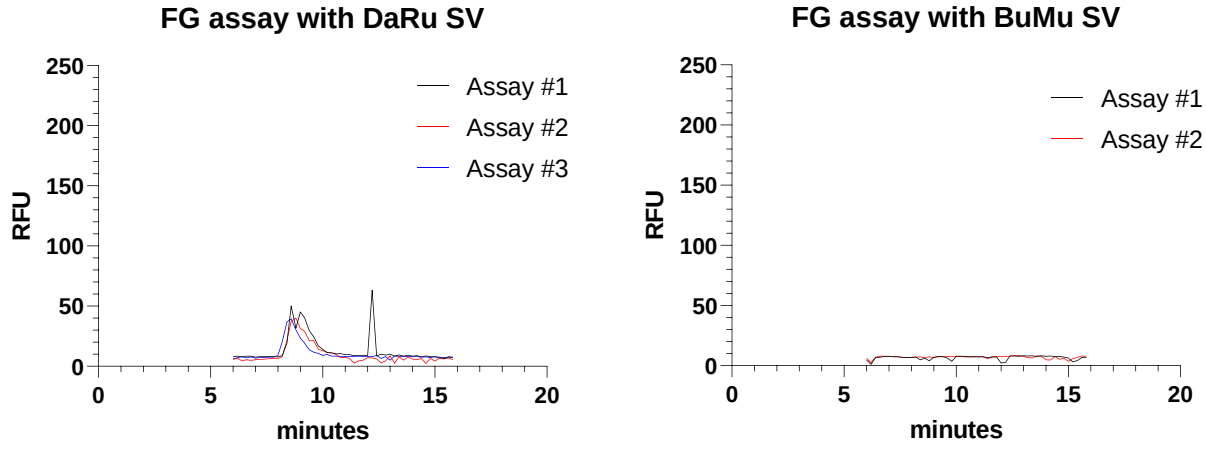


Figure 14|**FG assays using the DaRu and BuMu SV:** The parameters of those assays were 2.5 mg/mL SV, 10 µL of fractionated SV/well and 100 % DPBS. 3 assays were made for the DaRu SV and only two for the BuMu. A false positive well (only one point) can be seen on the DaRu SV graph with the Assay #1 at 12.2 minute.

They were performed three times for the DaRu and two times for the BuMu with different samples. A false positive well (only one point) can be seen on the DaRu SV graph with the Assay #1 at 12.2 minute. This is explained by a manipulation error. All those results were expected. Indeed, the DaRu is also a *Viperidae* snake, like the DeAc, and the BuMu is an *Elapidae* snake. The BuMu has no proteolytic activity, as expected, and therefore does not degrade at all the FG. The degradation activity of FG of the DaRu SV is lower than the DeAc SV. Indeed, the highest peak is 50.1 RFU (not considering the false positive peak). Therefore, it has a lower proteolytic activity and degrade less the FG than the DeAc SV, where the highest peak was 209.6 RFU. But it still has the ability of degrading this FG substrate.

Performing this substrate degradation assay with DaRu SV using different parameters can only bring positive impact for the future. A higher concentration of SV could be tested (5 mg/mL SV). Furthermore, testing this assay on other proteolytic SV can only improve the research. However, this substrate degradation assay using FG was a success, as well as the separation that precedes it. It is a repeatable assay, but further improvements can be done in the future, as well as in the separation.

5.4.2 Degradation of Fluorocollagen assay with Snake venom

The same parameter was taken from the FG assay results to perform this FC degradation assay. The SV was separated with 100 % DPBS, the concentration of the SV of 2.5 mg/mL and 10 µL of fractionated SV/well (see Figure 15). The assay #1 was lower than the two others. This could be because either the separation was not properly done, or either manipulation was not done perfectly as it was the first time the assay was performed.

The positive control on this FC was a mean of 1953.0 RFU (SD: 631.7) and the negative control was a mean of 472.2 (SD: 31.8). It is indeed not as high as the collagenase; however, the activity is clear. The highest peak is 1399.0 RFU. An RFU of about 470 would be the same as the negative control, which is only the FC and the buffer. Therefore, the activity is only detected 9.0 and 11.2 minutes. In comparison, the FG was between 8.8 minutes to 11.6 minutes. A second peak can also be seen, and is lower than the first and highest peak, just like the FG assay.

