

# Comparative Analysis of Venom of Two Geographically Distinct European Adders

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## Abstract

**Introduction:** The study compares the venom of species, *Vipera berus*, from two geographically distinct areas. Individuals living in Slovakia produce larger volumes of venom in a venom gland compared to individuals living in Poland.

**Methods:** The complex molecular structure of individual native adder venom was recorded by synchronous fluorescence fingerprint, atomic force microscopy and protein electrophoresis.

**Results:** Fluorescence spectroscopy revealed differences in Slovak and Polish male and female structure of venoms and showed higher fluorescence intensity in Polish adder venom compared to Slovak adder venom. The similar pattern of venoms of snakes living in distinct area was observed by atomic force microscopy. The qualitative and quantitative protein differences in both albumin-like and globulin-like regions in adder venoms were detected by gel electrophoresis.

**Conclusion:** Our results exhibited differences in venom molecular structure of species *Vipera berus* from the distinct area.

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## Introduction

Species *Vipera berus*, the common European adder (Linnaeus 1758), is a wild venomous snake of the Palaearctic region<sup>[1]</sup>. Species *Vipera berus* is the only venomous snake living in Central and North Europe<sup>[2]</sup>. The adders are not considered to be extremely dangerous to humans<sup>[3]</sup>, however, they only bite when they are disturbed and feel threatened<sup>[4]</sup>.

This snake has a wide, flattened, triangular head which differs from the neck and the rest of the body. The head takes up from 4.5 to 5.5% of the body length. Lips are short and blunt, rounded at the tips. Eyes are relatively small with vertically elliptical pupils. The maximum length of the male body is 700 mm, while female body length ranged between 800 and 1000 mm measured by Oliva., et al<sup>[5]</sup>. Kminiak and Kalúz<sup>[6]</sup> observed smaller values of overall length of the body ( $L_{tot} = \textit{longitudo totalis}$ ) in a population-based samples of male and female adders. The following results were recorded:  $L_{tot} = 440$  mm, mean = 530 mm, and maximum = 640 mm in 96 males; minimum  $L_{tot} = 440$  mm, mean = 579 mm, and maximum = 680 mm in 83 females investigated in the Slovak and Czech Republic<sup>[1]</sup>.



The adder venom is a multi component mixture of substances which contains more than 100 different components<sup>[7]</sup>. More than 90 % of the dry weight is composed of proteins comprising a variety of enzymes, nonenzymatic polypeptide toxins, and non-toxic proteins. It does not contain paralytic postsynaptic neurotoxins, cardiotoxins, myotoxins and necrotizing agents. PLA2 (phospholipase A2) is highly neurotoxic substance occurring in subspecies of the adder venom. PLA2 neurotoxins act presynaptically and can cause the innervation of the facial muscles.

Nonprotein compounds contain carbohydrates, metal ions (often part of glycoprotein, metalloprotein enzymes), lipids, free amino acids, nucleosides, and biogenic amines such as serotonin and acetylcholine, polysaccharides, low-molecular-weight substances and ions<sup>[8,9]</sup>.

Hemocoagulation and cytotoxic agents may be present in the adder venom, albeit in negligible quantities. Any venomous snake species contains clinically dangerous haemorrhagins and circulating toxins; if injected into the bloodstream they increase capillary permeability and cause haemorrhage. The constituent proteins of snake venom can affect the breakdown of tissue proteins and peptides.

The lethal dose for an adult human is estimated at 15 – 20 mg of adder venom, which is similar to the venom of cobra *Naja naja*<sup>[7]</sup>. L-amino acid oxidases originating from *Vipera berus* venom that can induce apoptosis and have ability to cause aggregation of platelets in conjunction with haemorrhagic effect<sup>[10,11]</sup>. The hyaluronidase, originating from the family Viperidae, enhances the toxicity of venom by increasing the influx of systemic toxins<sup>[12]</sup>.

