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Hydrophilic interaction chromatography coupled to high resolution mass spectrometry (HILIC-LC-HRMS): An approach to study natural peptides in *Viperidae* snake venom

Monica V. Falla [a], Ivo Lebrun [a], Marcos A. Pudenzi [c], Laudiceia A. Oliveira [c], Heloisa F. Almeida [a], Nathalia G. Santos [a], Mariana S. Rodrigues [d], Patrick J. Spencer [d], Marisa M. Rocha [b], Daniel C. Pimenta [a], Guilherme R. Coelho [a,*]

[a] *Laboratório Bioquímica e Biofísica, Instituto Butantan, São Paulo, Av. Vital Brasil 1500, São Paulo, SP 05503-900, Brazil*
[b] *Laboratório de Herpetologia., Instituto Butantan, São Paulo, 05503-900, Brazil*
[c] *Shimadzu, São Paulo, 06460-000, Brazil*
[d] *Instituto de Pesquisas Energéticas e Nucleares (IPEN), São Paulo, 05508-000, Brazil*

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ABSTRACT

Although proteins in snake venoms have been extensively studied and characterized, low-mass molecules remain relatively unexplored, mainly due to their low abundance, secondary role in envenomation, and some analytical technique limitations. However, these small molecules can provide new important data related to venom toxins' molecular structure, functions, and evolutionary relationships. This research aimed to characterize molecules below 10 kDa in the venoms of snakes from the *Viperidae* families (*Bothrops, Agkistrodon*, and *Bitis*) and compare two chromatographic approaches: reverse-phase chromatography (RP), a classic technique, and hydrophilic interaction liquid chromatography (HILIC), an alternative technique, both coupled with high-resolution mass spectrometry (HRMS). The results showed that the separation of the HILIC column provided a more efficient evenly distributed ion profile than RP, contributing to a 25.6% increase in the sequences identified. Homologous sequences for Bradykinin-potentiating peptides (BPPs) and fragments of major venom proteins, possibly cryptids, were found. In addition, BPP 13a, peptides rich in histidine and glycine (pHpG), and spacer sequences were identified in all snakes analyzed, especially with HILIC separation, suggesting that these sequences may be conserved within *Viperidae*. These findings indicate that the use of the HILIC column, compared to RP, is a promising approach for characterizing peptides in snake venom obtained by the ultrafiltration process. It contributes to the study of these still poorly understood molecules and is also a good option for studying other complex protein/peptide mixtures.

1. Introduction

The venoms of various animals exhibit a broad spectrum of activities and are composed of a diverse range of components, including alkaloids, steroids, peptides, and proteins. Venomics, the specific study of venoms, has enabled the biochemical characterization of these components and the understanding of their functions both in the venom and in the envenomation process by these animals. Furthermore, many of these components have been successful in the pharmaceutical industry, such as Captopril, derived from the venom of *Bothrops jararaca* and used to treat systemic hypertension; Prialt, a potent analgesic derived from the cone snail venom *Conus magus*; and Exenatide, an antidiabetic peptide found in the saliva of the Gila monster, *Heloderma suspectum* [1]

In snakes, the most studied components are enzymatic and non-enzymatic proteins, especially those that play relevant roles in the envenomation process [2]. Proteomics has been essential for the analysis of these protein components. Calvete et al [3] proposed the fractionation of crude snake venom by reverse-phase high-performance liquid chromatography (RP-HPLC) to characterize its components. This method, which combines N-terminal sequencing, SDS-PAGE, and mass spectrometry (MS/MS), enables a detailed characterization of the toxins present. Since then, the use of reverse-phase chromatography for the

* Corresponding author.
E-mail address: guilherme.coelho@butantan.gov.br (G.R. Coelho).

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analysis of venom components has become a classic and widely adopted approach in the characterization of snake molecules.

The bradykinin potentiating peptides (BPPs), discovered in *Bothrops jararaca* venom by Ferreira and collaborators [4], revealed a promising hypotensive activity that later led to the development of the drug captopril. This discovery brought a new perspective on the low molecular mass components present in snake venom, with an emphasis on BPPs, which have been the focus of many studies focused on small molecules [5–11].

With the advance of peptidomics studies on *Viperidae* snake venoms, other low-mass molecules have been discovered, such as natriuretic peptides, which act on the cardiovascular and renal systems [12], and tripeptides, which inhibit the proteolysis of venom in the gland [13]. There is a growing effort to identify more low-mass molecules in snakes, especially due to the pharmacological potential of these components [14]. However, the low abundance of these molecules and the lack of specific approaches for their study have been limiting factors in reaching a better understanding [15].

In peptidomics, chromatography coupled with mass spectrometry is an essential tool [16]. Traditionally, reverse-phase (RP) has been widely employed to analyze snake venom components due to its effectiveness in characterizing components, mainly proteins [3,17,18]. When observing the elution of snake venom components in the reverse phase, most low-mass molecules elute first than the major proteins [19], indicating a more polar characteristic.

In the literature, hydrophilic interaction liquid chromatography (HILIC) has been well-utilized for the study of polar molecules in general, being applied mainly in the pharmaceutical industry [20,21]. Originally introduced by Alpert in 1990, the HILIC column uses a stationary phase composed predominantly of silica or polymers, along with a mobile phase based on aqueous-polar organic solvents (buffers), which can be used with a great variety of polar/apolar solvents in isocratic or gradient conditions [21].

The HILIC column and Normal Phase (NP) chromatography use polar stationary phases, but differ in terms of the mobile phase: NP uses non-aqueous solvents, while HILIC employs an aqueous-organic phase, which improves the solubility of polar compounds and allows elution with a water gradient, optimizing the separation of hydrophilic substances [21–23]. The HILIC stationary phase, usually based on silica with functional groups, retains a layer of water that facilitates hydrophilic and electrostatic interactions, making HILIC an efficient and environmentally advantageous alternative to NP [21–23].