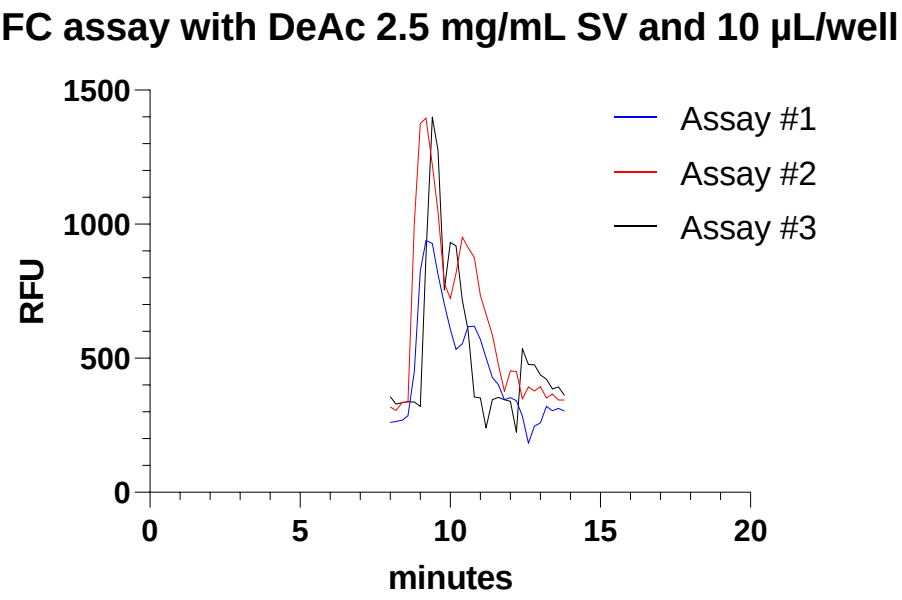
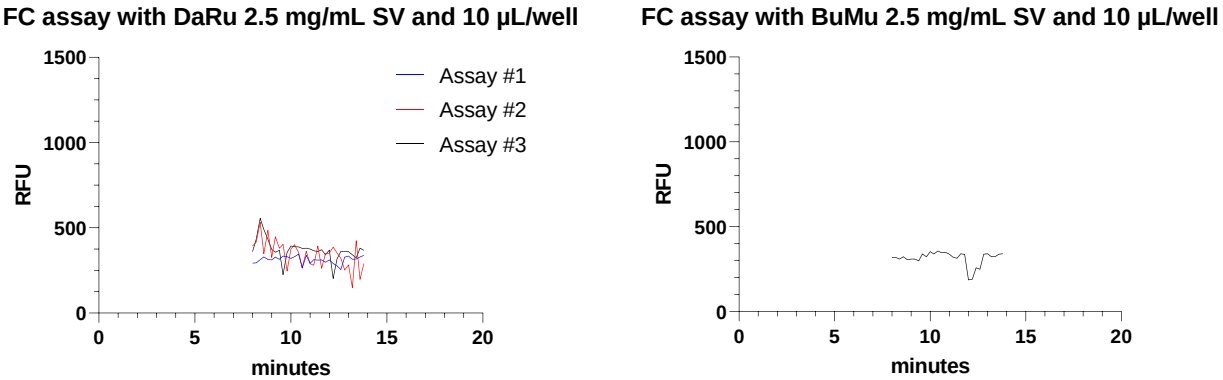


Figure 15| **FC assay using DeAc SV repeated at three different times with different samples but the same condition:** The three assays was performed using a 100% DPBS separation solvent with the DeAc SV and with a concentration of 2.5 mg/mL and 10 µL of fractionated SV/well

The same FC assay was performed with the DaRu and the BuMu SV with the exact same parameters (see Figure 16). Here again, no activity with the BuMu SV was expected, the same reason as with the FG assay. However, more surprisingly, no activity was detected with the DaRu SV. It cannot be from a deficit of activity from the sample as the same separation was taken to perform the FG assay and this FC assay. Furthermore, the DaRu is known to contain SVMPs(9).



*F*igure 16| **FC assays using the DaRu and BuMu SV**: The parameters of those assays were 2.5 mg/mL SV, 10 µL of fractionated SV/well and 100 % DPBS. 3 assays were made for the DaRu SV and one for the BuMu.

In the next graph, one assay of each SV snake was taken, and a negative control was made with 50 wells (See figure 17).