The fluorescence properties of the particular venom organic components are the consequence of their molecular structure and the presence of endogenous fluorophores e.g. aromatic amino acids, proteins, nucleosides, vitamins, and coenzymes<sup>[13,14]</sup>. All proteins and amino acids have not been revealed, yet. To understand the functions of proteins at a molecular level, it is often necessary to determine their structure. This is the topic of the scientific field of structural biology, which employs several analytical techniques such as X-ray crystallography, NMR crystallography, NMR spectroscopy, MS spectroscopy, HPLC, dual polarization interferometry, and other techniques.

Adders usually occur at high altitudes over 400 meters above sea level, however, our identified individuals were captured also at a lower altitude (115 – 130 meters above sea level) in Poland. Therefore, the aim of our study was to compare craniometric measurements of the adder of two distinct geographical areas (Slovak Republic and Poland), focusing on the length of fangs, the amount of venom in the venom glands, and then the findings were compared with the differences among adder venoms using fluorescence spectroscopy, atomic force microscopy, and electrophoresis of proteins. These unconventional methods were used in our previous research in a number of different African mambas of genus *Dendroaspis*<sup>[15]</sup> and two species of African cobras *Naja ashei* and *Naja nigricollis*<sup>[16]</sup> where we examined the components of snake venoms.

## Materials and Methods

### Venom Samples Preparation Technique

The venom of *Vipera berus* (male and female from Slovakia and Poland) was collected into the plastic vial<sup>[15,16]</sup>, stored in the transport cryogenic microtubes under liquid nitrogen ( $t = -196\text{ }^{\circ}\text{C}$ ) and kept in deep freezer ( $t = -70\text{ }^{\circ}\text{C}$ ). The volumes of obtained venom in individuals were too small (only 25 micro liters, in drops). The venom of 4 individuals was mixed within the same population-gender group of the same geographical area. The venom was centrifuged (Centrifuge EBA 21, HETTICH GmbH & Co. KG, Tuttingen, Germany) for 5 min at 600 x g prior each analysis. The pH of adder venom was determined by using potentiometry on Twin compact pH metre B-212 (HORIBA Europe GmbH, Sulzbach, Germany).

### Fluorescent Analysis of Crude Venom

Native venom samples from the adders were analyzed by Synchronous Fluorescent Fingerprint (SFF) and simple synchronous excitation fluorescence spectra  $\Delta = 70\text{ nm}$  of SFF in a quartz cuvette (500  $\mu\text{L}$ ) on a Perkin-Elmer LS 55 (Waltham, Massachusetts, USA) spectrophotometer with instrument settings and measurement conditions at scan speed of 1200 nm/s, slits were set to 5 nm for both excitation and emission wavelengths at laboratory temperature. Each synchronous excitation spectrum was measured from  $\lambda = 200$  to  $\lambda = 600\text{ nm}$ .

The resulting SFF was created from 20 scans of simple synchronous excitation spectrum at different excitation wavelengths with constant distance between individual spectra<sup>[20]</sup> called increment. The data were processed by WinLab software (PerkinElmer, Waltham, Massachusetts).

### Atomic Force Microscopy (AFM) of Crude Venom

The venom (25  $\mu\text{L}$ ) of *Vipera berus* was analysed on the microscopic slide by atomic force microscopy (ICON, Bruker, Berkeley, California, USA) in the tapping mode with silicon tips (Mikro Masch, NSC35 series), and with radius of curvature  $\sim 10\text{ nm}$ . The results were processed by Nanoscope software (Bruker, Berkeley, California, USA).

### Atomic Absorption Spectrophotometry of Crude Venom

The total protein concentration of adder venom was measured at  $\lambda_{\text{abs}} = 540\text{ nm}$  by using a diagnostic kit (Randox, Crumlin, United Kingdom) on automatic biochemical analyser Alizé (Lisabio, Pouilly-en-Auxois, France).