In general, the HILIC column has demonstrated significant results in the analysis of peptides [24–29], as evidenced in studies focused on di-, tri- and tetra peptides present in cow's milk [26,28] and human plasma [24] most of these studies use a sample preparation process that involves digestion with enzymes, protein precipitation and solid-phase extraction process to obtain very small peptides. However, its use in venom

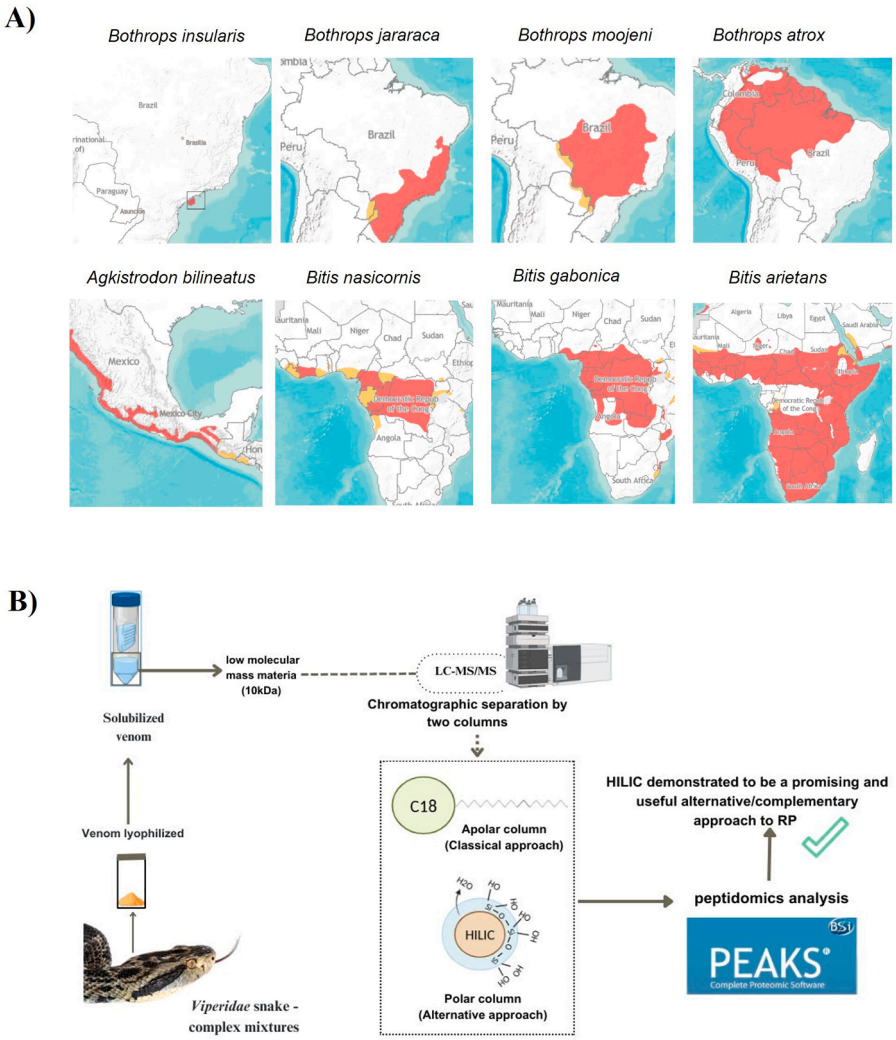

Fig. 1. Illustrates the geographic distribution of the snakes studied, showcasing the different localities where these animals can be found, including the *Bothrops* genus in South America, the *Agkistrodon* genus in Central America, and the *Bitis* genus in the African continent. The red regions indicate a high incidence of snakebites of the species, while the orange regions indicate a low incidence of snakebites. Figures adapted from World Health Organization (WHO) [56].

analysis is still limited, with few reports in the literature. HILIC has been applied as an accessory or secondary chromatographic step in separating peptides from scorpion venoms [30] and amphibians [31,32].

For snake venoms, HILIC has been used as a complementary approach in the identification of angiotensin-converting enzyme (ACE) inhibitors [33], as orthogonal chromatography in the screening of metalloproteinase inhibitors [34] and in proteomic preparation [35]. More recently, a study conducted by Briones et al. [36], using HILIC and RP as complementary approaches on different snake venoms from the *Elapidae* and *Viperidae* families, identified peptides and mainly secondary metabolites. However, RP is the gold standard for crude venom analysis, and the performance of HILIC as a single-step chromatography for peptides and other low-mass toxins identification was never evaluated in snake venoms compared with RP. Thus, the present work aims to perform the peptidomics of venom without enzymatic digestion process from different species of *Viperidae* snakes (Fig. 1), comparing both the classical RP approach and the HILIC column for the separation of peptides present in complex mixtures, to allow the detailed identification and characterization of these components.

2. Methodology

2.1. Venom collection and sample preparation

The lyophilized venom pools from *Viperidae* species (*Bothrops jararaca, Bothrops moojeni, Bothrops atrox,* and *Bothrops insularis*) were kindly provided by Dr. Marisa M. Teixeira da Rocha from the Herpetology Laboratory at Instituto Butantan. The lyophilized venom pools from *Bitis* species (*Bitis gabonica, Bitis nasicornis, Bitis arietans,* and *Agkistrodon bilineatus*) were kindly supplied by Venom Supplies (Australia).

