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Analysis of intact venom proteins with capillary zone electrophoresis - mass spectrometry

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ABSTRACT

In this work we demonstrated the favourable analytical performance of capillary zone electrophoresis coupled with mass spectrometry (CZE-MS) for the intact protein analysis of similar venom samples. Venom provides a characteristic feature (fingerprint) of a given snake. Using 1 M formic acid (pH = 1.9) as background electrolyte, minimal adsorption and narrow peaks shapes - thus good separation efficiencies - were obtained for the protein components of the venom samples. The precision of migration times and peak areas were 1.9–2.8 RSD% and 0.8–7.2 RSD%, respectively and the theoretical plate numbers were 32000–238000 for peaks having signal-to-noise ratio (S/N) larger than 50.

More than 250 different neuropeptides (7–10 kDa) were detected in the venoms obtained from snakes of 9 different subspecies (belonging either to *Naja* or *Dendroaspis* species). The protein contents of the venoms of the same subspecies collected from different geographical regions are similar and differ only in a few (less than 10 %) components. However, the venoms collected from different subspecies (but from the same species) exhibit very different protein patterns: no matches were found in the molecular weights of proteins.

1. Introduction

Snake venom is an intricate mixture containing a large number of components with a peptide/protein content exceeding 90 % dry weight. Effects such as neurotoxicity, haemotoxicity and cytotoxicity (which vary depending on snake species [1,2]) can be attributed to the peptide/protein components. The Food and Drug Administration (FDA, US) has approved several snake venom toxin-based life-saving drugs for chronic pain [3], hypertension [4], and heart attack treatment [5]. Additionally, these toxins also offer potential therapeutic agents, such as blood thinners [6] and anti-platelet agents [5] due to their bioactive potency and extremely high specificity towards their targets [7,8]. Antivenoms developed for one species may lack effectiveness to treat bites from other species due to interspecies variations in toxin composition [9]. In order to understand the mechanism of action of venoms, the determination of their composition is important.

Peptide toxins such as neurotoxins, which act postsynaptically, have a molecular weight in the range of 4.5–10 kDa [10]. The largest physiologically effective components in snake venoms are enzymes with molecular weights ranging from 13 kDa to 150 kDa [10] (e.g., phospholipase A_2 (PLA_2) have molecular weight 13–18 kDa). Snake venom also contains low molecular weight and less physiologically active compounds such as lipids, nucleoside, metal ions, carbohydrate and some peptides having molecular weight less than 1.5 kDa [10]. Around 80 % of the components in elapids belongs to the relatively small proteins (neurotoxins and PLA_2) [5]. Venom composition significantly varies across subspecies even within a species [9,11] due to very different factors as the geographical, environmental or living conditions or even the diet of snake [8,9]. Venom of a given snake could possess hundreds of bioactive toxins [7,11], which belong to any of ~ 30 protein

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Table 1
The venoms used for the analyses.

snake species (geographical origin)	sample
Dendroaspis polylepis (Eswatini)	A419
Dendroaspis polylepis (South Africa, KwaZulu-Natal)	A379
Dendroaspis viridis (Ghana)	A236
Dendroaspis viridis (Togo)	A260
Naja annulifera (South Africa, KwaZulu-Natal)	B38
Naja annulifera (South Africa, Pretoria)	A416
Naja annulifera (Eswatini)	A410
Naja haje haje (Egypt)	A676
Naja haje legionis (Morocco)	A872

families [12,13]. This is due to the fact that protein families present in venom may possibly have several hundreds of isoforms [14]. Nearly 3000 isoforms of snake toxin were retrieved in the reviewed entries of UniProt database [15]. It has been well studied that different isoforms of the same protein might have altered biological activity [10]. The most common cause of this protein complexity is the varying degree of glycosylation [16–18]. However, no additional published reports were found for the analysis of other post-translational modifications in snake venom proteins.

The progress made in the field of proteomics (driven by well-developed analytical methods, such as two-dimensional gel electrophoresis [19], size exclusion chromatography [20], reverse-phase chromatography [21], SDS polyacrylamide gel electrophoresis [18,22], ion exchange chromatography [23] and mass spectrometry [24,25]) has led to a more advanced understanding of venom composition. Bottom-up and top-down proteomics are the two main approaches for venom protein analysis. In the bottom-up approach, proteins are first digested with proteases and the peptide mixture is then analyzed using LC-MS/MS. The relatively small size of the resulting peptides facilitates their MS/MS sequencing and identification [26–28]. Most venomics studies utilize the bottom-up approach even though it often does not give sufficient information of the proteoforms present in the sample [29,30]. Additional (and direct) information, such as the molecular mass of intact toxins and their proteoforms can be often retrieved using top-down analysis of snake venom [31–35].