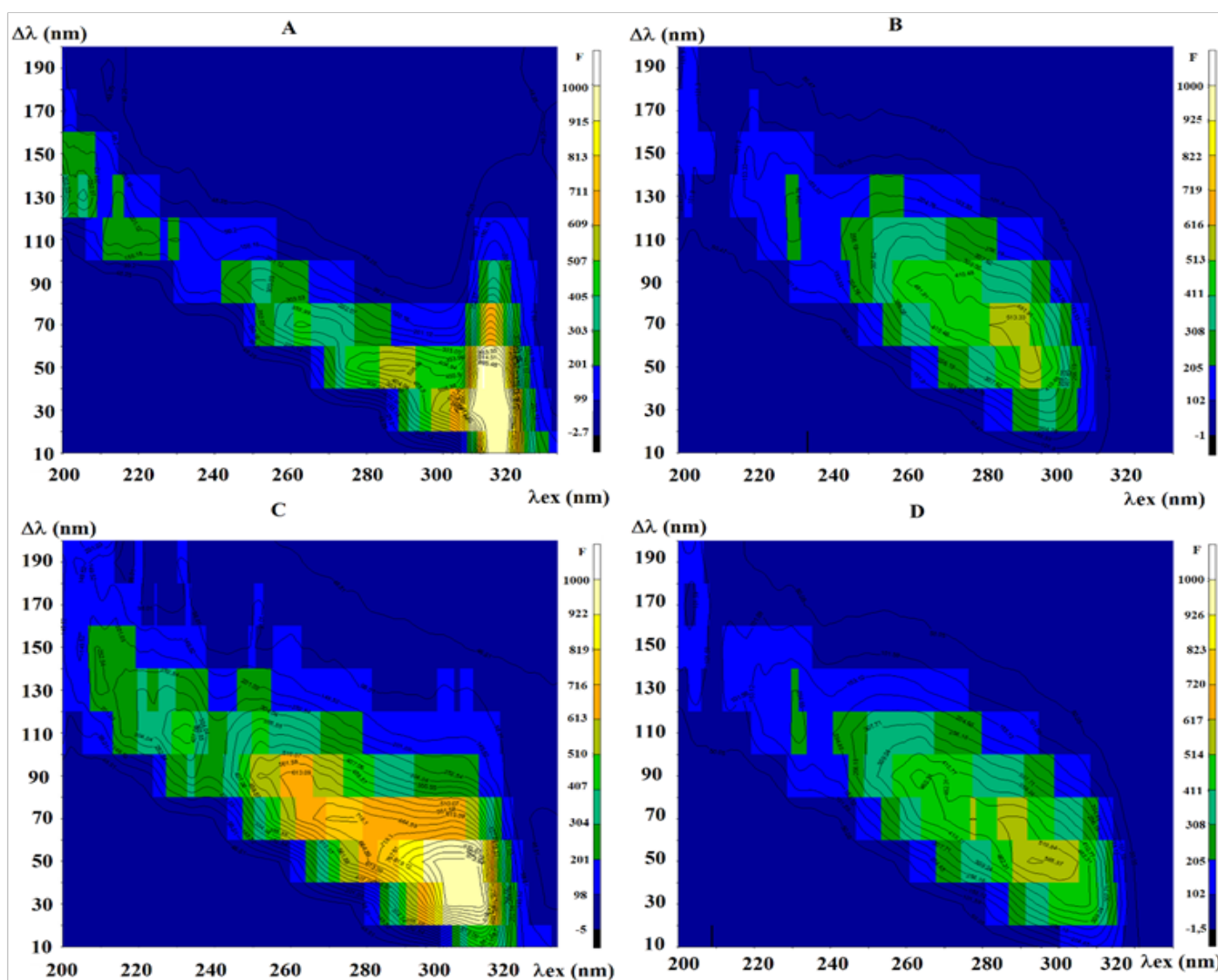
## Electrophoresis of Crude Venom

The proteins in adder venom (10  $\mu\text{L}$ ) was separated from crude venom by using Hydragel 15 protein kit (Ecomed, Žilina, Slovak Republic) on agarose gels (pH = 9.2) by electrophoretic Hydrasys equipment (Sebia, Lisses, France). The fractions were visualized by 0.4 g/dL Amido Black. The dried gels were evaluated by using Phoresis software (Version 5.50, 2009, Sebia, Lisses, France) on Epson perfection V 700 Photo ( $\lambda = 570 \text{ nm}$ ) and obtained results were visualized in electrophoretograms and densitometric curves.

