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A simple protocol for venom peptide barcoding in scorpions

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ABSTRACT

Scorpion venoms contain many species-specific peptides which target ion channels in cell membranes. Without harming the scorpions, these peptides can easily be extracted and detected by MALDI-TOF mass spectrometry. So far, only few studies compared the venom of different species solely for taxonomic purposes. Here, we describe a very simple protocol for venom extraction and mass fingerprinting that was developed for peptide barcoding (venom code for species identification) and facilitates reproducibility if sample preparation is performed under field conditions. This approach may serve as suitable basis for a taxonomy-oriented scorpion toxin database that interacts with MALDI-TOF mass spectra.

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1. Introduction

Scorpions belong to a group of arthropods which phenotype remained largely unchanged during the last 400 million years. The evolutionary success of these predators is largely associated with their very potent venom, which is used for deterring predators and for immobilizing their prey [1]. Major compounds of the venom are peptides [2] and many of these peptides interact with K^+ ion channels ("short chain" peptides [3]) and Na^+ ion channels ("long-chain" peptides [4]). It is hypothesized that the numerous scorpion venom peptides with their divergent effects may have evolved from a common ancestor gene [5].

As it is typical of venom toxins from other animals [e.g. 6–9], composition and efficacy of peptide toxins tend to be taxon-specific in scorpions, although basic types of peptides can be found in different clades [10]. The rapid diversification at the molecular level results from evolutionary adaptations of ion-channels of local prey and predators [11]. Scorpions are becoming helpless if predators resist the scorpions sting [12] and have to adapt their toxin efficacy or die out. For certain species it was even shown that different populations can be differentiated based on AA-substitutions within peptide sequences [13–16].

Information about specific peptide sequences can be used to explore the pharmaceutical potential [17] but might also be useful to confirm species identity. Most of the toxic peptides contain less than 100 AAs and are therefore well detectable by mass spectrometry, which is the method of choice for a fast venom screening [18,19]. For a number of scorpion species, particularly medically important species of Buthidae, comprehensive data exist already, that reflect the peptide complement of the venom [20]. The peptidome of the venom glands from these species consists of mature peptides but also many breakdown products of larger polypeptides [21]. Only few studies compared the venom of different species solely for taxonomic purposes [e.g. 22,23]. The general idea

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behind that strategy was described already by Dyason et al. [22] who pointed out that it is, for species-identification, not important to obtain a complete map of existing peptides but a typical venom profile of ion signals for a given taxon. In the chemotaxonomic studies, different types of mass spectrometers, different mass ranges, different settings (e.g. linear or reflectron mode) and also different protocols for venom extraction have been used.

In our study, we tested several scenarios to obtain mass fingerprints of venom peptides as easy and repetitive as possible. The protocol that turned out to be the most convincing one is described herein and may serve as suitable basis for a taxonomy-oriented scorpion toxin database that interacts with MALDI-TOF mass spectra.

2. Materials and methods

2.1. *Venom extraction*

Heterometrus cyaneus (Vietnam) were obtained from the Pet Factory (Hülsede, Germany); all other scorpions tested in this study were collected during field trips and released following second venom extraction. For electrical stimulation of the membrane anterior to the telson, we used the transcutaneous electrical nerve stimulation unit Promed tens 1000 s (Promed GmbH, Farchant, Germany) connected with modified tweezers to apply power. To improve stimulation, the tweezers were lubricated with conduct gel. The intensity of stimulation current was adjusted for each scorpion; usually a pulse width from 200 to 250 μs and a pulse rate of 60–130 Hz was applied. The extracted venom was collected with a glass capillary (inner diameter of 1 mm; Hilgenberg GmbH, Malsfeld, Germany) that was attached to a flexible silicone tube and a disposable syringe, and transferred into a 0.5 ml Eppendorf microtube containing 200 μl of 35% ethanol/0.1 TFA.