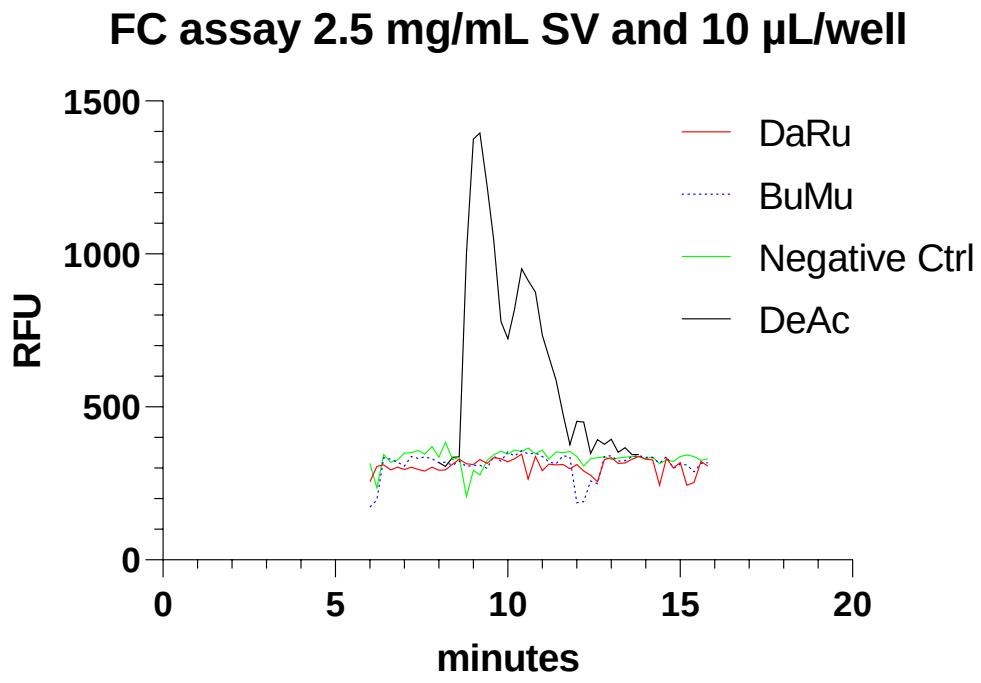


Figure 17|**Comparison graph of the FC assays with DaRu, DeAc and BuMu SV and a negative control:** These four assays were made with the exact same conditions. 2.5 mg/mL SV and 10 µL of fractionated SV/well (except the negative control that only has special buffer).

It is important to note that this FC substrate degradation assay is more sensible than the FG substrate degradation assay. Indeed, even among the negative control, a straighter line cannot be obtained. However, like the FG assay, it has shown an activity when it was degraded. Therefore, this assay can also be used for future research. Furthermore, it can be improved also with a better separation, that would thinner the peaks.

In conclusion, those two assays were a success. Activity was proved to appear as the substrate was degraded. The small amount of SV allows to perform a lot of fractionations and a lot of parallel bioassays. Indeed, 10 µL of SV was injected in the column. 62 µL of fractionated SV was programmed to elute in each well. This can be changed as needed with different analytical times and methods. From those 62 µL per well, only 10 µL (some assays were performed with only 5 or 2 µL but were shown too less activity with those snakes) were taken to perform the bioassays.

Performing this FC assay with a different SV concentration (5 mg/mL) and different fractionated SV amounts like 20 µL/well could be great. Maybe the activity wasn't seen on the DaRu with the FC assay because the toxins responsible for the collagen degradation are not present in a sufficient quantity. Indeed, activity was shown with the FG, sure with a lower RFU than the DeAc, but was still capable of degrading the gelatine. And the DaRu proteomic has

already shown that it contains a lot of SVMPs(36). This could lead to analysis of new species and the activity of their toxins.

As a future lead, it would be great to test an inhibitor like Marimastat. It is a matrix metalloproteinase inhibitor. Performing this assay with the Marimastat (or other inhibitors) would allow to test if it reduced the degradation of FG and FC by the toxins present in proteolytic active SV.

5.4.3 Paralleling the SV separation and the substrate degradation assay

The paralleling between the chromatograms and the two substrate degradation assays can be seen (Figure 18). The chromatograms show the separation of both the DeAc and DaRu SV with the same parameters: 100 % DPBS separation and 2.5 mg/mL SV. The same fractionated SV obtained after those separations is used for both the FC and FG assay. The four assays have the same amount of fractionated SV per well: 10 µL of fractionated SV/well. The red lines are made to prove the parallel between the separation of the SV and the assays.

Concerning the DeAc, the peaks from the FG and FC assay (graph #1 and #2, Figure 18) match 2 peaks from the separation. The only peak seen with the DaRu from the FG assay (graph #3, Figure 18) match with the first peak of the separation. Therefore, we can conclude that those peaks from the chromatograms obtained from the separation of SV correspond to a toxin or a toxin family that has the potential to degrade the substrate.