## Results

### Adder venom comparisons

The venom in male snakes showed maximal intensity of fluorescence spectra in the lipophilic region (at lower wavelength) and in the hydrophilic region (at longer wavelengths). The venom in female snakes exhibited the highest intensity of fluorescence at longer wavelengths (Figure 1). Each venom revealed an acidic pH value (pH = 5.5). The SFF (Figures 1 B and D) showed a similar structure and position of fluorescence centers of a venom in male snakes from Slovakia (70/289 nm, F = 546) and from Poland (50/294 nm, F = 573). However, detailed examination of the fluorescent centres showed differences. Female adder (Figures 1 A and C) from Slovakia ( $\Delta 50/\lambda_{\text{ex}} = 312 \text{ nm}$ , F = 1000) and Poland ( $\Delta 50/\lambda_{\text{ex}} = 303 \text{ nm}$ , F = 1000) showed different structures of SFF but had similar localization of centers with maximal fluorescence intensity (Figure 1).



**Figure 1:** The synchronous fingerprints and fluorescence spectra of adder venom (*Vipera berus*): (A) Female from Slovakia (B) Male from Slovakia (C) Female from Poland (D) Male from Poland. Constant differences between emission and excitation wavelengths correspond to the delta lambda ( $\Delta\lambda$ ).

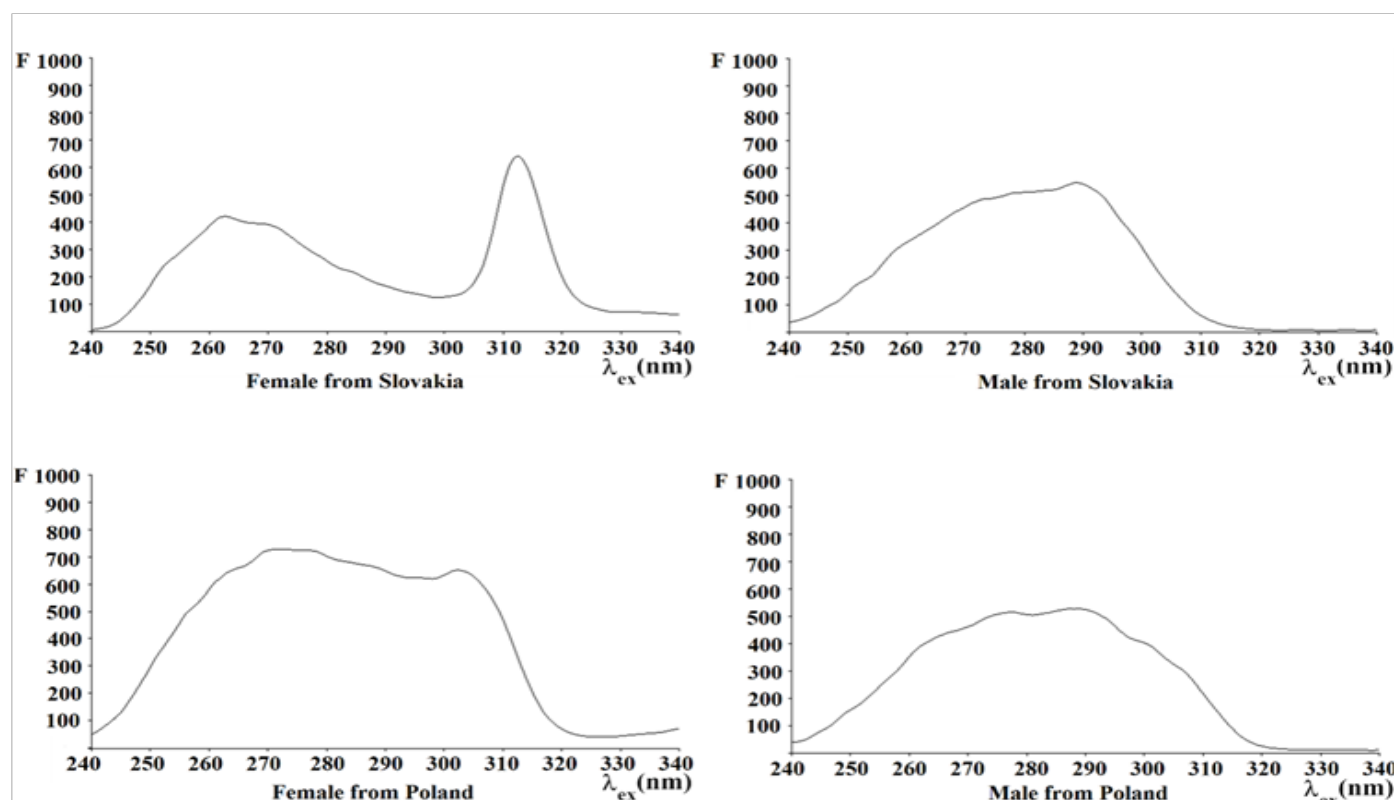
A horizontal cut of individual samples at ( $\Delta 70 \text{ nm}$ ) of SFF revealed simple spectra of SFF and showed differences in the structure of individual adder venom (Table 1; Figure 2). The venom from female snakes from Slovakia showed two maxima (Table 1; Figure 2 A), while the venom from male snakes also from Slovakia exhibited only one maximum (Table 1; Figure 2 B) at  $\lambda_{\text{ex}} =$