Complementary to chromatography, capillary electrophoresis coupled with mass spectrometry is also a powerful tool for the top-down analysis of intact proteins [36–38]. In our previous studies in the field of intact protein analysis, the efficient separation of various proteoforms was achieved [37]. In the present study, our goal is to demonstrate the beneficial analytical performance of capillary zone electrophoresis (coupled with mass spectrometry) using bare fused silica capillaries through the comparison of protein patterns of similar venom samples. To the best of our knowledge, there is no existing report on the intact analysis of snake venom proteins with CZE-MS.

1.1. Experimental section

1.1.1. Reagents and samples

All reagents used were of analytical grade. Methanol, formic acid, acetonitrile (ACN), propan-2-ol (isopropyl alcohol, IPA), sodium hydroxide (NaOH), and ammonium hydroxide solution were procured from Sigma Aldrich (St. Louis, MO, USA), and dilutions were prepared prior to use in ultrapure water (Millipore Synergy UV). For the preparation of background electrolytes (BGEs), formic acid was diluted to 1 M concentration or solid ammonium acetate was dissolved and its pH was adjusted to the desired value by titration with 25 % m/m ammonium hydroxide solution. A membrane filter (0.45 μm pore size) was used to filter all solutions and filtered solutions were stored at + 4 °C. BGEs were kept in ultrasonic bath for a minimum of 5 min to remove gases. For first time use, the fused silica capillary was conditioned with 1 M NaOH (20 min) followed by de-ionized water (10 min) and then running buffer (20 min).

Snake venom samples were collected by a specialist certified by SNTC/FAGASD following approval from the Ethics Committee at the UVMP in Košice, under permission No. EkvP/2023–09. The samples were taken at the breeding facilities VIPERAFARM with registration number CHNZ-01-TT (Tropical world with registration number CHEZ-TT-01, and UVMP with registration number SK NZ 0010/2022). Individuals in the breeding facilities were captured using standard methods with protective handling equipment. The different venoms were collected from different geographical sites from the different snake species (Table 1.). Snake venoms were individually extracted from six adult and subadult individuals of each species, of both sexes, directly into glass collection containers. Subsequently, the venom was transferred in 1.5 ml microcentrifuge tubes. During transportation, the tubes containing snake venom were initially stored at −20 °C and later placed in a deep freezer box at −80 °C for preservation. The venom samples were 5 and 20 times diluted with water before MS and UV detection, respectively.

2. Instrumentation and analysis

Samples were analyzed with a CE instrument (7100 model, Agilent, Waldbronn, Germany) using UV and MS (maXis II UHR ESI-QTOF MS, Bruker, Bremen, Germany) detection.

For CE-UV measurements, fused silica capillaries (Polymicro, Phoenix, AZ, USA) of 65 cm x 50 μm I.D., 370 μm O.D. (effective length of 56.5 cm) and on-capillary UV photometric measurement (λ: 200 nm) was used. Samples were injected hydrodynamically (50 mbar for 2 s) at the anodic end of the capillary. BGE contained 1 M formic acid (pH = 1.9). Capillaries were preconditioned with 1 M NaOH for 10 min, followed by 1 M HCl for 5 min and finally with BGE for 8 min. + 25 kV voltage was applied for the separations. OpenLAB CDS Chemstation (Agilent) software was used for both controlling the CE instrument and data processing.

For MS detection, a CE-ESI interface (G1607B, Agilent) provided online hyphenation to the CE instrument. An isocratic pump (1260 Infinity II, Agilent) was used for transferring the sheath liquid. MS instrument was operated by otofControl version 4.1 (build: 3.5, Bruker). For CE-MS measurements, the following parameters were used: fused silica capillary: 85 cm x 50 μm I.D. and 370 μm O.D.; voltage applied: +25 kV; sample injection (hydrodynamic): 50 mbar for 6 s; sheath liquid (SL): IPA:water = 1:1 with 0.1 % formic acid (v/v); SL flow rate: 7 μL min^{-1}. The capillaries were preconditioned with the BGE and post-conditioned with ACN and BGE for 2 min each. MS parameters: positive ionization mode; dry gas flow rate: 8 L/min; dry gas temperature: 220 °C; nebulizer pressure: 0.4 bar; capillary voltage: 4500 V; spectra rate: 3 Hz; end plate offset: 500 V; mass range: 800–2200 *m/z*. The syphoning effect generated by the nebulizer gas flow was decreased by turning off the nebulizer gas pressure for 5 min at the beginning of each run. For internal *m/z* calibration, Na-formate calibrant was introduced after each separation. Compass Data Analysis version 4.4 (build: 200.55.2969) was used for processing the mass spectra obtained.