2.2. *N-terminal peptide derivatization using 4-sulfophenyl-isothiocyanate (SPITC)*

For SPITC derivatization, 2 μl of the venom extract in 35% ethanol/0.1 TFA was mixed with 18 μl of 0.1 TFA. SPITC was dissolved in 20 mM $NaHCO_3$ (pH 9.0) at a concentration of 80 mg/ml. 15 μl of the diluted venom extract and 40 μl of the SPITC solution were mixed and the sulfonation reaction performed for 1 h at 55 °C and 300 ppm. After that, the sample was acidified by adding 2.5 μl of 10% acetic acid followed by 50 μl of 0.5% acetic acid. Peptides were loaded in aliquots of 20 μl onto an activated and equilibrated home-made StageTip Empore 3M (IVA Analysentechnik e.K., Meerbusch, Germany) C18 column. The column was then flushed with 2× 20 μl of 0.5% acetic acid. Retained peptides were eluted from the column with 1.3 μl of 10/20/25/30/40/50/60/70/80% acetonitrile in 0.5% acetic acid. Each elution step was performed three times and the eluates spotted onto a MALDI target, respectively. Before drying, matrix solution (CHCA) was added to the samples.

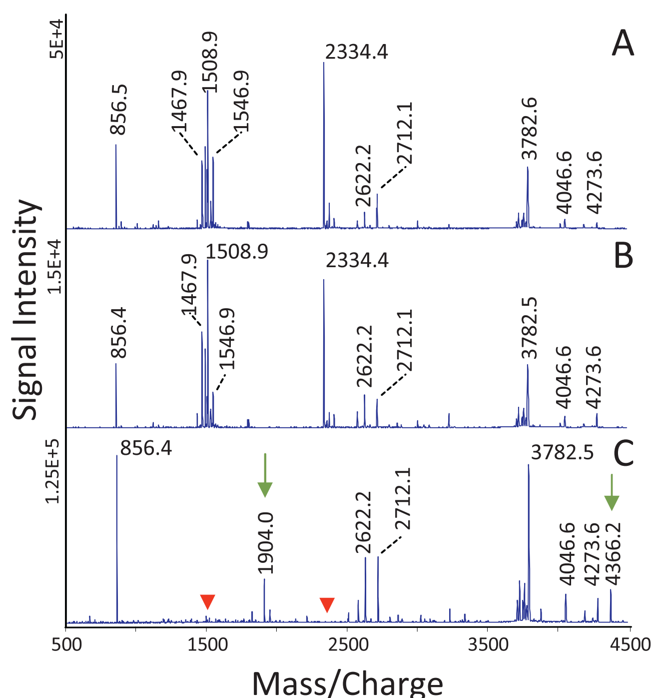

Fig. 1 – MFPs (*m/z* 500–4500) from a venom sample of *Heterometrus cyaneus* stored under different conditions. (A) Venom extract was diluted in 100 μl 0.1 TFA and 0.5 μl of this solution was immediately spotted on a sample plate. Matrix application has been accomplished after 4 weeks; (B) venom sample was diluted in 200 μl 35% ethanol/0.1 TFA and stored for 4 weeks at room temperature. About 0.5 μl of this solution was spotted on the sample plate and matrix application has been accomplished immediately; and (C) remaining venom solution in 0.1 TFA (see A) was stored for 4 weeks at room temperature. About 0.5 μl of this solution was spotted on the sample plate and matrix application has been accomplished immediately. MFPs of samples which were prepared as described in (A) and (B) are almost identical. The sample stored in 0.1 TFA was partially degraded; missing ion signals are marked by arrowheads; additional ion signals are marked by arrows.