289 nm ( $F = 546$ ) that was similar to the venom from male snakes from Poland (Table 1; Figure 2 D) at  $\lambda_{ex} = 289$  nm ( $F = 527$ ). The venom from the female snake from Poland visualized two maxima (Table 1; Figure 2 C) with higher fluorescence intensity than the venom from female snakes from Slovakia.

**Table 1:** The comparison of fluorescence maxima on the profiles of synchronous excitation spectrum of *Vipera berus* venom.

Maxima	Female SK		Male SK		Female PL		Male PL	
	$\lambda_{max}$ (nm)	F	$\lambda_{max}$ (nm)	F	$\lambda_{max}$ (nm)	F	$\lambda_{max}$ (nm)	F
1	263	420	289	546	271	726	289	527
2	313	641			303	651		

$\lambda_{max}$ , Maximal excitation wavelength at constant  $\Delta\lambda 70$  nm difference between emission and excitation wavelengths; F, Intensity of fluorescence; SK, Slovakia; PL, Poland.

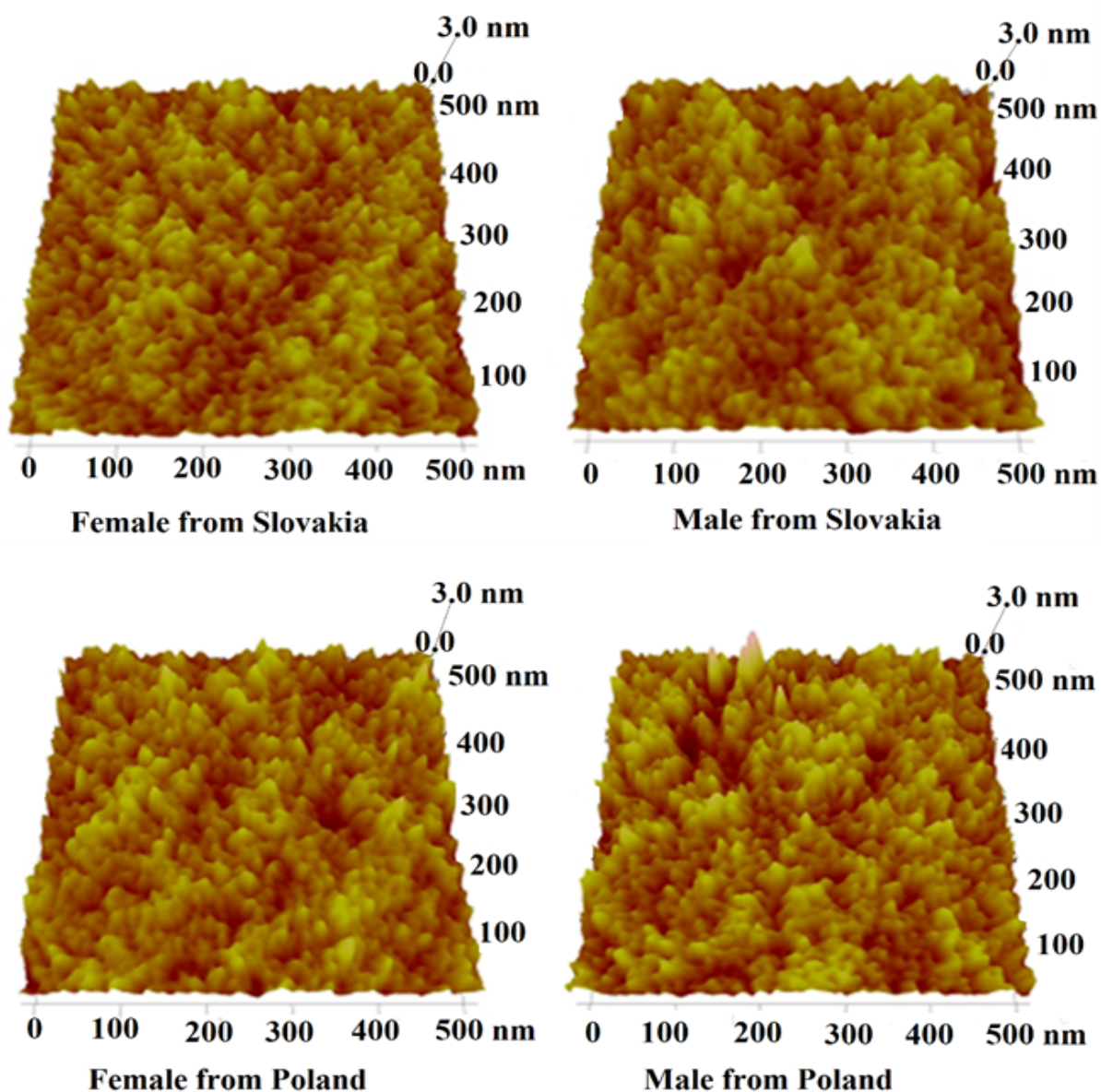


**Figure 2:** Fluorescence intensity of the simple synchronous excitation spectra in venom (*Vipera berus*) measured at the delta lambda ( $\Delta\lambda = 70$  nm).  $\Delta\lambda = 70$  nm corresponds to the constant difference between emission and excitation wavelengths;  $\lambda_{ex}$  (nm) is excitation wavelength and F is intensity of fluorescence.

The structure of selected adder crude venom (Figure 3) was investigated and compared by Atomic Force Microscopy (AFM). The present results of AFM showed that all individuals (male and female snakes living in Slovakia and Poland) have the similar pattern of