However, it is very important to mention that in this figure, the two chromatograms were manually translated to the right (about 0.4 minutes) to match the peak. This was made because the delay from the detector to the fractioner wasn't calculated. It must be the first thing to arrange with this method. It can easily be done by injecting a modified compound that has fluorescence properties, like a modified Bovine Serum Albumin, and let it go through the fractioner. After this, the eluent will split in the fractioner and it can be detected with the same method, using a plate reader with fluorescence property. The delay time would be the time of the corresponding well from the fractioner subtract by the time of retention of the compound.

The global objective of this *Thesis* was to parallel the separation of the proteolytic SV with the substrate degradation assays and it was a success. It has a positive impact on the SV research and understanding. Indeed, it can help other methods of research or other fields, like proteomic. By a lack of time, unfortunately, it couldn't have been done but it would reinforce the data obtained in this *Thesis*. A High Content Imager can also be envisaged after this method as it

would allow to study more deeply the toxicity on living cells. The most positive impact this parallel method has is the small amount of SV needed to be able to perform multiple assays. Only few leads can be given but it can be adapted to many species of snakes, and why not other animals.

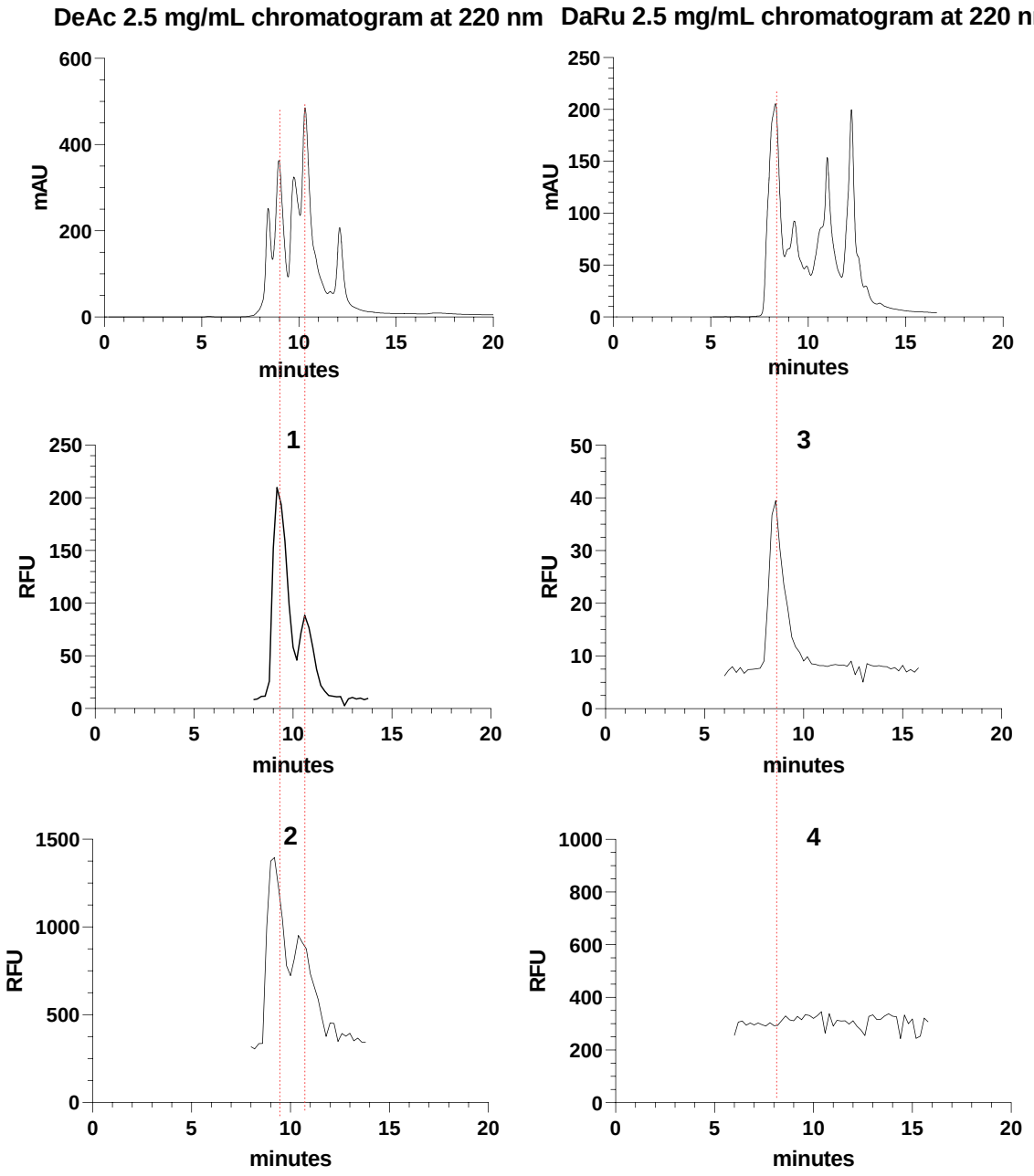


Figure 18|**Paralleling the chromatograms and the substrate degradation assays:** The graph 1 is the FG assay with DeAc. The graph 2 is the FC assay with DeAc. The graph 3 is the FG assay with DaRu. The graph 4 is the FC assay with DaRu. They have the same parameters: 2.5 mg/mL SV separation, separated with 100 % DPBS, and 10 µL of fractionated SV/well. 10µL of SV was injected in the SEC system for both. The chromatograms were both translated to the right to match with the line, to compare the peak. Indeed, the delay of time between the detector and the fractioner has not been calculated yet but a few tenth of a second is expected.

6 Conclusion

The three objectives of this *Thesis* had been fulfilled:

- to create and develop a method that is fast, reliable repeatable and global, meaning applicable for not only one specie but many families, and that would keep the proteins active
- to perform bioassays on modified gelatine and collagen type IV using the previously separated SV
- To make a parallel between the separation and the substrate degradation assay.

Firstly, the separation of SV was indeed a success. However, it had only been tested on a few species, compared to the tremendous number of venomous snakes, and some completely failed. The DPBS allows to keep the proteins active, therefore performing bioassays after the separation. But it has largely been seen in the results that the eluent sometimes goes after 12.5 minutes. The separation is not good for those. Separation of venoms is complex and a method that keep the proteins active is not easy to perform. Further method can be tried by changing the solvent and increasing the analysis time. It would also be great to try this method on other animal venom, like cone snail or spider. However, this is not the research field of this *Thesis* and might be completely unenforceable.