2.3. *MALDI-TOF mass spectrometry*

About 0.5 μl of matrix (10 mg/ml 2,5-dihydroxybenzoic acid [DHB; Sigma–Aldrich, Steinheim, Germany] dissolved in 20% acetonitrile/1% formic acid or 10 mg/ml α-cyano-4-hydroxycinnamic acid [CHCA; Sigma–Aldrich] in 60% ethanol/36% acetonitrile) was mixed with the same quantity of venom extract before air drying at room temperature. Samples of venom extract, which were spotted onto the sample plate long before mass spectrometric analysis, were dried without matrix application and matrix was added immediately before analysis.

MALDI-TOF mass spectrometry was performed in positive ion mode on an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). All spectra, which are discussed here, were acquired in reflectron mode within a range of *m/z* 500–10,000; the settings were optimized to *m/z*

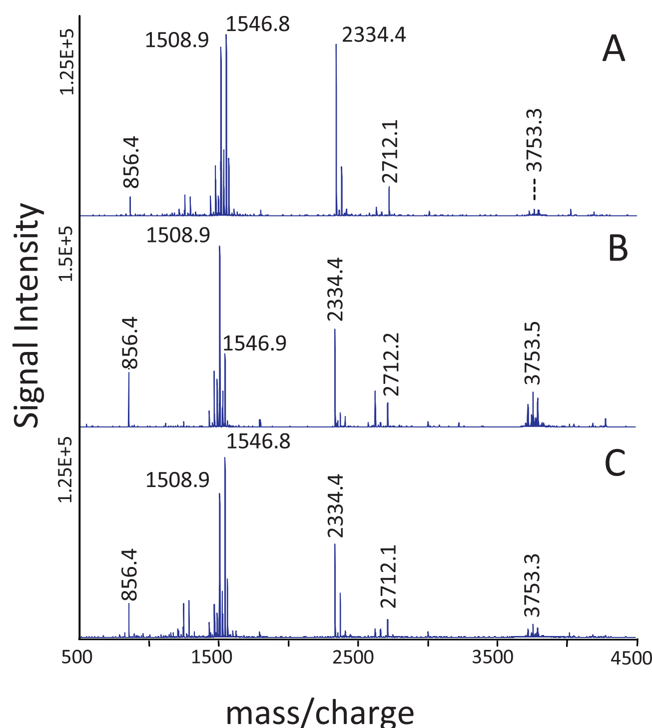

Fig. 2 – **Nearly identical MFPs (*m/z* 500–4500) from three succeeding venom extractions (extractions 2–4) of the same specimen of *Heterometrus cyaneus*. Venom samples were diluted in 100 μl 0.1 TFA and 0.5 μl mixed with DHB on the sample plate. Only prominent ion signals are labeled. (A) Venom extract 2 taken 24 h after the first extraction; (B) venom extract 3 taken 72 h after the second extraction; and (C) venom extract 4 taken a week after the third extraction.**

