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The Electrochemical and Statistical Evaluation of Isolation of Mellitin and Apamin from Honey Bee (*Apis Mellifera*) Venom

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We present in this manuscript for the first time the electrochemical and statistical evaluation of FPLC isolation of mellitin and apamin from honey bee (*Apis mellifera*) venom. Venoms are extremely complex blends of diverse substances that target a myriad of receptors or ion channels. Therefore, toxins, isolated from venomous organisms can be a valuable tool with diverse biological applications. In this study we decided to optimize the purification of honey bee venom by using fast protein liquid chromatography, to obtain biologically active peptide - melittin (2846.46 Da). Due to a presence of other compounds with similar molecular weight (apamin 2027.34 Da), we optimized a differential pulse voltammetry method with adsorptive transfer technique (AdT DPV), utilizing Brdicka supporting electrolyte for measurements. Typical voltammograms - fingerprints for each substance were obtained and numerical projections of voltammograms were employed to propose an artificial neural network. Our suggested neural network can simply predict the content of each peptide in fraction with following performance: 100 % for training and 100 % for testing.

Keywords: Antimicrobial peptides; *Apis mellifera*; Brdicka reaction; MALDI-TOF; Differential pulse voltammetry

Abbreviations:

FPLC	-	Fast protein liquid chromatography
AdT DPV	-	Differential pulse voltammetry with adsorptive transfer technique
MCDP	-	Mast-cell degranulating peptide
MALDI-TOF	-	Matrix-assisted laser desorption/ionization-time of flight
APS	-	Ammonium persulfate
TEMED	-	Tetramethylethylenediamine
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TRIS	-	Tris(hydroxymethyl)aminomethane
HCCA	-	α -cyano-4-hydroxycinnamic acid
HMDE	-	Hanging mercury drop electrode
ANN	-	Artificial neural network
RBF	-	Radial basis function
MLP	-	Multilayer perceptron
BFGS	-	Broyden-Fletcher-Goldfarb-Shanno training algorithm
RCSB	-	Research collaboratory for structural bioinformatics

1. INTRODUCTION

The honey bee is a valuable organism both in agricultural and also in biological research, especially as a social insect that have evolved unique defense systems, such as individual molecular defence mechanism - production of strong antimicrobial peptides, known to be expressed in an innate immune system and venom system [1].

Bee venom is a natural compound produced by the honey bee (*Apis mellifera* L. 1758), and has been reported as having the biological and pharmacological activities, including anti-bacterial, anti-viral and anti-inflammation. In traditional medicine bee venom has been employed for treating of broad spectrum of diseases, such as arthritis, cancer, skin diseases or rheumatism [2]. It contains a large number of biologically active peptides, including mellitin, apamin, adolapin and mast cell-degranulating peptide (MCDP) [3]. In particular, melittin constitutes approximately 40 - 60 % of the dry weight of bee venom and can be found also in *Apis cerana*, *A. dorsata* and *A. florea*.

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂) - a toxic, cationic, water soluble peptide is mainly composed of a hydrophobic amino-terminal region (residue 1 - 20) and an entirely hydrophilic carboxyl-terminal region (residue 21 - 26) [4]. Natural or synthetic melittin is one of the most studied antimicrobial peptides and many studies have focused on its membrane-disruptive ability [4-7], resulting from asymmetric distribution of polar and non-polar side chains, that leads to elevated affinity towards membranes. Melittin adopts predominantly a random coil conformation and exists as monomer in aqueous solution at low pH, low concentration and low ionic strength [8]. At high ionic strength or higher concentration (more than 4 mM) and at high pH melittin self-associates to form an α -helical tetrameric structure, driven by the formation of a hydrophobic core [9].

Depending on membrane properties and lipid/protein ratio, melittin was previously shown to be transmembrane [10] or oriented parallel to membrane surface [11]. Both mechanisms of action cause the leakage of cellular content through artificial transmembrane pores and destruction of cell [12,13].

To reveal the exceptional biomedical and biophysical properties, melittin may be produced synthetically or it can be obtained *via* purification of collected honey bee venom. Since a production of synthetic peptides is highly reproducible and rapid, it is compensated by high operating and production costs. On the other hand, purification of melittin from honey bee venom is complicated by its high heterogeneity and presence of other peptides - mainly apamin, which is the smallest neurotoxin in bee venom, composed of 18 amino acids [14].