Secondly, the bioassays had been a success. Both gelatine and collagen type IV bioassays have been tried and had shown good results. They both proved that:

- the separation of the SV with this method and with the in-home "fractioner" were a success. Indeed, the activity is limited to a separated part of the SV, which means that some toxins and toxin families are responsible for the hydrolysis of the collagen type IV and gelatine
- Some snake hydrolysis the collagen type IV and the gelatine and some not, as expected. This confirms the fact that venomous snakes have more cytotoxic activities and some not.

However, despite those great results, many improvements and further achievements can be done. By lack of time, only a few snake species were tried. Performing the substrate degradation assay on more cytotoxic snakes would reinforce the assay. Furthermore, inhibitors couldn't have been tested. For instance, Marimastat is a matrix metalloproteinase inhibitor, and it was planned to test it and see if it inhibits the substrate degradation assay. It would be great to

perform this assay and test the Marimastat. Would it inhibit the reaction occurring in the assay, this would mean that it can inhibit SVMPs present in SV. Other inhibitors can be tested. To finish about the assay, unfortunately the conservation condition and the price of those assays can also not be neglected. Indeed, they cannot be performed in poor laboratories where they lack cold chain conservation, money and tools.

Finally, the parallel between the separation of SV and the substrate degradation assay has been demonstrated. Some peaks from the chromatograms match the peaks of activity from the substrate degradation assay and therefore it was concluded that those peaks have proteolytic activity. Furthermore, with this method, it is possible to perform multiple assays with only one small sample of SV. This is the most important, as it allows researchers to perform bioassays with one sample of SV, after one separation. The small amount needed of fractionated SV to perform those bioassays is impressive. With only 10 µL of fractionated SV (x 50 as the fractioner was programmed to split the eluent 50 times), effects can be seen and compared.

However, with a more accurate separation, the results would be even better. The collecting time of the fractioner can also be changed as needed. Testing separation with longer collecting time might increase the repeatability and accuracy of the substrate degradation assay while getting even better chromatograms with thinner peaks.

In the future, a lot can be done with this method of parallel separation using SEC and bioassays. Optimizing the method for specific needs can be easily done, like changing the analytical time and trying other solvent or DPBS concentration. This would allow other SV from other species to be analysed, as the results has shown that some species didn't separate properly.