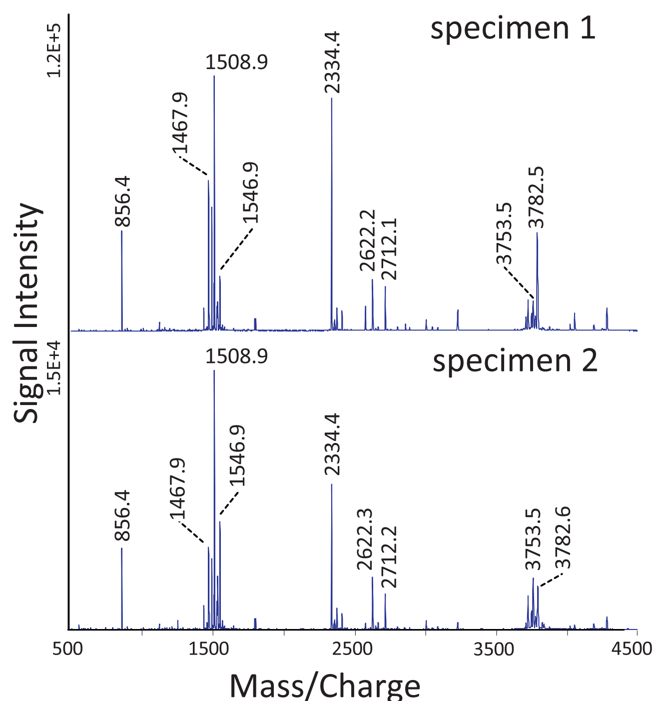

Fig. 3 – **Comparison of MFPs (*m/z* 500–4500) from venom extracts (2nd extraction) of two specimens of *Heterometrus cyaneus*. Venom samples were diluted in 100 μl 0.1 TFA and 0.5 μl mixed with DHB on the sample plate. MFPs of both extracts are nearly identical. Only prominent ion signals are labeled.**

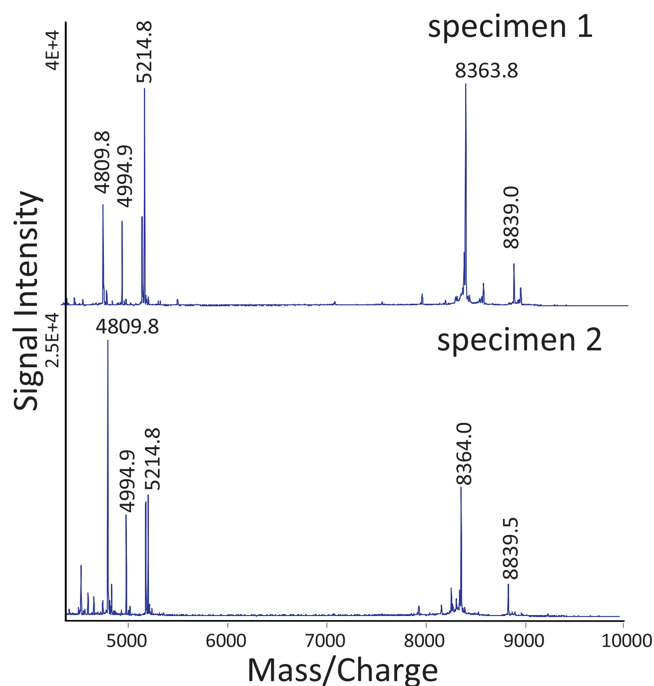

Fig. 4 – **Comparison of MFPs (*m/z* 4500–10,000) from venom extracts (2nd extraction) of two specimens of *Heterometrus cyaneus*. Venom samples were diluted in 100 μl 0.1 TFA and 0.5 μl mixed with DHB on the sample plate. MFPs of both extracts are nearly identical. Only prominent ion signals are labeled.**

500–3500 and 3000–10,000. Intensity, frequency, and number of laser shots of the smartbeam-II^{TM} Laser were adjusted to obtain an optimal signal-to-noise ratio; most data resulted from 3000 shots at 666 Hz. For calibration, Bruker peptide and protein standard kits were used. Data were processed with FlexAnalysis 3.0 (Bruker Daltonics). MS/MS experiments were conducted by using LIFT^{TM} technology without and with argon CID that also enabled assignment of isobaric Leu/Ile. Peptide sequences were identified by manual analysis of fragment ions and subsequent comparison of predictable (http://prospector.ucsf.edu) and experimentally obtained fragmentation patterns.

3. Results and discussion

The aim of the study was to describe an easy to use workflow for the compilation of taxon-specific peptide signals from the venom of single scorpions ("peptide barcoding"); practicable also for all those, who are without any knowledge in chromatography and mass spectrometry. Therefore, the method should be simple, cheap, not time-consuming, highly reproducible, non-fatal for the scorpions, and should also enable sample preparation under field conditions. Experiments were first performed by using *Heterometrus cyaneus* (Koch); simply because the specimens were readily available, large, and