Herein we report an electrochemical study of apamin (24345-16-2) and melittin (20449-79-0) mixtures in various ratios after purification by using fast protein liquid chromatography (FPLC) and confirmation by matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry. Our method comprised differential pulse voltammetry coupled with adsorptive transfer technique using Brdicka supporting electrolyte for measurements, which offer sensitive determination of both peptides in mixture. The results obtained from electrochemical measurements were statistically processed into artificial neuron network with ability to predict the ratio of peptides in FPLC fractions of honey bee venom.

2. EXPERIMENTAL PART

2.1. Chemicals and pH measurement

Working solutions like buffers (Tris buffer or Brdicka buffer) and standard solutions of mellitin and apamin were prepared daily by diluting the stock solutions. pH value was measured using inoLab Level 3 (Wissenschaftlich-Technische Werkstätten; Weilheim, Germany). Standards and Oother chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) meeting the specification of American Chemical Society (ACS), unless noted otherwise. Methyl cellosolve and tin chloride were purchased from Ingos (Prague, Czech Republic).

2.2. Honey bee venom collection

The venom was collected from honey bees *Apis mellifera* Linnaeus, 1758 by using their electrostimulation on elastic layer (0.25 mm wide), localized under a conductor (tinned wire). The elastic layer was placed on a glass board (390 × 240 mm). After a sting, venom was stacked on a glass board, protected by elastic coverage against contamination (parts of digestive tract or abdomen). Impulses were generated in following conditions: 1 kHz, impulse voltage 25 V, duration 0.3 - 1 s with pause (1 s). The whole venom was stored at -80 °C until used for purification.

2.3. FPLC purification of honey bee venom

Aliquots of venom (1 mg) were resuspended in 50 mM Tris buffer (1 mL) and fractionated on fast protein liquid chromatography (FPLC) system Biologic DuoFlow (Biorad, Philadelphia, PA, USA). FPLC system consisted of two chromatographic pumps for carrying of elution buffers, gel

filtration column (Bio-Gel® P-10 Gel, Biorad, Philadelphia, PA, USA), injection valve with 0.5 mL injection loop, UV-VIS detector and fraction collector (Biorad, Philadelphia, PA, USA). As the mobile phase was used 50 mM Tris-HCl adjusted to pH 7.4. Flow rate of mobile phase was set to 0.5 mL.min⁻¹. Separation of melittin was done using isocratic elution. Before separation started, column was washed with mobile phase for 15 minutes. UV detection was carried out at 280 nm. Fractions were collected approximately in volume of 1 mL. After purification, the fractions were lyophilized (Christ Alpha 1-2 LD Freeze Dryer, Martin Christ, Osterode am Harz, Germany) and stored (-80 °C) for further experiments.

2.4. Tris Tricine-SDS PAGE

Tris-tricine gels were prepared from 5 mL 30 % acrylamide; 1 % BIS, 3.34 mL 3M TRIS (pH 8.45); 0.3 % SDS, 0.64 mL dH₂O, 2.12 mL 50 % glycerol, 100 µL 10 % APS, 3 µL TEMED for separation gel and 0.52 mL 30 % acrylamide; 1 % BIS, 1 mL 3M tris pH 8.45; 0.3 % SDS, 2.48 mL dH₂O, 40 µL 10 % APS, 2 µL TEMED for stacking gel. Tricine sample buffer (Bio-Rad, Philadelphia, PA, USA) with 2-Mercaptoethanol in ratio 19:1 was mixed with sample in ratio 1:1 and heated for 3 min at 95 °C. Polypeptide SDS-PAGE Molecular Weight Standards (Bio-Rad, Philadelphia, PA, USA) was used as a weight marker. 4 µL of weight markers and 10 µL of samples with sample buffer were pipeted into the wells and run for 80 min, 120 V in 1× running buffer, prepared from 12.1 g TRIS, 17.9 g tricine and 1 g SDS to 1 L. After electrophoresis, gels were stained with Coomassie brilliant blue.

2.5. Determination of total protein in fractions

Total proteins were established by using Bradford protein assay, according to [15]. For measurements a BS-400 automated spectrophotometer (Mindray, Shenzhen, China) was employed.