3. Results and discussion

3.1. CZE analysis of snake venoms

Capillary electrophoresis has a variety of techniques available for intact protein analysis, however, due to MS-compatibility issues, CZE is most often the method is choice. In the context of intact protein analysis, the application of coated capillaries is generally considered imperative for avoiding adsorption effects. However, efficient separations are also possible using bare fused silica capillaries provided that the conditions in the capillary do not allow the occurrence of electrostatic or hydrophobic interactions between the proteins and the bare fused silica wall, which can be achieved by using BGEs of very low pH. Using moderate pH (e.g., ammonium acetate) the adsorption effects are increased due to

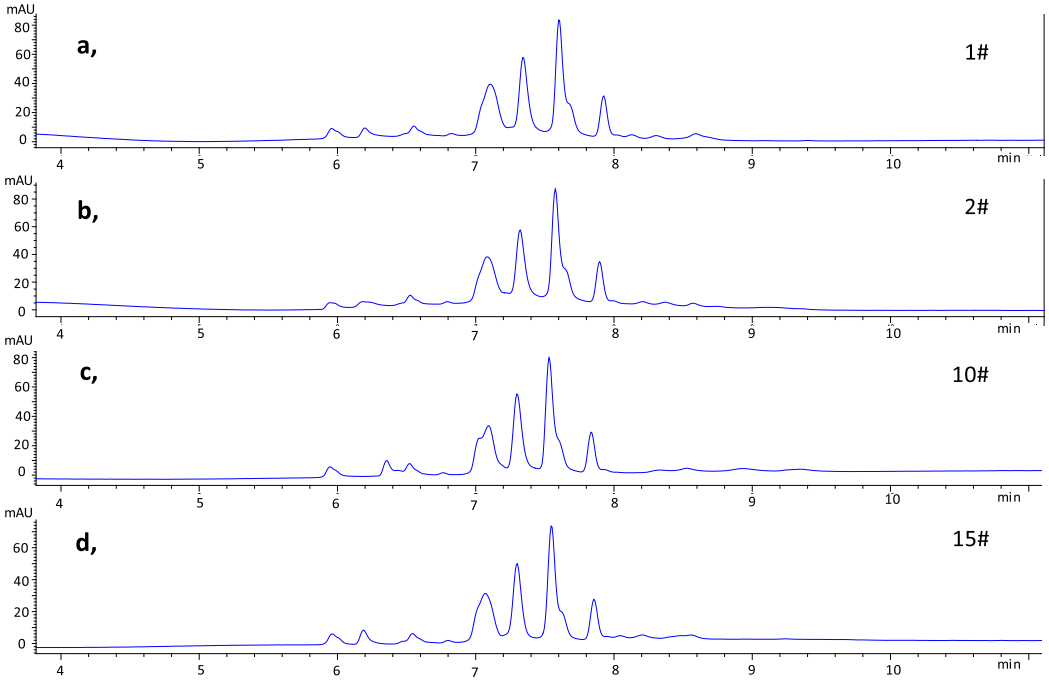

Fig. 1. CZE electropherograms of snake venom (Naja annulifera B38) obtained for consecutive runs (#1 (a,), #2 (b,), #10 (c,), #15 (d,)). Conditions: capillary: l_{tot}: 65 cm; l_{eff}: 56.5 cm; i.d.: 50 μm; BGE: 1 M HCOOH, pH = 1.9; U: 25 kV; injection: 50 mbar x 2 s; preconditioning: 5 min with 1 M NaOH, 5 min with 1 M HCl and 8 min with BGE, λ: 200 nm.

the strong electrostatic interactions and at high pH (e.g., 0.1 M NH_3) the aggregation/denaturation of proteins can occur (Fig. S-1). Around or below pH = 2 the siloxide groups are completely protonated leading to a neutral charge of the silica surface (silanol groups), while the proteins possess a large net positive charge [37]. Using 1 M formic acid (pH = 1.9) as BGE, minimal adsorption and narrow peaks shapes, thus good separation efficiencies were obtained for the protein components of the venom samples. Even if a small fraction of the protein content is adsorbed, the proper pre/postconditioning of the capillary (5 min with 1 M NaOH, 5 min with 1 M HCl and 8 min with BGE for UV and 10 min with BGE for MS) can renew the capillary surface. Fig. 1 demonstrates excellent separation efficiency and reproducibility of 15 consecutive runs of a venom sample. The precision of migration times and peak areas were 1.9–2.8 RSD% and 0.8–7.2 RSD% determined for peaks having signal-to-noise ratio (S/N) larger than 50. Changing the concentration of the BGE has only little effect on the separation efficiency since at this