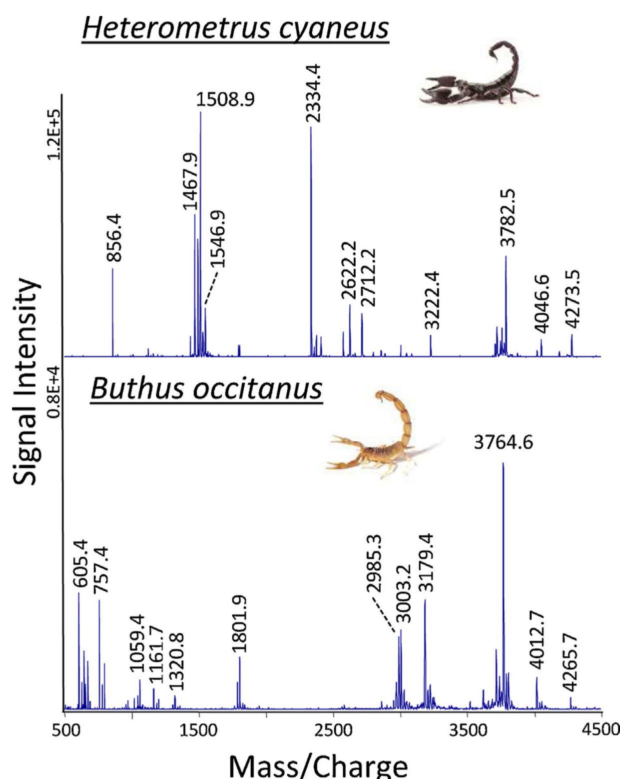

Fig. 5 – Comparison of MFPs (*m/z* 500–4500) from venom extracts (2nd extraction) of two scorpion species. Venom samples were diluted in 100 μl 0.1 TFA and 0.5 μl mixed with DHB on the sample plate. MFPs of both extracts do not contain any ion signal which is detectable in both samples. Prominent ion signals are labeled. (A) MFP of diluted venom extract from *Heterometrus cyaneus* and (B) MFP of diluted venom extract from *Buthus occitanus* (San Jose, Spain).

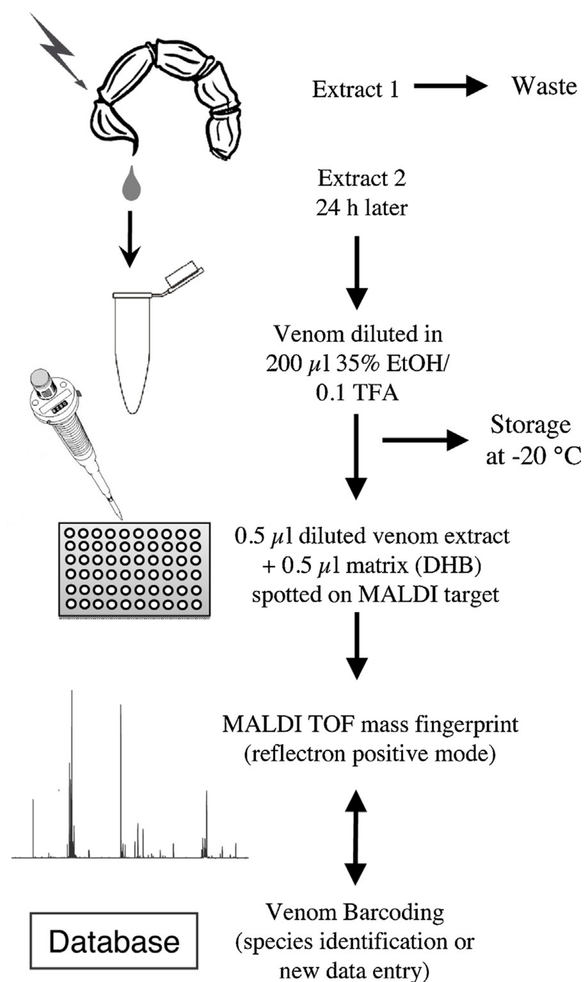

Fig. 6 – Overview of the steps necessary to obtain highly reproducible MFPs for the assignment of scorpion taxa (venom peptide barcoding).

quite harmless. Venom peptides from different species of *Heterometrus* were recently analyzed [24–26]. Later on, the practicability of the optimized protocol was tested for venom extraction under field conditions.