2.6. MALDI-TOF/TOF mass spectrometry

For confirmation of the presence of melittin/apamin MALDI-TOF/TOF mass spectrometry was applied. The experiments were performed using MALDI-TOF/TOF mass spectrometer Bruker Ultraflextreme (Bruker Daltonik GmbH, Bremen, Germany) equipped with a laser operating at wavelength of 355 nm with an accelerating voltage of 25 kV, cooled with nitrogen and a maximum energy of 43.2 µJ with repetition rate 2000 Hz in reflector and positive mode, and with software FlexControl version 3.4 and FlexAnalysis version 2.2 for data acquisition and processing of mass spectra respectively. α -cyano-4-hydroxycinnamic acid (HCCA) was used as the matrix. The matrix was prepared in TA30 (30% acetonitrile, 0.1% trifluoroacetic acid solution, w/w). Working standard solutions were prepared daily by dilution of the stock solutions. The solutions for analysis were mixed in ratio of 1:1 (matrix/substance). After obtaining a homogeneous solution, 1 µL was applied on the target and dried under atmospheric pressure and ambient temperature. External calibration was

performed with the Bruker Standard Peptide Calibration kit (m/z 1000–3500). The MS spectra were acquired by averaging 2500 sub-spectra from a one spot [13].

2.7. Electrochemical measurements of fractions isolated from honey bee venom

The isolated fractions composed of melittin/apamin were analysed by differential pulse voltammetry coupled with adsorptive transfer technique (AdT DPV). All measurements were performed with an AUTOLAB Analyzer (EcoChemie, Utrecht, Netherlands) connected to a 663 VA Stand instrument (Metrohm, Herisau, Switzerland). System was equipped with a standard cell consisting of three electrodes, measurement cell and sample holder set cooled at 4 °C (Julabo F25, JulaboDE, Seelbach, Germany). The three-electrode system consisted of a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm² as the working electrode, an Ag/AgCl/3M KCl reference electrode and a platinum electrode, acting as the auxiliary one. Software GPES 4.9 was used for data evaluation. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %) saturated with water for 60 s. The Brdicka supporting electrolyte contained 1 mM Co(NH₃)₆Cl₃ and 1 M ammonia buffer (NH₃(aq) and NH₄Cl, pH = 9.6). The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: accumulation time of 120 s; initial potential of -0.7 V; end potential of -1.8 V; modulation time 0.057 s; time interval 0.2 s; step potential 0.005 V and modulation amplitude 0.025V.

2.8. Development of artificial neural network

Automated neuronal networks were used as a predictive model. Classification analysis network was employed for the estimation of categorical data. The dataset was randomly divided as follows 67 % for learning, 33 % for testing. Following network types were tested using automated network search: multilayer perceptron (MLP) network, and radial basis function (RBF). Number of hidden units to search was determined as follows: 8 to 24 and 8 to 11 for MLP and RBF, respectively. Total 1000 networks were trained, following activation functions were searched for: identity, logistic, tanh, exponential, sinus. Weight decay was used as follows 0.0001 to 0.001 for hidden layer and output layer. Unless noted otherwise, p -level 0.05 was considered significant. Software Statistica 12 (StatSoft, San Francisco, CA, USA) was used for analysis [16].

2.9. Descriptive statistics

Analysis of the data and their graphical interpretation were made using Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®. Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise.

3. RESULTS AND DISCUSSION

Previously, the honey bee venom was collected manually, but it resulted in a rather different composition of venom, especially in its volatile constituents. Currently, a gold standard is formed by venom collection by using electrostimulation of bee workers [17]. Hence, we employed this technique to force the honey bees (*Apis mellifera*) to release their venom between glass slide and elastic platform. The depiction of entire collection process is shown in Fig. 1A. After stopping of electrostimulation, the venom was gathered from a glass board with specially adapted blade and stored at $-80\text{ }^{\circ}\text{C}$. Although melittin and apamin easily form protonated molecules in low pH solutions, due to their basicity and polarity [14], of which can be employed for ion-exchange separation, we employed fast protein liquid chromatography for fractionation, which is based only on molecular weight of target molecules (calculated mass of apamin = 2027.34 Da and melittin = 2846.46 Da). In contrast to most commonly utilized reverse phased-HPLC [14,18-20] or ion-exchange chromatography [21] our FPLC method works without application of organic solvents or denaturing agents. Prior to fractionation on FPLC, harvested venom (1 mg) was resuspended in 1 mL of 50 mM Tris buffer, which formed also a separation mobile phase. The fractions were collected as approx. 1 mL aliquots.