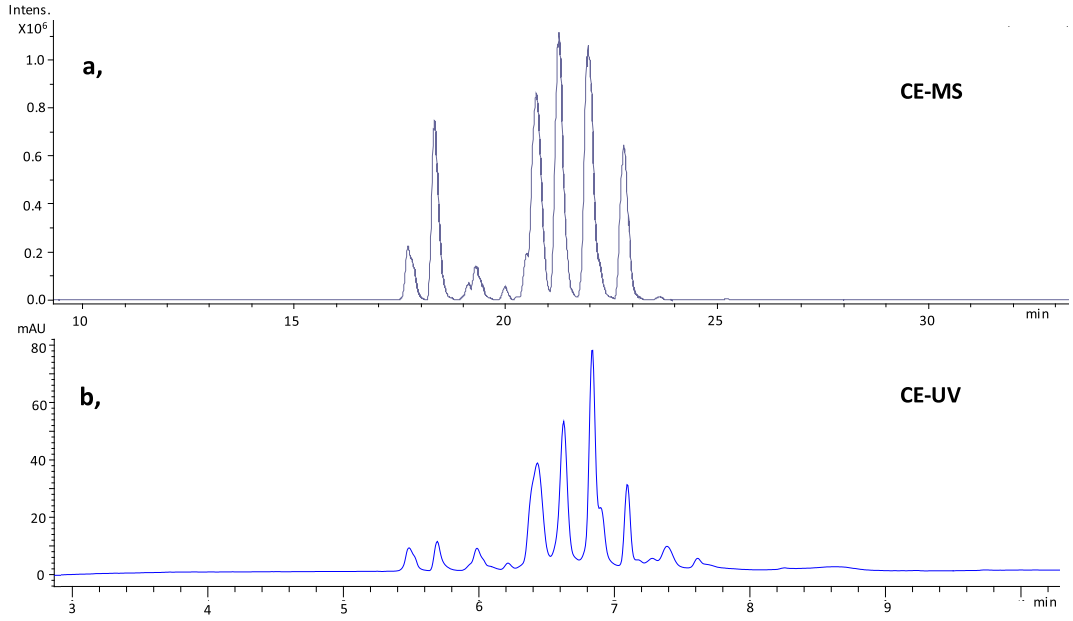

Fig. 2. CZE electropherograms of snake venom (Naja annulifera B38) with MS (a,) and UV (b,) detection. Conditions: BGE: 1 M formic acid (pH = 1.9). For MS detection: capillary: l_{tot}: 85 cm, i.d.: 50 μm, sheath liquid: 0.1 % formic acid in 1:1 IPA-water. For UV detection the parameters were the same as in Fig. 1. MS parameters: positive ionization mode; nebulizer pressure: 0.6 bar; dry gas temperature: 200 °C; dry gas flow rate: 4 L min^{-1}; capillary voltage: 4500 V; end plate offset: 500 V; spectra rate: 3 Hz. Venom samples were diluted with 5 and 20 times for MS and UV detection, respectively.