Proteomic can be tested with a better accuracy and performing totally different assays in parallel could be a great idea that would lead to a better understanding of the SV toxins and toxin families.

Using other assays than the substrate degradation assay could also help the research on other SV than are not particularly cytotoxic. Also, increasing the separation efficiency would be there optimal to get the assay (or proteomic) even more accurate.

Calculate the exact delay between the detector and the fractioner would also be positive. It wasn't done because of a lack of time.

As a last lead, High Content Imager could be used as it has already been done to study venom. *In vivo* toxicity of the SV and separated SV could therefore be performed and the effects of toxicity could directly be seen.

In conclusion, the research and the studies are complex. Studying with precision a mixture as complex as snake venom (or other venoms) demands a lot of techniques and tools, that are not necessarily available all around the world. However, a new approach to study and understand more the cytotoxicity of SV by SVMPs was made in this *Thesis* and a positive impact is therefore expected on the global research. Furthermore, improving the knowledge about snake venoms can lead to new methods, better understanding and hope against snakebite envenoming and therapeutical and medical potential. In the future, it is expected to have a better global view of the snake venom actions and mechanisms.

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9 Appendix

9.1 Supplementary data

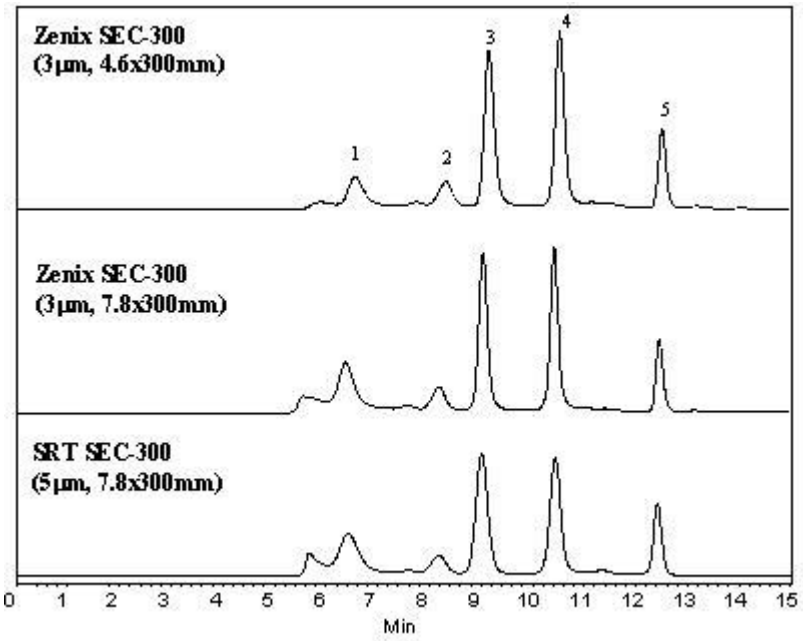

Figure 19|**Separation of protein mixture A by Zenix SEC-300 and SRT SEC-300 columns.** *(53)* Here the results that matter for our method is the 1st chromatogram: Zenix SEC-300 (3µm, 4.6x300mm). The pic **1** matches with Thyroglobulin (670 kD) but it wasn't put in our protein mixtures.