The venoms were weighed (10 mg) and dissolved in ultrapure water, and a 10 kDa molecular mass cutoff membrane filter (Amicon® Ultra-10 k) was used to obtain the low-molecular mass fraction. The samples were centrifuged for 1 hour at 8 °C and 3000 x g. The filtrate was collected and lyophilized (Liotop L101 lyophilizer). Quantification of the low-mass material was performed by dry weight measurement.

The low molecular mass fractions were subjected to SDS polyacrylamide gel electrophoresis [37] with silver staining. 80 μg of material with reducing buffer (5% *w/v*Tris–HCl buffer 0.5 M pH 6.8, 25% *v/v* glycerol, 40% *v/v* sodium dodecyl sulfate at 10% *w/v* stock solution, 10% *v/v* 2-mercaptoethanol, and 5% *v/v* bromophenol blue at 0.05% *v/v* stock solution) were applied to a 12% *v/v* polyacrylamide resolving gel and a 5% *v/v* stacking gel, with a constant current of 100 V. The gels were stained with silver nitrate.

2.2. Mass spectrometry

2.2.1. MALDI-TOF

The MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) analysis was performed on an AXIMA MALDI-TOF instrument (SHIMADZU BIOTECH), using α-cyano-4-hydroxycinnamic acid (Sigma) as the matrix, prepared in a solution of 50% acetonitrile (ACN), 50% ultrapure H_2O, and 0.1% trifluoroacetic acid (TFA). Sample and matrix were pipetted onto the plate at a 1:1 ratio (1 μL of sample and 1 μL of matrix were added). Analyses were conducted in reflector mode, with mass detection between 300 and 3000 Da, with a preference for the 1200 Da mass range. Parameters were set to a laser power of 65, with 1000 profiles, and shots set to off. The most intense masses, excluding those corresponding to the matrix, were collected for each snake sample.

2.2.2. LC-HRMS – RP and HILIC

The low-mass fractions were analyzed using a binary HPLC system (Nexera 40D X, Shimadzu Co., Japan) coupled with the LCMS-9050 Q-TOF, equipped with an ESI ion source. A volume of 20 μL at 0.8 μg μL^{-1} was analyzed on C18 Phenomenex columns (Kinetex, 2.6 μm, 50 mm x 4.6 mm i.d.) and HILIC Phenomenex columns (Kinetex, 2.6 μm, 50 mm x 4.6 mm i.d.), both with the same size of particle, length and internal diameter and the same amount of sample injected.

The solvents for the C18 column were ultrapure H_2O with 0.1% formic acid (A) and 90% ACN + 0.1% TFA (B), with a temperature of 40 °C and a constant flow rate of 0.4 mL min^{-1}, using the following gradient: 0% B for 12.5 min, 0–100% B over 67.5 min, and 100% B for 7 min. For the HILIC column, the solvents were ultrapure H_2O with 0.1% formic acid + 5 mM ammonium formate (A) and 95% ACN + 5 mM formic acid (B), with a temperature of 50 °C and a constant flow rate of 0.4 mL min^{-1}, using the gradient: 100% B for 12.5 min, 100–60% B over 67.5 min, and 60% B for 7 min. Both methods have equal times of running.

For MS/MS analyses, the mass spectrometer interface voltage was maintained at 4.0 kV, and the detector voltage at 1.98 kV; interface and DL temperatures were 300 and 250 °C, respectively. Fragmentation was achieved by argon collision, with a collision energy parameter of 25V. The mass range for MS was 200–1500 *m/z*, with an event time of 0.1 s, while for MS/MS, the mass range was 50–1800 *m/z*, with 10 dependent events and an event time of 0.2 s. Interface parameters included a nebulization gas flow rate of 3 L min^{-1}, heating gas flow of 10 L min^{-1}, drying gas flow of 10 L min^{-1}, a heating block temperature of 400 °C, and an interface voltage of 4 kV. Instrument control and data acquisition were conducted via Insight Explore Software. Initial data processing and MZML export were carried out with LabSolutions software.

2.2.3. Data processing analysis

Peptide identification was performed using Peaks Studio 7.0 software (BSI), where a detailed review of the mass spectrum quality for each peptide sequence was conducted. For sequence identification, the National Center for Biotechnology Information (NCBI) database was used, specifically querying the "snakes" and "*Bothrops*" databases. For a more precise analysis of *Bitis* and *A. bilineatus* species, the specific databases "*Bitis*" and "*Agkistrodon*" were employed.

The search parameters were configured as follows: no enzyme specificity, N-terminal pyroglutamic acid or Glu as post-translational modifications, with error tolerance for the precursor and fragment ion of 0.07. For sequence identification, only data with Log >100 and containing at least one unique peptide were considered. For de novo sequence selection, sequences with at least 90% ALC and five or more amino acids were chosen, as recommended by Kusalik et al [38]

3. Results and discussion

3.1. Strategies for the study of small molecules in Viperidae snakes

The study of snake venom components has led to the characterization of various enzymatic and non-enzymatic proteins, many of which play essential roles in the envenomation process [2]. RP chromatography has been commonly used to characterize these components [3,17,18]. However, smaller, less abundant venom molecules tend to have amphiphilic characteristics compared to the major proteins, making their isolation alongside more abundant components challenging and poorly resolved [19]. To address this, our study used the more polar HILIC column approach to better separate and identify these molecules.

Most peptidomic studies focus primarily on BPPs-related components to understand their biochemical and biological properties. Common techniques, such as solid-phase extraction (SPE) [5,6,15] and size-exclusion chromatography [7–10], are widely used for isolating and analyzing these components. In contrast, this study focused on characterizing low-mass molecules more broadly, using a 10 kDa cut-off filter to isolate smaller molecules. Furthermore, considering the biochemical differences between individuals of the same species [39], a pool of venoms was used to mitigate these variations.