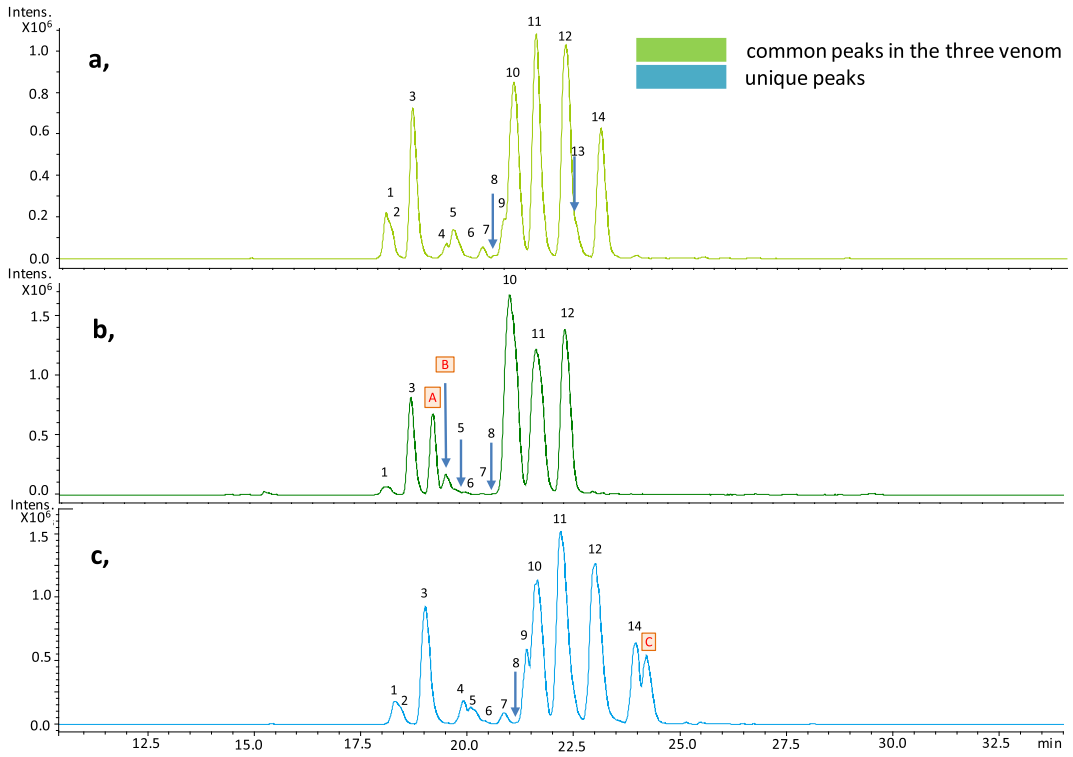

Fig. 3. CZE-MS analysis of three different snake venoms obtained from Naja annulifera species (a,: South Africa, KwaZulu-Natal, b,: South Africa, Pretoria and c,: Eswatini). Peaks with identical numbers represent the same components and the peaks with identical components of 2 or 3 venoms were represented in green. The peaks were shown in blue if those contain other component than the relevant peaks of the other snake venom (peaks are denoted with letters). The detailed monoisotopic mass list of the peaks are given in Table S-1. CZE-MS parameters are given in Fig. 2.b. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

low pH there is no EOF that could be influenced by the different ionic strength and the pH only slightly changes in the range of 0.1–2 M formic acid (Fig. S-2). The separation efficiency can be increased with a longer capillary (e.g., 120 cm as in Fig. S-3) but this leads to prolonged analysis time (>30 min) (even if a small adsorption is acting the peak broadening would be significant). The (5-100x) dilution of the venom sample did not result in a remarkable enhancement of the resolution (Fig. S-4).

3.2. MS and UV detection of separated intact proteins

Both MS and UV spectroscopy are considered as methods that are applicable for the detection of different compounds. While UV is quite universal since most of compounds contain chromophor group(s) and the response signal depends on the absorption characteristics and the molar concentration of the compounds, in the case of ESI-MS, the obtained signal is influenced by several factors (ionizable groups, degree of ion suppressing effects and adduct formation and other interferences of the matrix). In UV detection the larger the protein molecule, the larger the molar absorption coefficient (as the number of the chromophore groups increase approximately in a linear fashion) that is the signal/mass ratios are similar for proteins of different sizes. However, in MS detection the increased size of the protein generally results in larger number of charged forms, adducts and wider isotopic envelopes, therefore the overall analytical signal is shared by the different forms of the same protein, leading to considerable signal dilution. This is the reason why MS detection sensitivity obtained for larger proteins (e.g., human serum albumin, MW: 66.5 kDa) is often not better than UV detection [37]. When the electropherograms of a venom sample (*Naja annulifera* species) obtained by MS and UV detection are compared, the peak patterns look quite similar: 8–10 high intensity peaks appear and the most intense 3–4 peaks are of the same components in both cases (Fig. 2.). There is good chance that if a protein has a high UV absorbance then its ESI-MS response is considerable as well, however, this is not to be taken as a general rule (for instance the a second high intensity peak in the MS electropherogram (at 18.3 min) appeared as a relatively low intensity peak in the UV electropherogram (5.7 min)).

Since UV detection can have better sensitivity when it comes to large proteins, it can be presumed that the small, broadened peaks of the slowest migrating components seen only with UV detection (7.2–9.5 min) are large proteins (e.g., serine proteases ranging from 28 to 60 kDa or snake venom l-amino acid oxidase (LAAO) with 120–150 kDa molecular weight [10]). These results acquired for snake venoms confirm our earlier findings [37] in that when real samples are analysed, proteins larger than 25 kDa can be detected only with a very poor sensitivity.

The separation efficiencies in the case of both detection methods look very similar (e.g., the theoretical plate numbers were 22000–142000 and 32000–238000 for UV and MS detection in the electropherograms of Fig. 2, respectively). Although the efficiency of the CE-MS system often lags behind that of CE-UV due to i) the laminar flow generated by the small vacuum at the outlet of the CE capillary in the ESI interface and ii) the 4 times larger sample concentration, the longer separation length in the case of MS detection compensated for this disadvantageous “siphon effect” (Fig. 2).