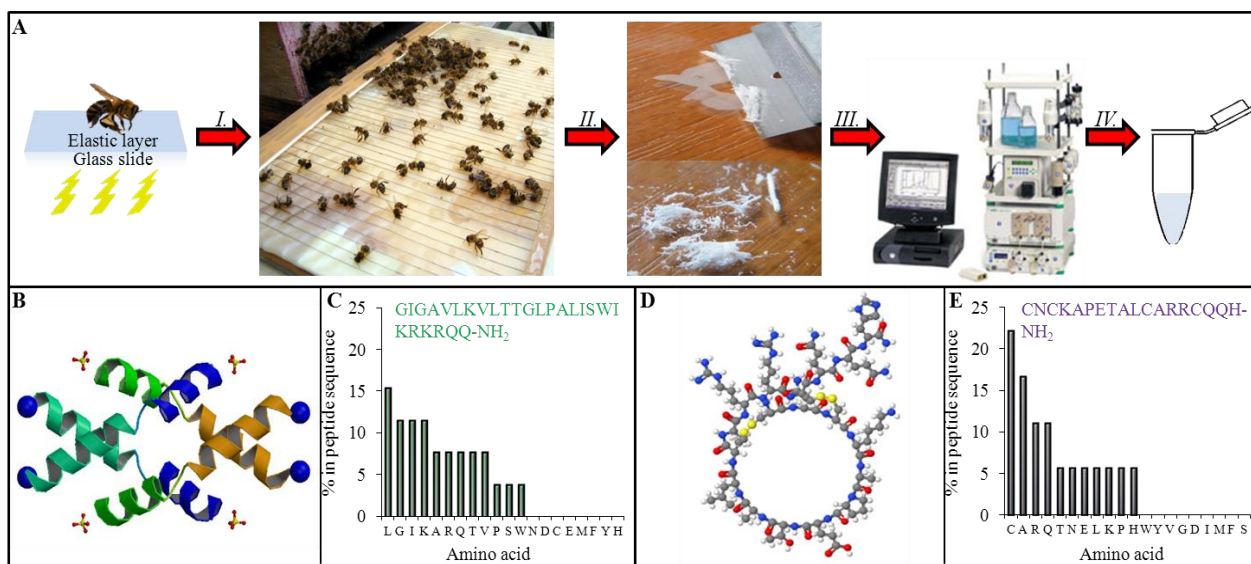


Figure 1. (A) Overall depiction of bee venom collection for melittin/apamin isolation. Honey bee venom was collected using by electrostimulation (I). The impulses for stinging were approx. 1 kHz, impulse voltage 25 V, duration 0.3 - 1 sec, with 1 sec pause (More details are mentioned in chapter 2.2. *Honey-bee venom collection*). After removal of elastic layer, the venom was collected from glass board with specially adapted blade and stored at $-80\text{ }^{\circ}\text{C}$ for further application (II). For purification, honey-bee venom was resuspended in 50 mM Tris buffer (III) and various fractions were collected (IV). The purity of crude isolate and presence of melittin was confirmed using by MALDI-TOF/TOF and Tris tricine-SDS PAGE. (B) Representation of melittin biological assembly forming homo 4-mer molecule, and (C) its percentual amino acid composition, with its sequence (single letter notation). (D) Representation of secondary structure of apamin and (E) its percentual amino acid composition, with its sequence (single letter notation). Structures of peptides were adapted from RCSB Protein Data Bank (www.rcsb.org) or Jsmol (www.chemapps.stolaf.edu).

We focused on isolation of the biologically interesting peptides - melittin and apamin. Melittin - the primary allergen in bee venom [22] is amphiphilic peptide, with a predominantly hydrophobic *N*-terminal region and a hydrophilic *C*-terminal region, with the characteristic structure of membrane bound cytolytic, channel-forming peptides and trans-membrane protein helices [23]. As it is shown in Fig. 1B, melittin biologically self-assemble to form α -helical tetrameric molecule, which is induced by increased peptide concentration [24]. However, in neutral pH and lower concentrations melittin is in a random coil configuration [25]. In Fig. 1C is shown the single letter notation of melittin amino acid sequence. The second molecule of interest was apamin - 18-amino acid peptide, comprising 4 cysteines with two disulfide bonds (Cys1-Cys11 and Cys3-Cys15) [26] with structure depicted in Fig. 1D and single letter notation in Fig. 1E. Apamin is believed to be a blocker of the calcium-activated potassium channels that mediate the long-lasting after-hyperpolarization in many cells [27]. In contrast to melittin, its antimicrobial activity has never been tested [20] and its exact structure is still unknown.