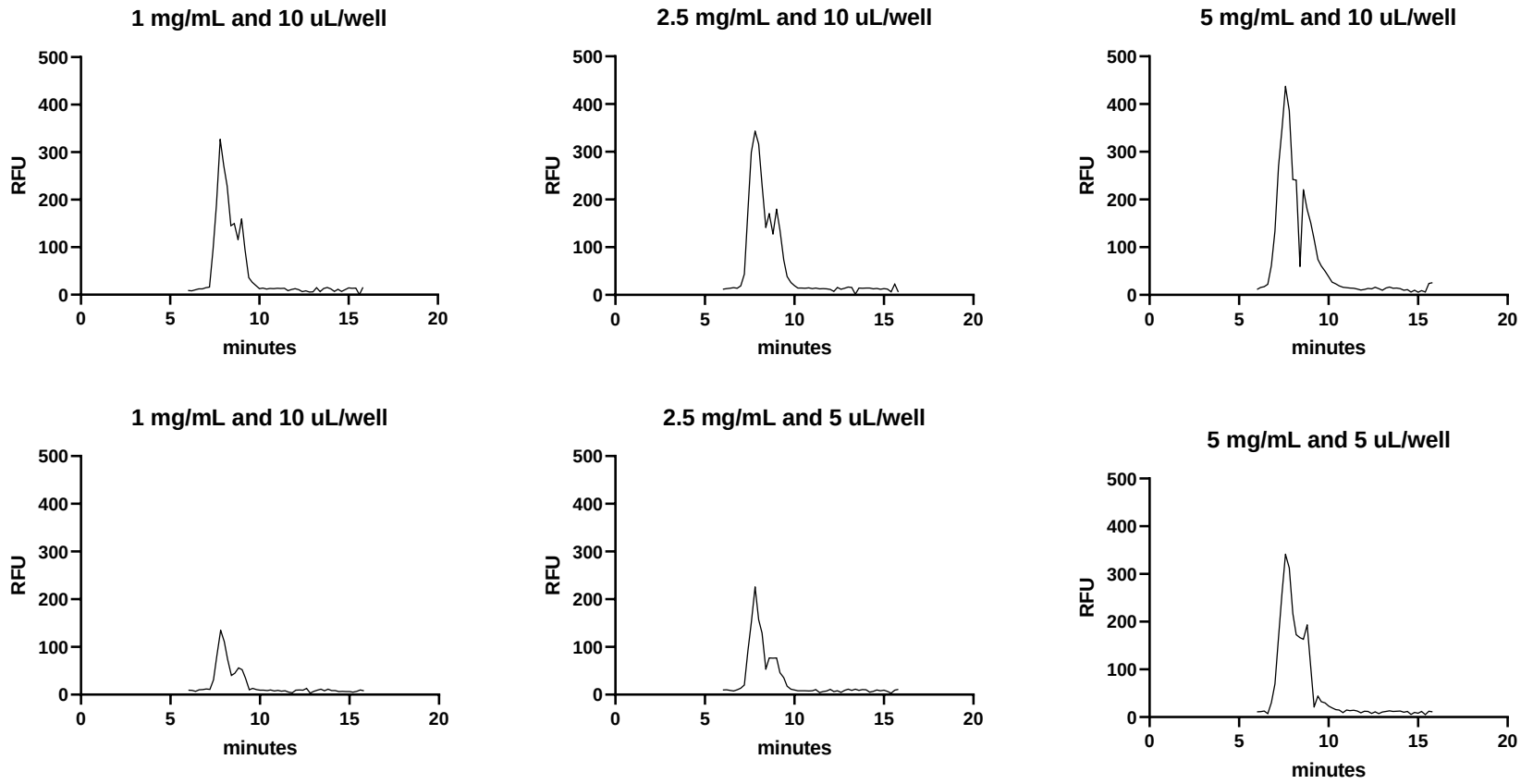

Figure 20|**FG assays with different parameters using the DeAc SV and separated with 20 % DPBS:** The degradation of FG was really high; however the separation is not good enough to parallel the separation with those assays