3.3. Comparison of protein contents of different snake

There is an exceptional large variety among the proteins of venoms. The venoms of elapids (snake family including several hundreds of species and subspecies (e.g., *Naja, Dendroaspis*)) includes eight protein families: natriuretic peptides, three-finger toxin (3FTx), phospholipase A_2 enzyme (PLA2), Kunitz-type serine protease inhibitor (KSPI), snake venom metalloproteinase (SVMP), cysteine-rich secretory protein (CRiSP), L-amino acid oxidase (LAAO) and snake venom serine protease (SVSP) [5]. The 3FTx (6–8 kDa) is the most abundant protein family

Table 2

Monoisotopic molecular masses of peptides/proteins determined by intact CZE-MS in different snake venoms. Molecular masses are shown in different shades of green depending on the degree of deviation from the masses of the other one or two venoms (in peaks marked with the same number) and the molecular masses in black largely differ. In each peak only the highest intesity mass is shown and low intensity components are not shown. (The components with low intensity or co-occuring in the numbered peaks are listed in Table S-1.) CZE-MS electropherograms of the relevant venoms are demonstrated in Fig. 3, Fig. S-11, Fig. S-12 and Fig. S-13.

Dendroaspis polylepis		
Peak no.	A379	A419
1	6555.2291	6555.2371
A		6609.1971
2	6552.2224	
3		6367.9748
4	7128.4683	7128.4564
B		7204.4984
5	6628.165	6628.1642
6	6847.2812	6847.2717
C		6794.0808
7	7170.4713	
8	6902.0748	
9	8069.7028	
10	8110.6805	
D		7970.6298
E		7985.6448
F		8500.9603
11	7345.2336	
12	7464.2364	
13	7981.6627	
14	8009.5999	
15	7031.1665	7031.1681
G		6804.0106
16	6351.8907	6351.8907
17	6760.8974	6760.9024
18	7435.3276	
19	7434.38	7434.374
H		7666.3292
20	6464.9618	6464.9625
I		6592.9287
21	7376.3451	7376.3413
22	7394.4249	7394.4246
23	7540.314	7540.3117
24	7287.2159	
25	7305.2287	
J		4088.9127
K		7692.2741

Dendroaspis viridis		
Peak no.	A236	A260
1	6563.1769	6563.1812
A		7005.3566
2	7645.8143	7645.8171
3	7742.8623	7742.8723
4	7205.4410	7205.4514
5	7044.3569	7044.3813
6	6614.1036	6614.1127
7	14171.3650	14171.4026
8	6729.9846	6729.9833
9	14278.4856	14278.4734
10	6774.1466	6774.1576
B		6549.9399
11	7281.2677	
C		6423.8933
12	8514.9303	8514.9441
13	7027.0601	7027.0698
14	7408.2045	7408.2143
D		7507.4522
15	6395.8393	6395.8488
16	7980.5371	
	7169.1726	7169.1822
17	8038.5403	
E		6692.9664
18	6660.2432	6660.2543
19	6197.8143	
F		6216.8651
20	7199.1868	7199.1965
21	7217.2017	7217.2107
22	7756.5048	
23	7336.2835	
24	6202.8012	
G		7541.1657
H		7543.1463
I		7729.4618
25	6740.8448	6740.8525
26	7331.3456	7331.3563
27	7676.2902	7676.2999

Naja annulifera			
Peak no.	B38	A416	A410
1	7576.4440	7576.4529	7576.4468
2	7128.4728		7128.4648
3	6829.4914	6829.5002	6829.4943
4	6412.9814		6412.9857
A		6900.2292	
B		6831.0181	
5	7515.3421	7516.346	7515.3372
6	7740.4268	7740.4274	7740.4202
7	6380.871	6380.8788	6380.8780
8	6849.0889	6849.0854	6849.1010
9	6789.3139		6789.3222
10	6684.1990	6684.1940	6684.1897
11	6844.2111	6845.2139	6844.2123
12	6858.1878	6858.1954	6858.1934
13	7806.3675		
14	6639.0953		6639.0978
C			6681.1465

Deviation in ppm
2 >
2 to 5
5< 150
>150

Naja haje haje		
Peak no.	A676	A872
1	7691.5988	
A		7638.5677
2	6875.1418	
B		7528.4701
C		7557.4808
3	6829.4932	6829.4931
4	6735.0481	
5	6848.1301	6847.1325
6	6831.0039	6831.0047
7	6875.4473	
D		6692.9103
8	6380.8715	
E		7301.1243
9	7301.1399	
F		7375.4329
10	7761.4364	
G		7207.0363
11	6812.2364	
H		6655.1263
12	6760.1557	
I		7806.369
J		6655.0901
14	6822.8334	
15	7877.4252	
16	7895.4439	7895.4407
K		7278.0914
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18	7652.3032	7652.3025
L		6376.6545
19	6371.1614	