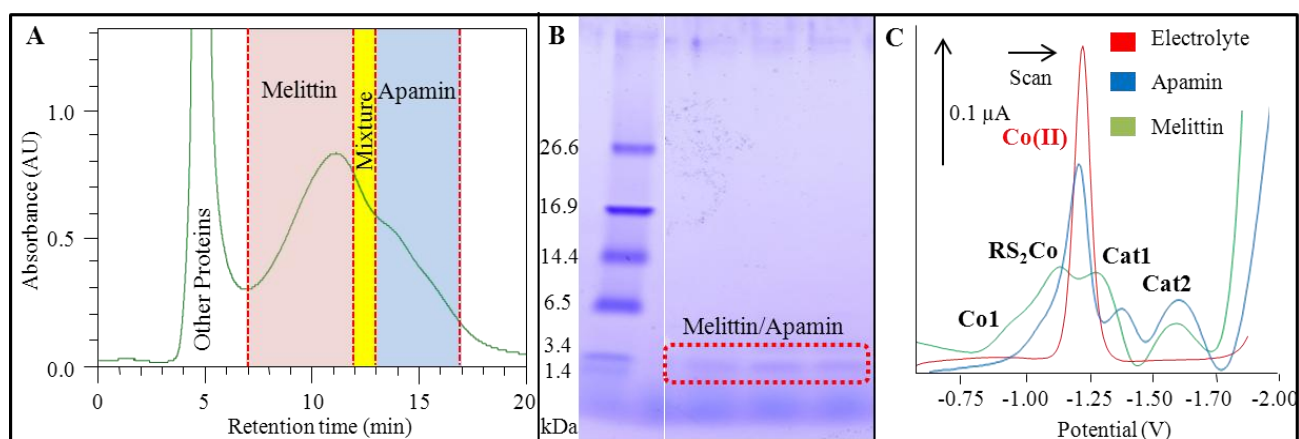


Figure 2. (A) Representation of FLPC-UV-Vis chromatogram obtained during purification of honey bee venom (applied detection wavelength - 280 nm). In red is highlighted the area of collection of fraction, composed mainly of melittin and in blue a fraction composed mainly of apamin. Between them was observed the elution of peptides mixture (yellow). After purification, fractions were further dried using by lyophilization, as is mentioned in chapter 2.3. *FPLC purification of honey bee venom* and used for experiments. (B) Tris-tricine-SDS PAGE electrophoretogram of fractions, comprising both - melittin and apamin, expressing the impossibility of their recognition, caused by their similar molecular weight ($M_{\text{melittin}} = 2846.46$ Da and $M_{\text{apamin}} = 2027.34$ Da). (C) Expression of the typical differential pulse voltammograms of melittin and apamin (both $200 \mu\text{g}\cdot\text{mL}^{-1}$) overlaid with Brdicka supporting electrolyte (1 mM $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ and 1 M ammonia buffer ($\text{NH}_3(\text{aq}) + \text{NH}_4\text{Cl}$, pH = 9.6).

For monitoring of composition of eluent after FPLC separation, Vis detection was employed ($\lambda = 280$ nm). As it is shown in representative chromatogram in Fig. 2A, our separation method (20 min) starts with elution of higher-molecular mass molecules, composed in honey bee venom (5 min). Since apamin exhibits lower mass, when compared to melittin, its molecules move through the porous beads in column slower, increasing its retention time. In 7 minute starts the collection of fractions composed of melittin, until approximately 12 min, where occurs elution of melittin/apamin, caused by mass

similarity of both peptides. In 13 min, the elution of mainly apamin was determined, till 17 min. As a first step of a fractions distinction, we employed Tris Tricine-SDS PAGE for small proteins (ladder 1.4 - 26.6 kDa). This method is the preferred electrophoretic system for the resolution of proteins/peptides smaller than 30 kDa [28]. However, as it can be seen in Fig. 2B, similar to FPLC fractionation, due to similar mass of both peptides only one band was observed between 1.4 - 3.4 kDa.