Chromatograms of different SV using 100 % DPBS at 220 nm

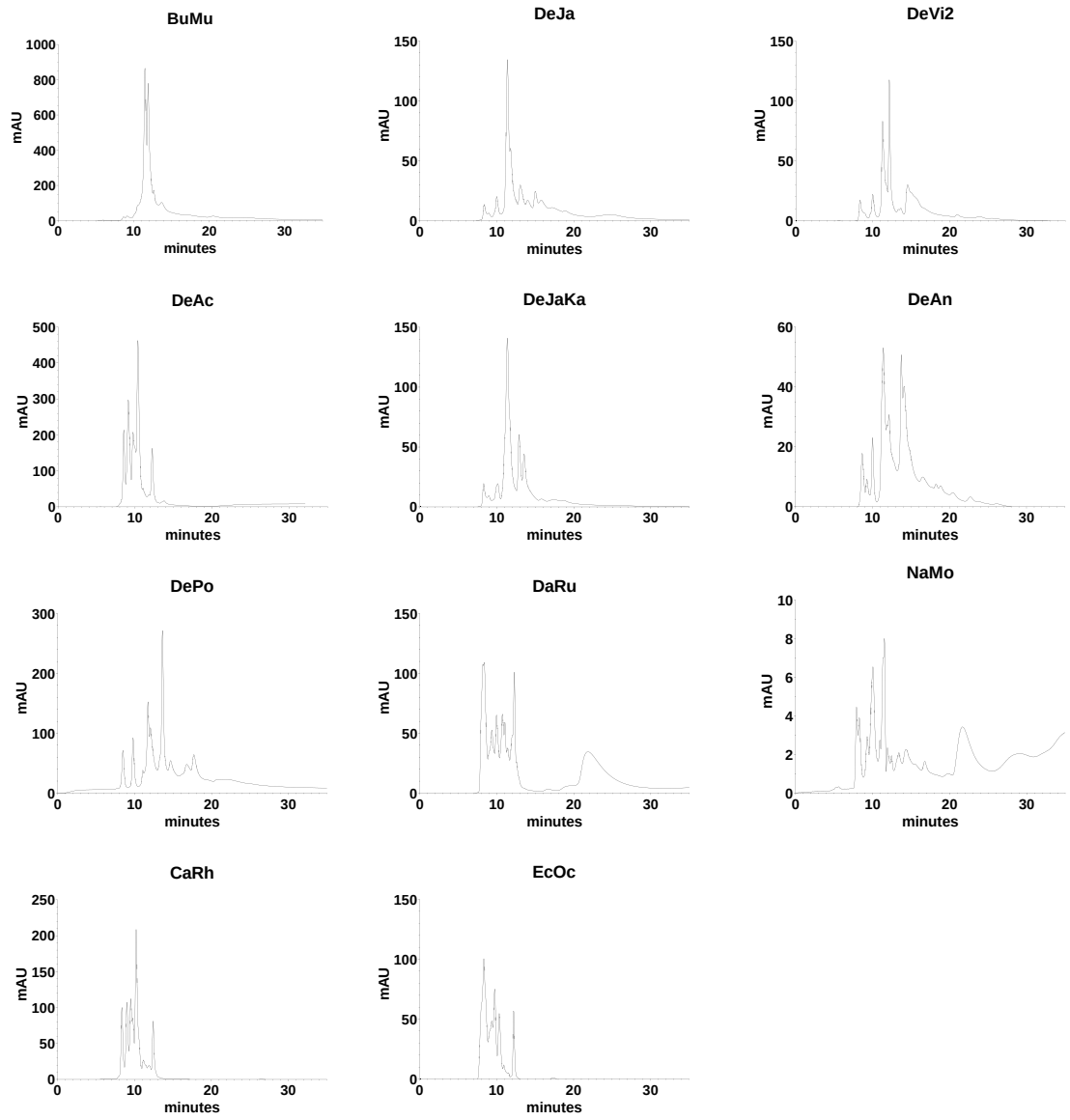

Figure 21|**Chromatograms of different SV using 100 % DPBS at 220 nm:** The analyses were continued up to 35 minutes for those tests. Ten µL were injected with every SV. They clearly show that having a global method to separate the SV is hard to develop. The DaRu chromatograms show a peak that comes far after the normally end of the method (20 minutes is normally settled).

Université de Liège, Faculté de Médecine, Master en sciences pharmaceutiques

"Proteolytic venoms: using size-exclusion chromatography and parallel bioassays to study proteolytic activity of snake venom toxins"

Axel de Monts de Savasse, Année 2021-2022

Snakebites are a major problem worldwide. Although it has always existed, the World Health Organisation (WHO) only recognised snakebites as a Neglected Tropical Disease (NTD) in 2017. By the same time, they acknowledged that they have been underestimating the mortality and morbidity caused by snakebites.

Snake Venom (SV) is known for many diverse types of activity as its compounds are complex and numerous. Different protein classes such as Phospholipase A2 (PLA2), Snake Venom Metalloproteinase (SVMPs) and Three-Finger Toxins (3-FTx) compose SV and each of them has complete different properties, targets and mechanisms of action. Some venoms are known for their high cytotoxicity, others for haemotoxicity or even neurotoxicity. Each family, sub-family and genus has its particularities, as evolution has selected the snakes according to their environment and their specific needs. That is why, through the years, various strategy of studying and curing SV have appeared.

In this Thesis, enhanced efforts have been done to study proteolytic venoms. A Size-Exclusion High-Performance Liquid Chromatography (SEC-HPLC) has been performed on different samples of venoms to separate them while doing parallel substrate degradation assays. The aim is to learn more about the proteolytic activity of SV toxins. Substrate degradation assays allows to study the degradation of Extracellular Matrix (ECM) by using in vitro fluorescent compounds like modified collagen and gelatine. Those assays can be done in parallel of the separation using an in-home modified fractioner that collects the eluent after the SEC-HPLC.

Impressive results have emerged, however further improvement can be done to improve the repeatability of the assays. Moreover, inhibitors of specific protein classes and further assays such as egg-yolk micelles degradation assay, showing the disruption of the cellular membrane, have yet to be performed.

Snake venom; toxin; SVMPs; bioassay; SEC;