venom. Clusters of globular particles with a diameter of 10 nm were associated into formations with irregular shapes and diameter of 50 nm. The adder venom obtained from male specimens ( $r = 0.5$  nm) and female specimens ( $r = 0.4$  nm) showed minimum differences in surface roughness between the genders living in Slovakia and Poland.

All adder venoms showed different concentration of total proteins. Separation of proteins resulted in qualitative and quantitative differences in fractions of proteins (Table 2). The highest concentration of total protein was recorded in the female snakes from Slovakia. The lowest protein content was determined in male snakes from Slovakia on the contrary with the male snakes from Poland which showed higher concentration of protein in comparison with the female snakes from Poland. The highest number of globulin-like fractions was recorded in male snakes from Slovakia ( $n = 8$ ; Table 2; Figure 4 B) and female snakes from Slovakia ( $n = 7$ ; Table 2; Figure 4 A) in comparison with male snakes from Poland ( $n = 5$ ; Table 2; Figure 4 D) and female snakes from the same country ( $n = 5$ ; Table 2; Figure 4 C). The lowest albumin-like fractions showed female snakes from Poland ( $n = 0$ ; Table 2; Figure 4 C) and male snakes from Slovakia ( $n = 1$ ; Table 2; Figure 4 D) contrary to female snakes from Slovakia ( $n = 2$ ; Table 2; Figure 4 A) and male snakes from Poland ( $n = 2$ ; Table 2; Figure 4 B) with similar number of albumin-like fractions.