Our aim was an electrochemical characterization of both peptides and their mixtures, obtained from FPLC fractionation. To determine the differences between electrochemical behaviors of target molecules we employed differential pulse voltammetry coupled with adsorptive transfer technique (AdT DPV), utilizing Brdicka reaction for observation of target electroactivity. In Fig. 2C are expressed typical differential pulse voltammograms of melittin, apamin ($200 \mu\text{g}\cdot\text{mL}^{-1}$) and Brdicka supporting electrolyte. In Brdicka reaction are evaluated catalytic signals of hydrogen evolution, provided by peptides/proteins on the mercury electrode in the presence of ammonium buffer with content of the cobalt salt [29]. Proteins and peptides with thiol/amino groups give four distinct signals - Co1 (- 0.9 V), RS_2Co (approx. - 1.15 V), Cat1 (approx. - 1.3 V) and Cat2 (- 1.55 V), however Brdicka electrolyte provides only one Co(II) peak (- 1.15 V) [30]. As it is apparent from Fig. 2C, large differences between electrochemical behaviors of both peptides were observed, in particular in RS_2Co (potential shift of about -0.4 mV and decrease of current in melittin peak of about $0.1 \mu\text{A}$) and Cat1 (potential shift of about -0.2 mV and decrease of apamin peak of about $0.05 \mu\text{A}$).

Since AdT DPV was shown to be potentially suitable for determination of content of both peptides in samples, based on differential pulse voltammogram shape, we carried out a comparison with MALDI-TOF/TOF mass spectra (Fig. 3). In Fig. 3A are shown mass spectra of apamin, melittin and their mixtures (m/z range 1900 - 3300). As it is obvious, differential pulse voltammograms (Fig. 3B) exhibit various shapes of typical peaks. Since apamin contains cysteines in its structure, large catalytic RS_2Co peak was observed. That is in agreement with the fact that Co^{2+} in Brdicka electrolyte is a borderline type of cation preferably binding to the soft ligands like SH-groups [31], according to following equation (Eq. 1):



However, the ability of certain organic molecules to cause catalytic hydrogen evolution is connected with the presence of unshared electron pairs also at nitrogen, phosphorous or arsenic atoms and the possibility to exist in two forms, the acid and the base, according to the Brønsted-Lowry acid-base theory [31]. As it can be seen in melittin voltammograms, their shape is significantly changed, increasing the Cat1 and Cat2 peaks (Fig. 3B), with subsequent decrease of RS_2Co . Cat1 and Cat2 signals correspond to the reduction of hydrogen on mercury electrode [32]. The voltammograms of various melittin/apamin mixtures show the various changes in typical potentials (mainly RS_2Co , Cat2) in dependence on melittin/apamin ratio (when compared with MALDI-TOF/TOF).

Because it is obvious that each peptide is represented by its fingerprint - voltammogram, influencing also the composition of peptides mixture, we decided to propose an artificial neural network (ANN) with ability to describe the voltammograms shapes. From 2000 artificial neural networks 5 were retained and one was used for further final custom neuronal network.