The absence of bands in the silver-stained SDS-PAGE gel (Supplementary Material 1) indicates that the major venom molecules were

excluded. Furthermore, MALDI-TOF analyses (Fig. 2) reveal the diversity of masses between the different materials, demonstrating that sample processing was effective.

3.2. LC-MS/MS analyses showed differences between the RP and HILIC approaches

To compare the approaches, the experiments were conducted under the same chromatographic conditions (time of running), using columns with identical specifications (size of particle, length, and internal diameter) for RP and HILIC, samples injected at the same concentration and volume, and analyses carried out on consecutive days. The LC-MS/MS analysis revealed significant differences in ion distribution: while the RP separation showed a concentrated ion profile over a limited interval of 20 to 40 minutes, the HILIC separation showed more diverse and evenly distributed ion profiles throughout the analysis (Fig. 3).

Furthermore, the use of the HILIC column favored the identification of peptides in most species of *Viperidae* venoms, except in *B. gabonica* (Fig. 4). *A bilineatus* showed a 52.3% increase in the number of sequences identified. The efficiency of HILIC in identifying peptide sequences can be attributed to its better separation of molecules, avoiding the accumulation of ions observed in RP separation, which facilitated the detection and identification of these components.

In addition, 24 sequences were identified by De novo using the RP column (the longest sequence found with 12 amino acids) and 39 with HILIC (the longest sequence found with 14 amino acids) (Supplementary Material 2). Sequences with ALC of 90% and at least five amino acids were considered, following the recommendation of Kusalik et al [38], due to the biological relevance of sequences with this length.

3.2.1. Peptidomic analysis shows a high diversity

Several sequences homologous to BPPs, SVMP/DIS fragments, and LAOO were identified following separation by RP (totaling 679 sequences) and HILIC (totaling 1.302 sequences) (Table 1). The use of the HILIC column significantly favored the identification of BPPs, with a total of 428 sequences (compared to 191 BPP sequences identified by the RP column), demonstrating the presence of this class of peptides in all the snake species analyzed. Fragments of SVMP/DIS and LAOO were predominantly identified in snakes from the *Bothrops* and *Agkistrodon* genera, using both RP and HILIC. However, in the genus Bitis, SVMP fragments were only detected in *B. nasicornis* using the RP column (the sequences are available in Supplementary Material 3).

The results highlight the remarkable performance of the HILIC column in retaining and separating amphiphilic compounds with better resolution. This efficiency is driven by its polar stationary phase and the dominance of hydrophilic interactions, enabling effective separation of hydrophilic molecules. Consequently, the improved sensitivity in ion detection significantly enhanced the identification of peptide sequences, underscoring the HILIC column's value in advancing peptide analysis.

Finally, it is important to note that the peptidomic analyses were carried out without enzymatic digestion of the material, a common practice in proteomics. In this study, the analyses were carried out directly with the natural molecules present in the venom, passing only through the fractionation stage to obtain low molecular mass material (<10 kDa).

3.2.2. Sequences homologous to bradykinin potentiating peptides

The sequencing of the *B. jararaca* genome carried out by Almeida et al. (2021) revealed that the precursor gene BPP/CNP is among the most abundant transcripts in the venom glands. The study demonstrated that the gene is composed of a first exon responsible for encoding the signal peptide and the region containing the BPP repeats, while the second exon encodes the spacer and the C-type natriuretic peptide (CNP) and also present sequence with high similarity with pHpG suggesting that this gene is also the origin of these peptides. In the present study, we identified sequences corresponding to the signal peptide, BPPs, spacer sequences, and C-type natriuretic peptides in the different snakes studied (Table 2).

The sequences identified in this study, related to the BPPs, were found in their whole, as fragments, and with additional amino acids (supplementary material 4). These fragments may result from proteolytic processes in the venom and may have functions that are still unknown [15]. Furthermore, for the first time, we found homologous sequences of different BPPs in *Agkistrodon* (BPP 10c, BPP 11a, BPP 11b, BPP 13a) and *Bitis* (BPP 11b, BPP 11c, BPP 13a, BPP 13d, BPP 6a, BPP 12b), which had already been described in snakes of the genus *Bothrops*. We also found sequences related to pHpG, BPP-13a, and the spacer region in all snakes analyzed.

The literature indicates the use of RP with specific strategies for the detection of these peptides [6,40,41]. These strategies include the identification of precursor ions characteristic of BPPs, such as Pro-Pro sequences (C-terminal) and post-translational modifications of pGlu (N-terminal), which are effective in detecting several BPPs in *B. moojeni* [6,40]. In addition, the authors identified sequences homologous to BPP-13a and BPP-10a, which were detected in this work using the HILIC column. In turn, Cleopatra et al [41] investigated antimicrobial peptides in *B. atrox* using RP after a 3 kDa filtration, identifying BPPs such as BPP-Bax11, BPP-Bax12, and BPP-Bax10, which were not found in this study.

Unique peptides were identified in *A. bilineatus*, such as the bradykinin inhibitory peptide (TPPAGPDVGPR) and its fragments, using RP and, mainly, HILIC approaches. The bradykinin inhibitory peptide was first described by Graham et al [10] in the venoms of *A. bilineatus, Crotalusviridis* and *Lachesis muta*. Calvete et al [42] also identified this sequence in *Crotalus atrox*. Later, Lomonte et al [43], in a proteomic analysis of different species of *Agkistrodon*, detected this sequence in several species of the genus, evidencing the conservation of these peptides within the group. In addition, we identified a sequence homologous to BPP POL-236 exclusively through the HILIC column, which was reported by Calvete et al [42] in the snake *C. atrox*.