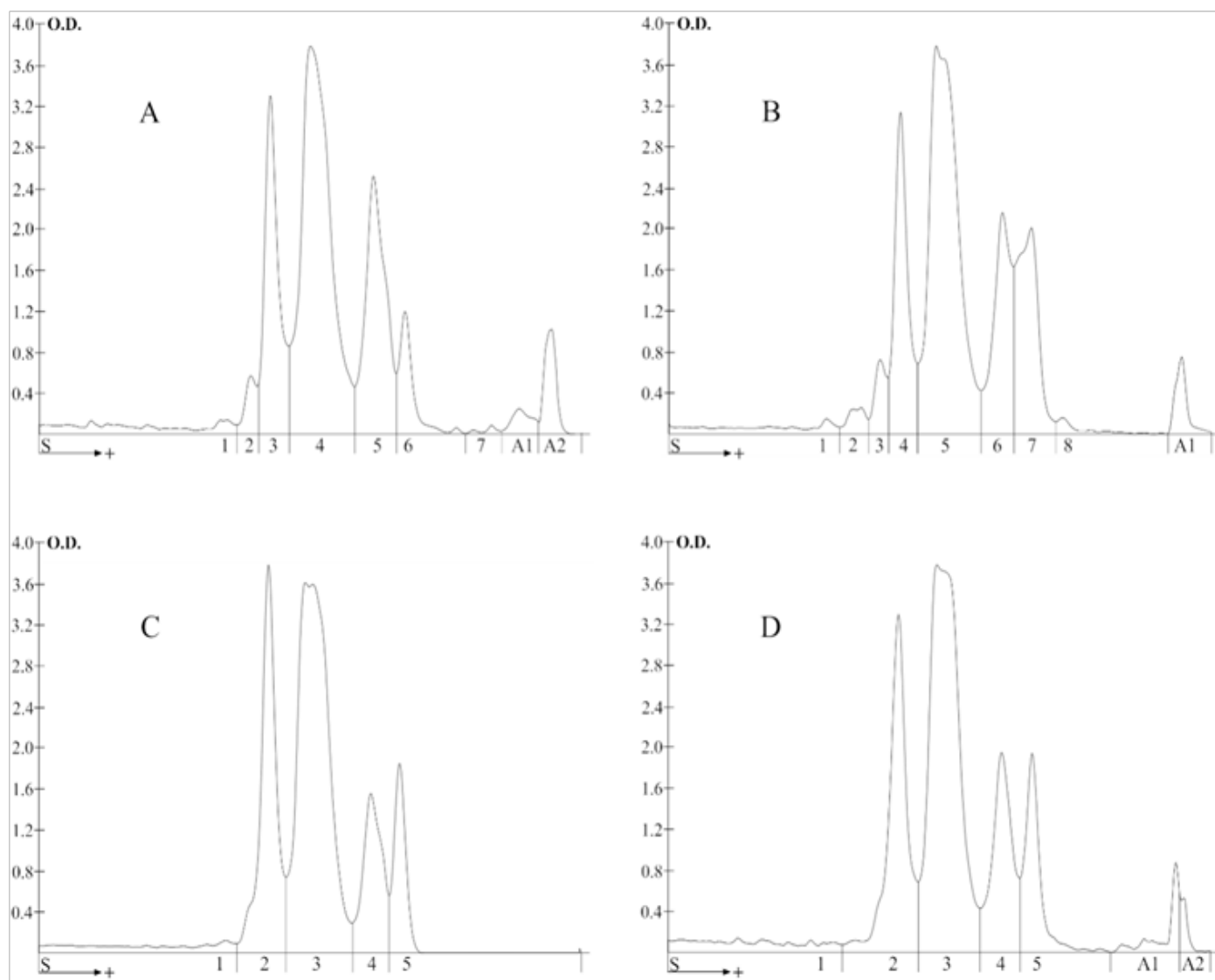


**Figure 3:** Atomic force microscopy of adder venom (*Vipera berus*). “0 nm” and “3 nm” indicates (z axis) minimal and maximal height of each globule.