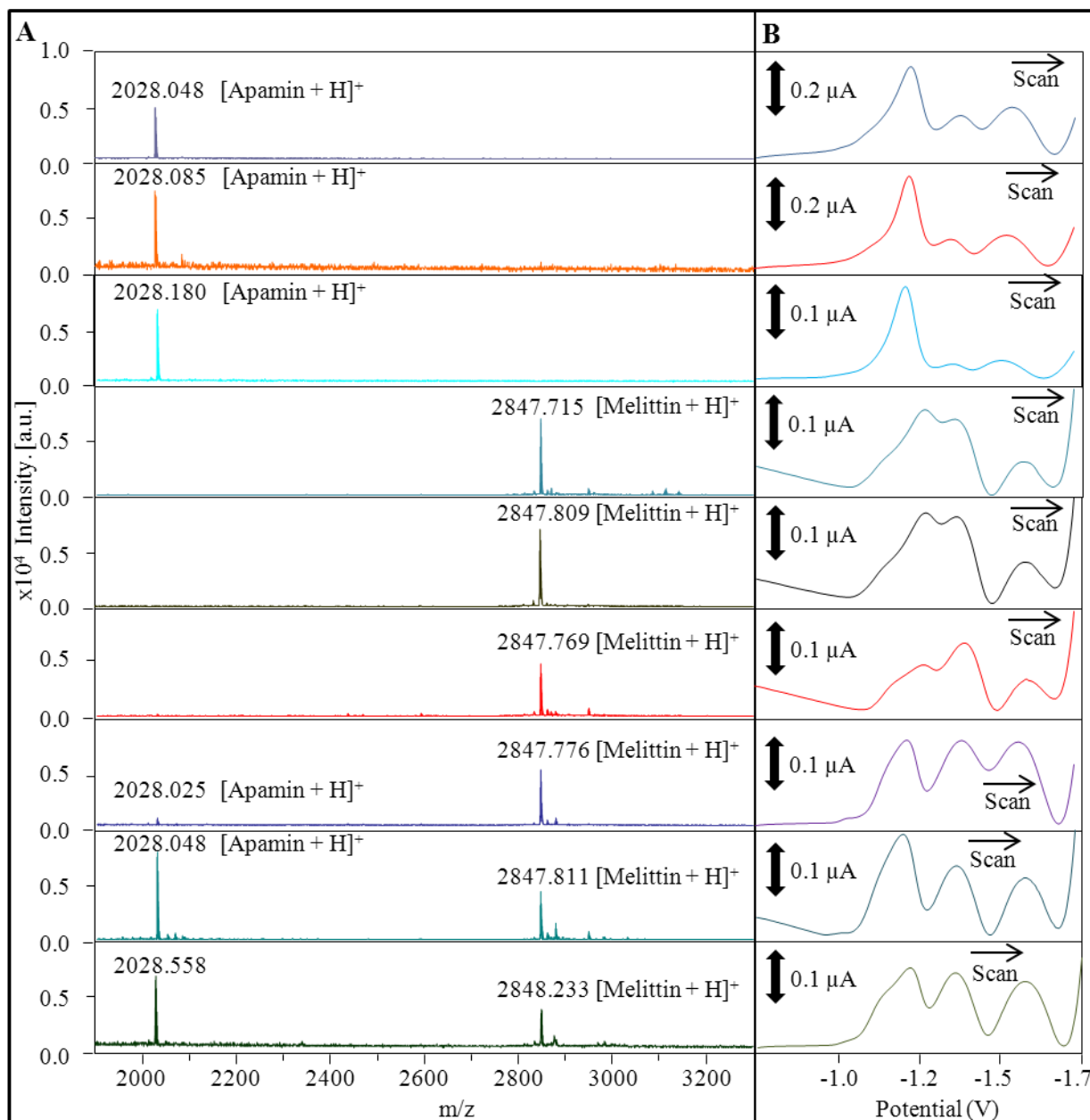


Figure 3. Expression of (A) MALDI-TOF spectra of honey bee venom fractions, obtained after FPLC isolation. Fractions are composed of apamin, melittin and their mixtures in different ratio. Spectra of melittin (calculated mass = 2846.46 Da) and apamin (calculated mass = 2027.34 Da) were obtained using reflector positive mode, HCCA as a matrix and laser gain of 45 %. 2500 averaged subspectra were evaluated on one spot. (B) Differential pulse voltammograms of apamin, melittin and mixture. Voltammograms were obtained by measurements of samples, standardized on the same concentration of total proteins ($200 \mu\text{g}\cdot\text{mL}^{-1}$).

The settings of the network created using automated algorithm and used for the custom final learning was as follows: Multilayer perceptron 206-16-3 (input-hidden-output neurons), Broyden-Fletcher-Goldfarb-Shanno (BFGS) training algorithm, sum of squares error function, identity function for hidden layer and logistic for output layer. With stopping conditions enabled, a final network was created in the 8th training cycle with following performance: 100 % for training and 100 % for testing.

The design of the network is displayed in the Fig. 4 and the performance of the network is depicted in Tab. 1.