3.1. Step 1: collection of venom

Venom extraction by electrical stimulation of the telson generally yielded higher reproducibility of ion signals in mass fingerprints (MFPs), likely due to elimination of the scorpions ability to secrete pharmacologically different venoms. Without separating possible prevenom fractions [27], released drops were collected into a glass capillary (see suppl. movie). For treatment of species of Buthidae we used a device to fix the scorpions tail (adapted from [28]). Occasionally, we found differences in MFPs of venoms from the initial venom extraction and those from the second extraction. Venom samples from the second and subsequent extractions, however, resulted in nearly identical MFPs. Therefore, the first venom extract of scorpions collected in the field was discarded and only the second extract, harvested 24 h later, was used for analysis. Venom extraction did not cause mortality in any case, even if repeated up to 10 times in 4 weeks under laboratory conditions (*Heterometrus cyaneus*). In the field, milked scorpions were released following the second venom extraction. Only a single specimen of local populations was kept as voucher specimen, respectively.

3.2. Step 2: dilution and storage of venom

Without considering the total volume, the extracted venom was diluted in 200 μl of 35% ethanol/0.1 TFA. Dilution in 50–1000 μl did not change the MFPs significantly (not shown). A small amount of the diluted samples (ca. 0.5 μl) can be spotted directly on a sample plate for MALDI-TOF mass spectrometry; the plate provides 384 sample spots. If prepared under field conditions, dried samples remain intact for more than 4 weeks if the sample plate was kept in dry and dark conditions. Extracted venom can also be diluted in 0.1 TFA, which yielded identical MFPs if immediately prepared for mass spectrometry. However, venom peptides kept in 0.1 TFA partially degrade over time (Fig. 1). Therefore, samples diluted in ethanol/0.1 TFA were kept in any case for subsequent analyses.

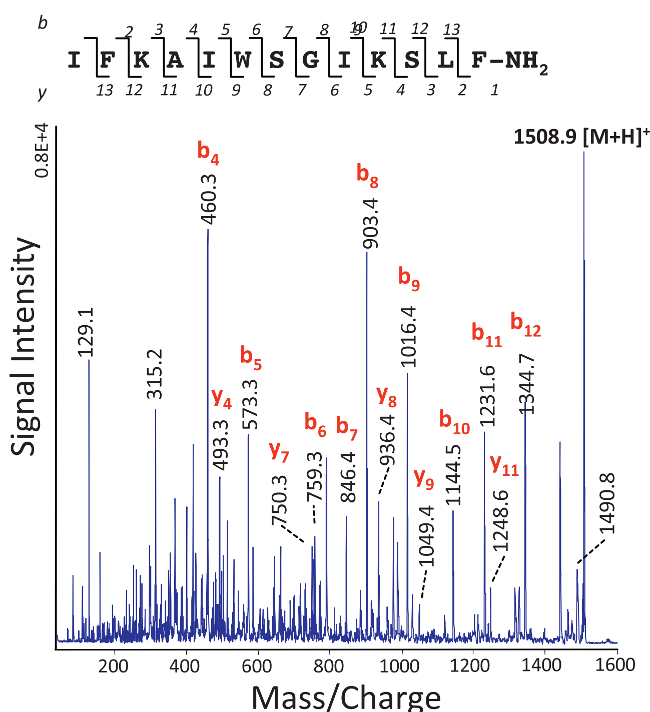

Fig. 7 – MS/MS fragment spectrum (CID off) of a peptide (*m/z* 1508.9) from *Heterometrus cyaneus* that is mass-identical to a venom peptide described from *Heterometrus petersii* [25]. Fragments were obtained from the same sample spot that yielded the MFP in Fig. 2B and confirmed the predicted peptide sequence (upper line).