**Table 2:** Total protein content and the results of electrophoretic separation of proteins from adder venoms.

Separated fractions		The concentration of proteins (g/L)			
		Female SK	Male SK	Female PL	Male PL
Globulin-like fractions	1	3.2	1.7	2.5	3.2
	2	1.9	0.8	12.5	12.9
	3	12	1.6	25.1	25.4
	4	28.9	7.4	6.5	8.5
	5	13.3	19.7	4.7	6.2
	6	4	6.8		
	7	0.3	7.4		
	8		0.5		
Albumin-like fractions	A1	1.2	3.3		2.1
	A2	4			0.8
<b>Total protein</b>		68.7	49.2	51.3	59.1

SK, Slovakia; PL, Poland.



**Figure 4:** Gel photograph shows the electrophoretic separations of the venom sample of *Vipera berus* of: (A) female from Slovakia, (B) male from Slovakia, (C) female from Poland, (D) male from Poland. S, start (junction between the stacking and separation gels); O.D., optical density; the x axis represents the scanned area of the gel strip; 1–8, labelling of globulin-like fractions; A1–A2, labelling of albumin-like fractions.

## Discussion

The mixture of organic fluorophores present in natural snake venom is a multi-fluorescent viscous liquid and can be studied by Fluorescence Spectroscopy (SFF) and atomic force microscopy as new alternatives for monitoring and the first characterization of adder venom structure<sup>[16,17]</sup>. The main organic fluorophores responsible for the final fluorescence of SFF include enzymes, amino acids and proteins. Obviously, serotonin, nucleosides and biogenic amines can influence the fluorescence intensity. Most venom contains fluorescent l - amino acid oxidase with prosthetic cofactor FMN or FAD<sup>[18]</sup> which is responsible for the yellow colour of many types of venom. It is known from the literature that riboflavin (vitamin B2) and cofactors (FMN and FAD) are active fluorophores<sup>[13,14]</sup>.

Electrophoresis is a specific method with widespread use<sup>[19]</sup>. According to Oh-Ishi and Maeda<sup>[20]</sup> the advantage of using this method is the high permeability for substances with high molecular weight, because it has a larger pore diameter compared to polyacrylamide gel. Our work was also focused on the comparison of electrophoretograms of adder venoms from male and female snakes. Gel electrophoretograms (Figure. 4) indicated the differences between qualitative and quantitative protein fractions in both albumin-like and globulin-like regions in adder venom. Similar nomenclature was also used in the study of Göçmen., *et al*<sup>[21]</sup>. They studied and compared the differences in morphology and electrophoretic types of venom proteins in compared individuals of Levantine vipers, *Macrovipera lebetina* (Linnaeus 1758), from Cyprus and southern Anatolia. They made a conclusion that the southern Anatolian population should not be appointed as the exclusive subspecies of *M. l. lebetina* that lives in Cyprus. Arıkan., *et al*<sup>[22]</sup>. studied the venom proteins of *Vipera ammodytes* (Linnaeus 1758), and *Vipera kaznakovi* (Nikolsky 1909) and they revealed considerable differences between mentioned species using polyacrylamide gel electrophoresis. The snake bites could cause a wide range of pathophysiological effects for which it is probably responsible complex molecular structure originating from snake venom<sup>[23]</sup>. Our results of all adder venoms showed different concentration of total proteins and electrophoretic separation resulted in

qualitative and quantitative differences in fractions of proteins (Table 2, Figure 4). The highest concentration of total protein was recorded in the female snakes from Slovakia. The lowest protein content was determined in male snakes from Slovakia on the contrary with the male snakes from Poland which showed higher concentration of protein in comparison with the female snakes from Poland. The amount of protein in snake venom can be affected by various factors which include sex, feed, genetic factors, habitat and distribution area of snakes<sup>[24]</sup>.

## Conclusion

Differences between species *Vipera berus* studied from distinct areas showed that individuals living in higher altitudes in Slovakia in comparison with those living in lower altitudes in Poland were observed. The different composition of all studied crude adder venoms was determined by fluorescence spectroscopy, atomic force microscopy and protein electrophoresis that might be influenced by various biotopes. These methods can be important tools for the analytical identification of novel and unknown venoms taken from different species of snakes.

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

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