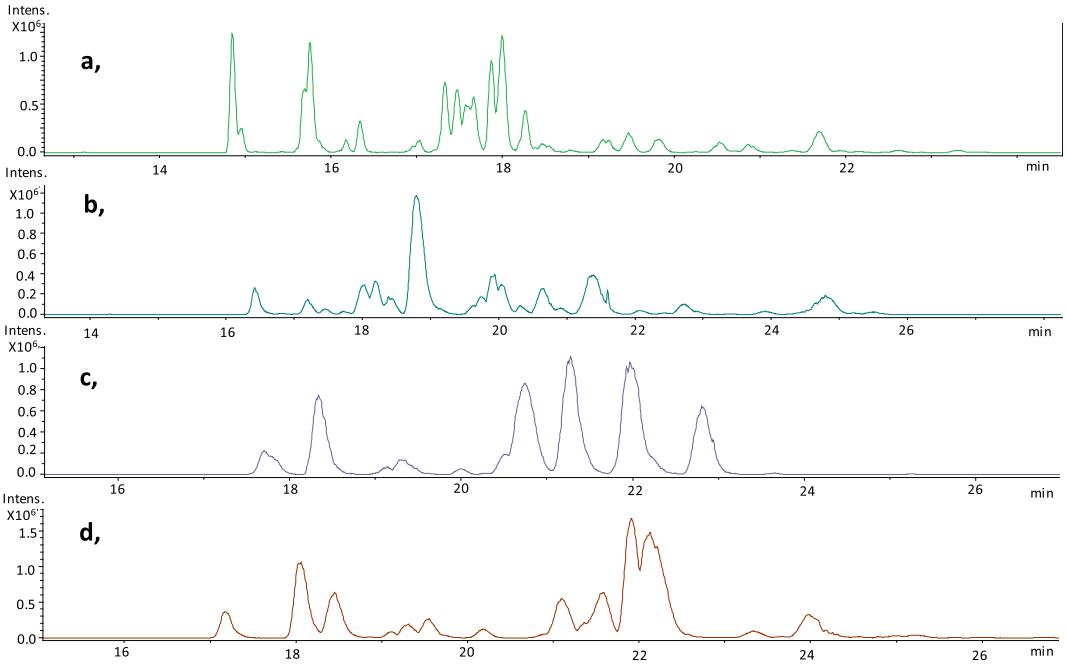

Fig. 4. CZE-MS electropherograms of venom samples obtained from different snake species (Dendroaspis polylepis A419 (a,); Dendroaspis viridis A236 (b,); Naja annulifera B38 (c,) and Naja haje haje A676 (d,)). The conditions were the same as in Fig. 3.

which together with the PLA2′s (13–15 kDA) make up 80 % of elapid's venom [5]. That is the majority of the proteins is smaller than 25 kDa, thus presumably those are efficiently detectable by intact ESI-MS.

In Fig. 3 the CZE-MS electropherograms of the venoms of the same subspecies (*Naja annulifera*) collected from different geographical regions (the venoms originated from South Africa and Eswatini) can be seen. The protein patterns are similar and those only differ in a few peaks regarding the high intensity ones. These slight differences could be expected since venom composition varies due to geographic location, environmental conditions and even the type of target prey available [9]. The peaks at the same migration times of the three electropherograms mainly contain the same protein components, but in a few cases very small differences (smaller than a few Da) could be found in molecular weights, which can be attributed to post-translational modifications or other slightly differing variants (Table 2). Some peaks contain more than a single component, the detailed monoisotopic mass list of the peaks are given in Table S-1. In the case of species *Naja annulifera* snake venoms, 17 peaks - but in case of the species *Dendroaspis viridis* snake venom 35 peaks - could be separated.