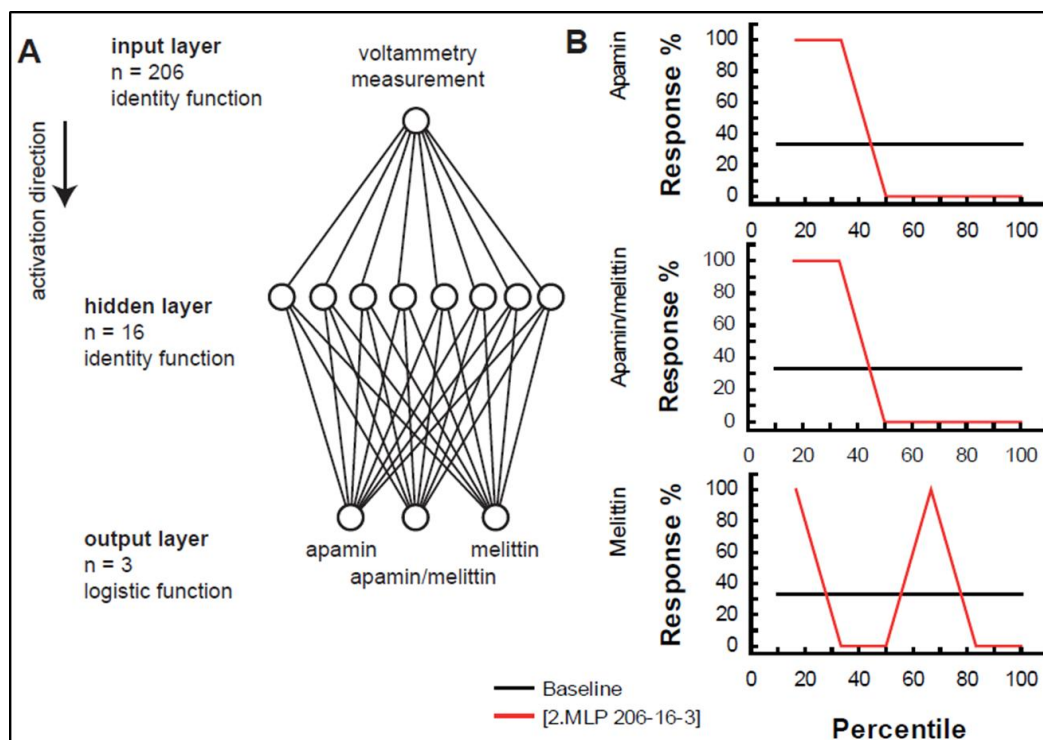


Figure 4. Design and performance of the neuronal networks. (A) Design of classification network for the prediction of the content of peptides in the sample using AdT DPV approach. The number of neurons/inputs is indicated by *n*. Note the number of input and hidden neurons is not displayed exactly. (B) Liftcharts for the estimation of individual compounds in the sample. Displayed as percentile vs. response of the network compared to the baseline.

Table 1. Prediction and accuracy of apamin and melittin AdT DPV measurements using neuronal networks.

Sample	Substance (Target) (200 ug.mL ⁻¹)	Substance - Output (MLP 206-16-3)	Substance Accuracy (MLP 206-16-3)	Substance Confidence levels (MLP 206-16-3)
Train	Apamin	Apamin	Correct	0.58
Train	Apamin	Apamin	Correct	0.58
Test	Apamin	Apamin	Correct	0.58
Train	Melittin	Melittin	Correct	0.35
Train	Melittin	Melittin	Correct	0.50
Test	Melittin	Melittin	Correct	0.37
Train	Apamin/Melittin	Apamin/Melittin	Correct	0.45
Train	Apamin/Melittin	Apamin/Melittin	Correct	0.53
Test	Apamin/Melittin	Apamin/Melittin	Correct	0.36

MLP - multilayer perceptron

Input-hidden-output neurons perceptron 206-16-3 shows perfect accuracy for all testing substances (Correct in all cases) with confidence levels between 0.35 - 0.58. Hence, it is obvious that numerical representation of voltammogram curves projection can be employed as an input for determination of melittin/apamin presence in FPLC fractions and any other sample.

4. CONCLUSION

Based on above mentioned facts, it was shown that FPLC fractionation can be sufficient method; however purification efficiency is not 100 %, and thus cheap and rapid method is required for peptides qualification and quantification. MALDI-TOF/TOF offers sensitive analyses, nevertheless the due to different ionization ability of peptides; quantification is complicated, combined with high acquisition costs. We have successfully developed a method using differential pulse voltammetry coupled with adsorptive transfer technique which gives very accurate and rapid results that can be employed for fractions characterization in combo with suggested artificial neural network. Due to typical peptides voltammograms - fingerprints, our ANN can simply predict the content of each peptide in fraction with following performance: 100 % for training and 100 % for testing.

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Conflict of interest:

The authors have declared no conflict of interest.

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