In the present study, the RP approach favored the identification of unique peptides in the snakes *B. gabonica* and *B. arietans*. We found the full sequence and fragments of BPP 10 g-AP (APQERGPPEIPP) exclusively in *B. gabonica*, which was first identified in the venom of *B. gabonica rhinoceros* [44]. BPP IIb (QGRAPHPPIPP), identified exclusively in *B. arietans* in this study, was described in a cDNA analysis of *B. jararaca*, which encodes BPPs and C-type natriuretic peptide [45]. It is interesting to note that the literature has identified BPPs in *Bitis* that are homologous to the BPPs of *B. jararaca*, as evidenced in the work of Kodama et al [11].

The study by Mladic et al [33] used a combined RP and HILIC approach for the rapid screening and identification of ACE inhibitors in the venoms of *Crotalus* and *Cerastes* snakes. Reverse-phase chromatography enabled the initial screening of venom components, while HILIC improved resolution, facilitating the precise identification of bioactive peptides and contributing to the discovery of new ACE inhibitors. In this work, we observed that HILIC, as the primary approach, significantly favored the identification of sequences corresponding to bradykinin-potentiating peptides (BPPs) in various snake species. This technique proves to be an effective tool for the isolation and characterization of these components, contributing to the discovery of new inhibitors of angiotensin-converting enzyme (ACE).

3.3. Identification of conserved peptides (pHpG, BPP-13a and spacer region) in Viperidae

Sequences homologous to pHpG, BPP-13a, and those related to the spacer region were identified in all snakes analyzed, as shown in Table 2. These peptides have already been described in several studies involving snakes of the *Viperidae* family. The pHpG peptides, for example, were identified in *Atheris* [46], *Echis* [47], and several species of *Bothrops* [8, 15]. Diverse sequences of the spacer region were reported by Nicolau

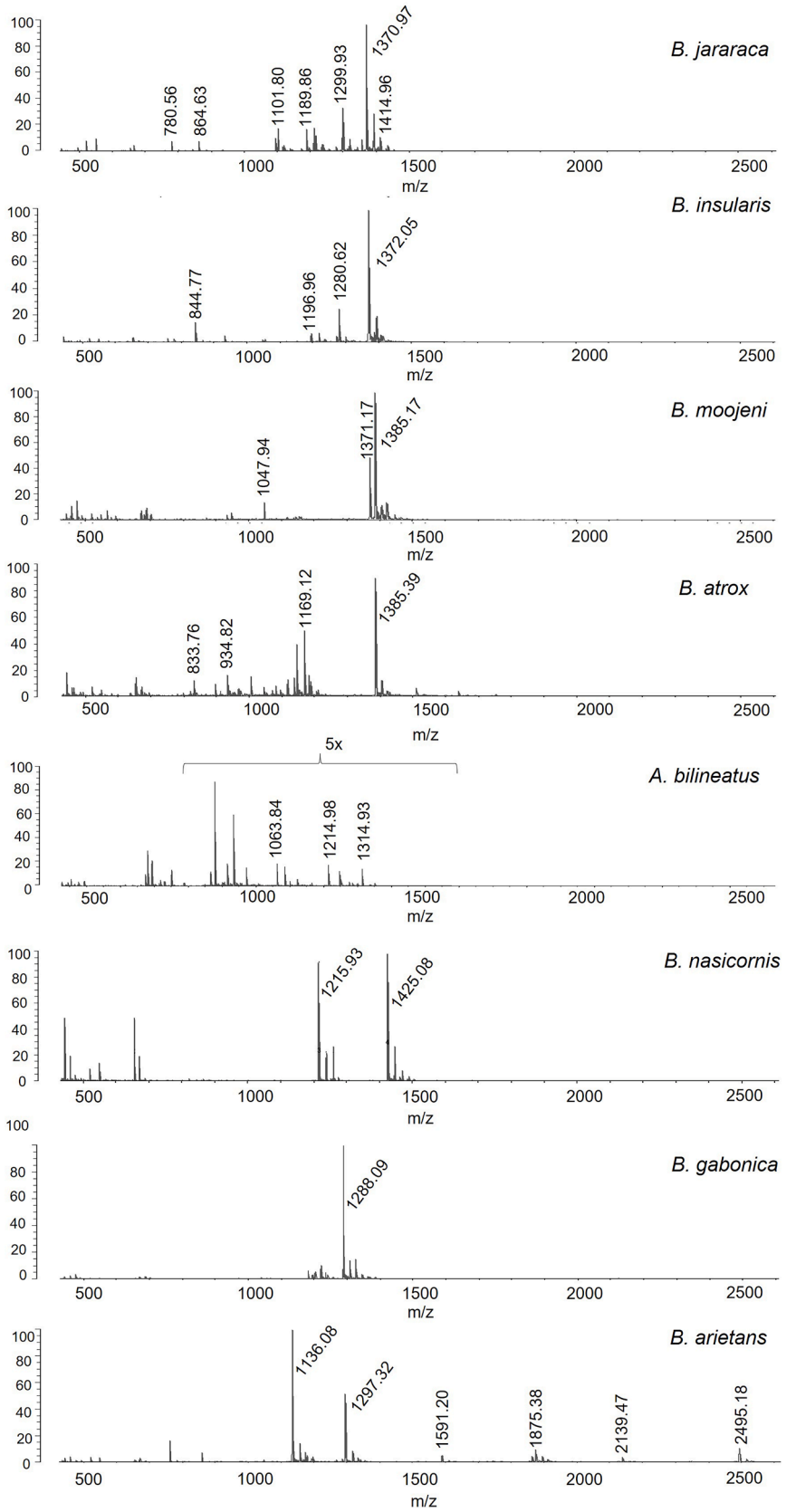

Fig. 2. MALDI-TOF/MS spectra of low-mass fraction venom samples from *Bothrops, Agkistrodon*, and *Bitis* snakes. A preliminary analysis was performed to select the 10 most intense peaks for each species, highlighting only the peaks that did not correspond to the α-cyano matrix in the figure. Mass detection was conducted in reflector mode, covering the range of 300 to 3000 Da, with a preference for the 1200 Da mass range.