When the CZE-MS electropherograms of venoms collected from different subspecies (but still from the same snake species (*Naja haje*)) are compared, very different peak patterns are obtained: the peaks mainly appeared in different migration times and the signal intensities are not similar (Fig. 4). There was no match found in molecular weights of protein components between the different subspecies, although the molecular weights of the majority of the detected 250 proteins ranged between 6.3 and 7.9 kDa, which indicates the presence of highly abundant 3FTx (6–8 kDa) in elapids. The other components of 13–15 kDA molecular weight constituting ~ 5–10 % of total protein we could separate, point at low amount of PLA2, which occurs in some species (e.g., *Dendroaspis* genus lacks PLA2 enzyme [5]). These differences seen in PLA2 ratio was also described in other snake species: among many other venom components PLA2 isotypes shows a high contribution to geographical variabilities [39]. The amount of PLA2 in the total venom changes with age: in a viperid genus of Mexico (Metlapilcoatlus nummifer) it was totally absent in juvenile individuals but abundantly expressed in the adults [40]. We also reported before that three different Naja species show very different PLA2 percentages in the total venom (Naja ashei: 27 %, Naja pallida: 5 % and Naja mossambica: 13 %) [41]. As suggested by others, the PLA2 content of venom can complement the phylogenetic classification of snake species. Some of the molecular masses (eg. 7331.3456 or 6367.9748) obtained in our measurements for the Dendroaspis polylepis venom agreed very well with the results of Petras et al. (7331.35 and 6367.97, respectively [34]) analysed the snake of same subspecies, the deviations were less than 0.6 ppm (the mass of 7331.35 was identified as Andrenergic toxin rho-elapitoxin-Dp1b, P25518 protein [34]).

4. Conclusions

In our work we demonstrated that a large number of venom proteins can be separated with CZE-MS in a bare fused silica capillary using 1 M formic acid as BGE. Minimal adsorption of components, good precision and separation efficiency were obtained (the efficiency was not remarkably better in coated capillaries like linear polyacrylamide (LPA), which are expensive or an intricate multi-step production is needed). Venom samples contain a large number of peptides/proteins along with other matrix components. High efficiency separation could be achieved even without resorting to sample pretreatment (only dilution with water).

Although the proteins have similar UV spectrum apart from their size, these proteins form different numbers of charge forms (and several adducts, as well) in ESI leading to different detection sensitivities. The larger the number of charged forms, the smaller the overall signal intensity for that particular electrophoretic peak. While ESI-MS is sensitive for peptides and smaller proteins (<25 kDa), poor detection sensitivities might be obtained for larger proteins, which appear in a wide range of charge state distribution (to which adduct formation also contributes). This is the reason why larger proteins (e.g., metalloproteinases) could not be detected with MS. Presumably these larger venom proteins were detectable with UV but not identifiable. The applied method has proven effective for the profiling of peptides and low molecular weight proteins in Elapidae venoms. The venom analyses of other snakes (eg. viperid snakes), which contain a higher proportion of large molecular weight proteins and enzymes [5], probably would be more difficult (limited) due to the smaller ESI-MS detection sensitivity of these large components. Future study should aim to validate analytical methods for large molecular weight components present in other (viperid) snake venoms.

In these intact analyses of venoms obtained from snakes of 9 different subspecies (but the same genus) more than 250 different neuropeptides (7–10 kDa) were detected. The protein contents of the venoms of the same subspecies snakes collected from different geographical regions are similar and those differ only in a few components (less than 10 % of the components). However, the venoms collected from different subspecies (but from the same species) exhibit very different protein patterns: no identical proteins (same molecular weights) were obtained. Therefore, the CZE-MS electropherograms of the venoms can be considered as a characteristic feature (fingerprint) of a given snake. This method can also be useful in the phylogenetic analysis of the same species to unveil procedures leading to geographic distribution of today's existing phylogroups.

The identification of proteins in a complex sample like snake venom can be challenging, especially since there can be variability in protein isoforms even within the same subspecies that differ only in their geographical origin. In cases when accurate monoisotopic masses can be acquired (i.e. small proteins), it might be possible to infer the identity of the protein, however, MS/MS measurements would definitely help in substantiating the amino acid sequence or the presence of potential post-translation modifications (e.g., different glycosylation profiles of the isoforms). However, many of the neuropeptides in snake venoms contain a large number of disulfide bonds especially compared to the relatively low molecular weight of these components. Cleave of the disulfide bonds does not occur during collision-induced dissociation (CID) fragmentation type, only the peptide backbone fragments into b and y type ions. The still intact disulfide bonds are very often keeping these backbone fragmented ions together resulting in no change in mass of the precursor peptide and the usual fragment patterns can not be seen. Here other fragmentation techniques like electron transfer dissociation (ETD) should be applied [42].

CRediT authorship contribution statement

Gayatri Vishwakarma: Writing – original draft, Investigation, Formal analysis, Data curation. **Melinda Andrasi:** Formal analysis, Data curation. **Ruben Szabo:** Investigation, Data curation. **Peter Hajdu:** Data curation. **Vladimir Petrilla:** Data curation. **Monika Petrillová:** Data curation. **Jaroslav Legath:** Data curation. **Attila Gaspar:** Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.110290.

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