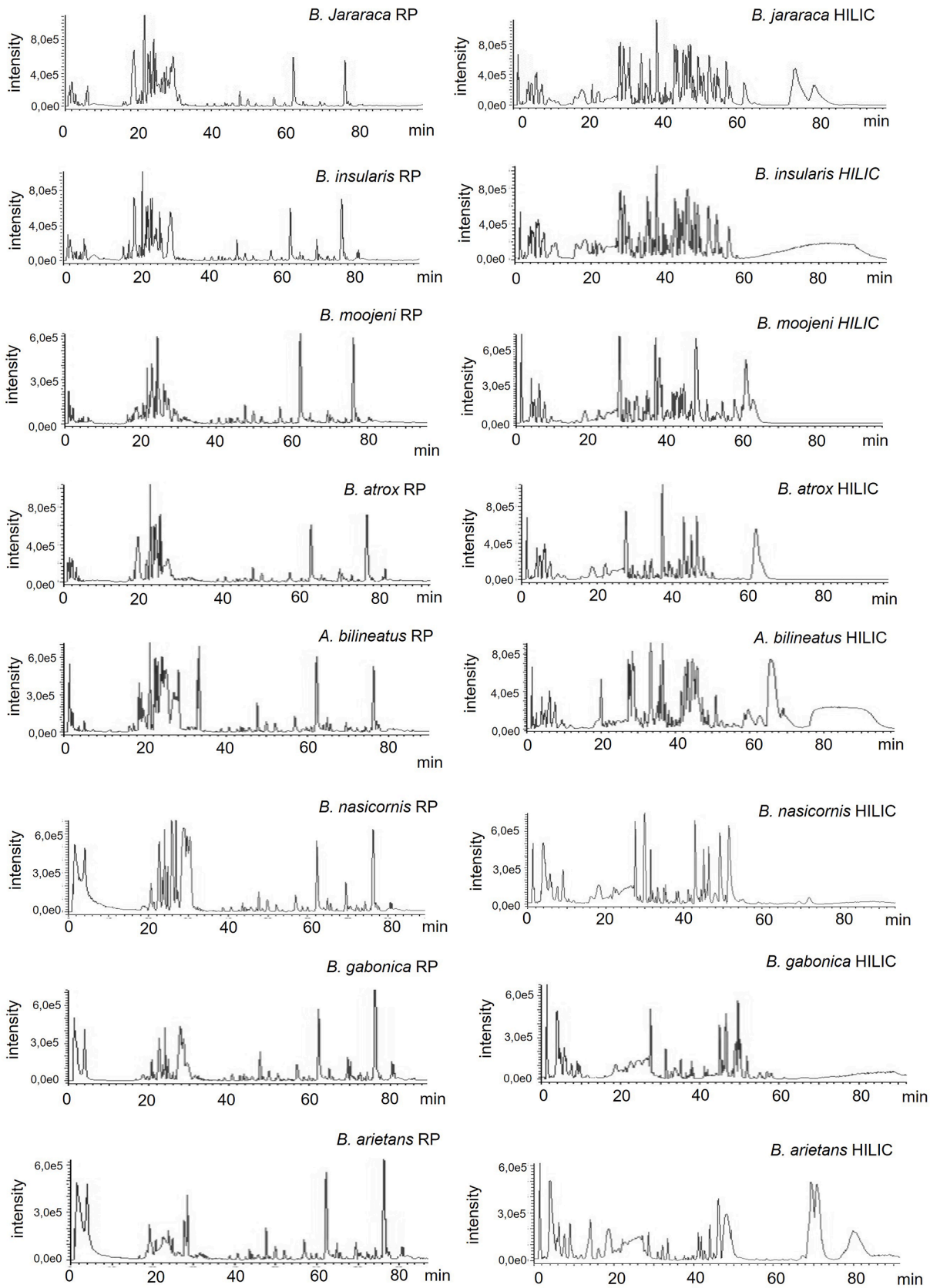

Fig. 3. Base peak chromatogram (BPC) of low-mass fraction after RP and HILIC separation. The figure shows the distribution of the most intense ions as a function of retention time, following chromatographic separation on RP and HILIC columns.

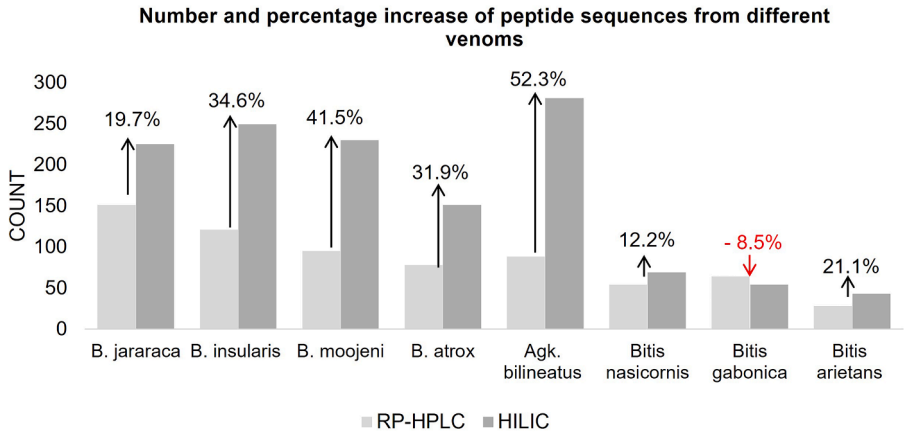

Fig. 4. Percentage increase in identified sequences in the peptidomic analysis of each species after HILIC separation compared to RP. Upward arrows indicate the percentage increase in sequences identified with HILIC column separation, while the downward red arrow for *B. gabonica* highlights that more sequences were identified using RP separation.

Table 1
Number of peptides identified in the database, classified by precursor family, after separation by RP and HILIC.

Species	RP		HILIC	
	Precursor family	Number of peptides Total/BPPs/SVMP-DIS/LAOO	Precursor family	Number of peptides Total/BPPs/SVMP-DIS/LAOO
B. jararaca	BPPs, SVMP and LAAO	151/63/58/30	BPPs, SVMP and LAAO	225/109/70/46
B. insularis	BPPs, SVMP and LAAO	121/25/10/86	BPPs, SVMP and LAAO	249/42/76/131
B. moojeni	SVMP and LAAO	95/0/8/87	BPPs, SVMP and LAAO	230/11/64/155
B. atrox	SVMP and LAAO	78/0/41/37	BPPs, SVMP and LAAO	151/51/49/51
A. bilineatus	BPPs, SVMP and LAAO	88/11/28/49	BPPs, SVMP and LAAO	281/49/174/58
B. nasicornis	SVMP	54/0/54/0	BPPs	69/69/0/0
B. gabonica	BPPs	64/64/0/0	BPPs	54/54/0/0
B. arietans	BPPs	28/28/0/0	BPPs	43/43/0/0

Table 2
Peptides related to the bradykinin potentiating peptide class identified by the peptidomic approach, with comparison between RP and HILIC separations.

Description BPPs		
Species	RP	HILIC
B. jararaca	BPP-10a; BPP 10c; BPP 11b; BPP 11c; BPP 13a; BPP 13b; BPP 13c; BPP 13d; BPP 5a, BPP 6a, BPP AP; CNP; pHpG; sequence spacer	BPP-10a; BPP 10c; BPP 11b; BPP 11c; BPP 13a; BPP 13b; BPP 13d; BPP 5a; BPP AP; CNP; peptide signal; pHpG; sequence spacer
B. insularis	BPP 10c; BPP 11c; BPP 13a; pHpG; sequence spacer	BPP 10c; BPP 11b; BPP 11c; BPP 13a; BPP 5a; CNP; pHpG; sequence spacer
B. moojeni	Not found	BPP 10a; BPP 13a; pHpG; sequence spacer
B. atrox	Not found	BPP-10a; BPP 10c; BPP 11b; BPP 11c; BPP 13a; BPP 13d; BPP 6a; CNP; peptide signal; pHpG; sequence spacer
A. bilineatus	BPP 10c; pHpG; Bradykinin inhibitor peptide; sequence spacer	BPP 10c; BPP 11a; BPP 11b; BPP 13a; peptide signal; Bradykinin inhibitor peptide; Angiotensin converting enzyme inhibitor; POL-236; pHpG; sequence spacer
B. nasicornis	Not found	BPP 10c; BPP11b; BPP 11c; BPP 13a; BPP 13d; BPP 6a; CNP; Peptide signal; pHpG; sequence spacer
B. gabonica	BPP-10a; BPP 10c; BPP 11b; BPP 11c; BPP 12b; BPP 13a; BPP signal; pHpG; BPP 10 g-AP; sequence spacer	BPP-10a; BPP 10c; BPP11b; BPP11c; BPP 13a; CNP; Peptide signal; pHpG; sequence spacer
B. arietans	BPP-10a; BPP 12b; BPP 13a; BPP 13d; CNP; Peptide Signal; pHpG; BPP 10c; BPP IIb; sequence spacer	BPP 10a; BPP 11b; BPP11c; BPP 12b; BPP 13a; BPP 13d; BPP 5a; CNP; pHpG; sequence spacer

et al. (2017), associating these fragments with "propeptides". As for BPP-13a, it has already been widely described in the venom of different snake species, as in the study by Pimenta et al [48], who identified these peptides in specimens of *B. jararaca*, and in the work of [49], who detected BPP-13a in several species of the genus *Bothrops*.

It is important to discuss the sequences related to pHpG and spacer, as although these fragments are present in snake venom, little is known about the function of these molecules in venom and envenomation. However, in an analysis of *Echis* ocellatus venom, pHpG peptides showed the ability to inhibit SVMP and reduce hemorrhagic action [47]. This result suggests that these peptides, although poorly understood, may play important roles in venom, reinforcing the need for further research.

The use of the HILIC column proved to be a promising tool for the identification of these peptides, which may contribute to a better understanding of the functionality of pHpG and spacer region fragments, for which there is little information in the literature. Furthermore, the presence of these fragments in all analyzed snakes may be characteristic of the *Viperidae* family, and potentially serve as taxonomic markers.

3.4. Venom protein fragments may be possible cryptids compounds

The detection of venom protein fragments has recently become a topic of discussion and still requires further investigation [6,8,15,41,50,51]. Currently, there is limited information about the functional importance of these components. Studies have increasingly reported the presence of these fragments in venom, particularly proteins from the SVMP/DIS and LAOO classes [8,15,41,46,51]. This raises questions about the origin of these fragments: Are they generated in the venom gland, or do they result from the extraction and manipulation processes? In this study, we ensured that the manipulation and solubilization of the venoms were performed with extreme care, using low temperatures to

obtain low-mass material while minimizing proteolysis as much as possible.

Studies that use peptidase inhibitors together with venom demonstrate a lower complexity of the material when compared to venom without inhibitors [8,15]. However, immunochemical [52] and proteomic [53] studies have revealed that venom proteins, especially metallopeptidases, are processed even before secretion, starting in the secretory vesicles and continuing in the gland lumen. These findings suggest that protein fragments may originate both during the venom collection and handling process and during storage in the snake's glands.

According to the literature and our interpretation, these fragments, even if they originated during venom handling, may play important biological functions that are still unknown and may be potential cryptids [54]. The relevance of exploring these fragments was highlighted in the research by Myamoto [55], where the authors demonstrated the inhibitory activity of a metallopeptidase fragment on an angiotensin-converting enzyme (ACE). This reinforces the importance of investigating these components to better understand their biological potential, or unknown effects since the target proteins present in the venom were also processed and degraded by plasmatic and tissular enzymes.

3.5. HILIC shown to be a more promising tool than RP for peptides mixtures

The experiments were conducted under similar conditions (column, equipment, solvents, and consecutive-day analyses), demonstrating that chromatographic separation using the HILIC column is highly effective for studying peptides. This approach enhanced chromatographic resolution, reducing ion accumulation compared to the RP column and significantly improving peptide identification efficiency. Consequently, there was a 25.6% increase in the number of sequences identified using the HILIC column compared to RP (Fig. 5).

When analyzing the classes of molecules, it is clear that the HILIC column exhibited a greater ability to identify certain types of molecules, particularly those with greater polarity. Sequences homologous to BPPs (bradykinin-enhancing peptides) were notably more abundant, with an increase of 53.5%. In contrast, RP chromatography was less effective in resolving these molecules, probably due to the hydrophilic nature of these molecules. In addition, fragments of LAOO (11.8% increase) and SVMP/DIS (18.1% increase) were also identified more frequently after separation with the HILIC column. These results highlight HILIC is ability to separate a wider range of molecules, especially those that are challenging to isolate with RP, which tends to favor more hydrophobic compounds.

The application of the HILIC column to separate small molecules from *Viperidae* venoms has proved to be a promising tool for isolating components that are not effectively resolved by RP chromatography. While RP is useful for separating hydrophobic molecules, HILIC is effective for separating small compounds with higher polarity in snake venoms, such as BPPs. This improved sensitivity is crucial for advancing our understanding of the chemical composition of snake venom. The methodology presented in this study can also be used for future investigations into other classes of compounds in venom mixtures, contributing to the study of venom evolution and functionality.

4. Conclusion

In conclusion, the application of the HILIC column for separating peptides from *Viperidae* venoms proved to be a valuable tool for isolating components not resolved by RP chromatography. In addition, the methodology presented in this study can be used for future investigations into other classes of bioactive compounds present in venoms, contributing to advances in the understanding of the chemical composition, evolution, and functionality of snake venoms. This approach not only provided novel insights into the composition of *Viperidae* venoms but also presented a promising strategy for analyzing other complex mixtures.

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CRediT authorship contribution statement

Monica V. Falla: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ivo Lebrun:** Writing – review & editing, Visualization, Supervision, Formal analysis. **Marcos A. Pudenzi:** Validation, Methodology. **Laudiceia A. Oliveira:** Validation, Methodology. **Heloisa F. Almeida:** Validation, Data curation. **Nathalia G. Santos:** Formal analysis, Data curation. **Mariana S. Rodrigues:** Validation, Resources, Investigation. **Patrick J. Spencer:** Resources. **Marisa M. Rocha:** Resources. **Daniel C. Pimenta:** Writing – review & editing, Visualization, Supervision, Formal analysis, Data curation. **Guilherme R. Coelho:** Writing – review & editing, Visualization, Supervision,

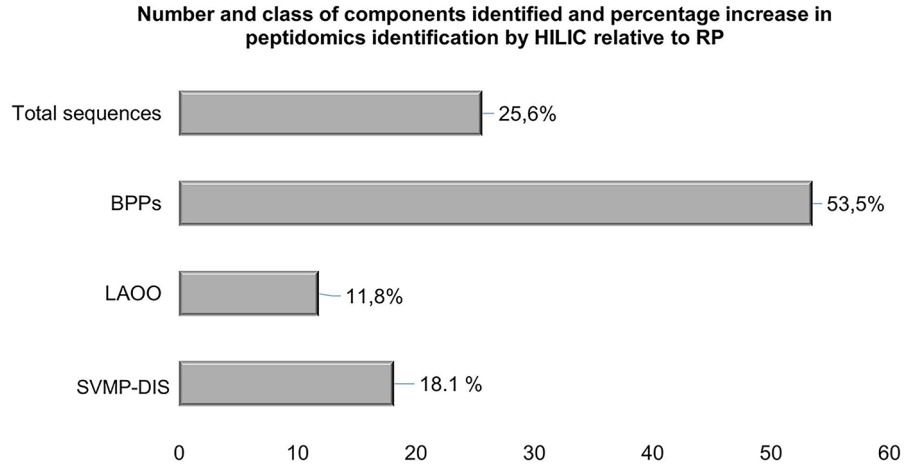

Fig. 5. Total percentage increase in peptidomic identification by HILIC relative to RP. The chart shows the percentage increase in the identification of total sequences, including specific classes such as BPPs, LAOO, and SVMP-DIS, when comparing HILIC separation with the traditional RP approach.

Project administration, Funding acquisition, Formal analysis, Data curation.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.465715.

Data availability

Data will be made available on request.

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