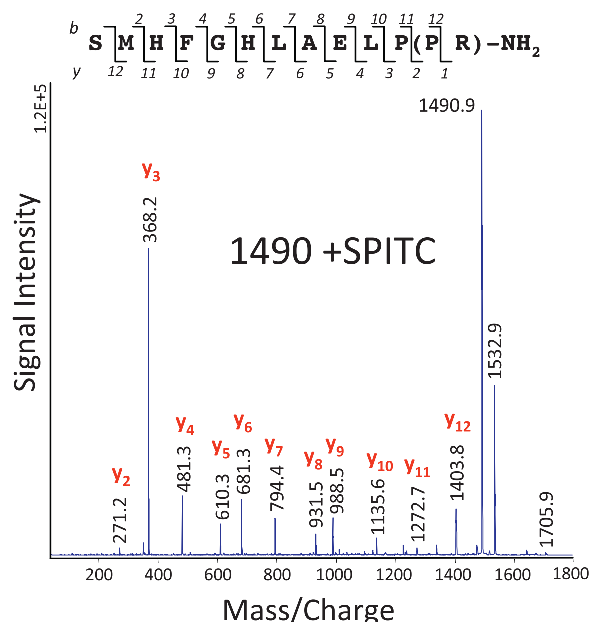

Fig. 8 – MS/MS fragment spectrum (CID off) of an unknown SPITC-derivatized peptide at *m/z* 1490.9 including the neutral losses of the modifying group (−173 Da, −215 Da). Assigned y-fragments are labeled and revealed the sequence SMHFGHLAELP(PR)-NH_2.

3.3. *Step 3: matrix application and mass fingerprinting*

The majority of peptides in scorpion venoms cover a mass range of *m/z* 800–10,000. For this mass range, DHB is the matrix of choice for a consistent detection of peptides in MALDI-TOF mass spectrometry. For various reasons, most studies on scorpion venoms were performed with CHCA as matrix, which favors the detection of smaller peptides. We tested both matrices and found a better coverage of peptides by using DHB, which was then used in all subsequent analyses. Mass spectra were generally recorded in the (positive) reflectron mode, which involves high resolution and the clear detection of similar-mass ions.

As expected, multiple MFPs from the same extract were found to be identical (not shown). Moreover, analyses of successive venom extractions from the same specimens yielded nearly identical MFPs (Fig. 2). Nearly identical MFPs were also obtained when analyzing venom extracts of different specimens from the same population (Figs. 3 and 4). On the other hand, no mass match was observed when comparing MFPs of two not closely related scorpions (Fig. 5). The simple workflow to obtain highly reproducible MFPs for the assignment of species is summarized in Fig. 6. For those readers not familiar with details of mass spectrometric analyses, time and costs were estimated in a recent manuscript, which describes the use of insect neuropeptides for similar purposes [29].

Ideally, the collection of data should be linked with a database that imports the MFPs of scorpion venoms and assigns new datasets to already incorporated taxa. Such programs exist for e.g. MFPs from microorganisms of medical importance (e.g. Biotyper, Bruker Daltonik GmbH) and operate with data obtained in linear mode. We are currently testing similar programs that will be adapted to reflectron mode. Using such programs for a high-throughput screening will also facilitate the recognition of possible population-specific ion signals and physiological changes in the peptidome of venom glands. Once saved as peptide complement of a certain species, which venom was extracted after electrical stimulation, subsequent analyses do not necessitate an identical procedure for venom extraction. Very few out of the numerous peptide mass signals will already be sufficient for species recognition.

3.4. *Facultative step 4: peptide identification*

From each venom sample we used 2× 0.5 μl to obtain MFPs. Since the venom was diluted in 200 μl of ethanol/0.1 TFA, more than 99% of the extract from single specimens was left and could be stored for further analyses such as identification of hitherto unknown peptides which were found in the MFPs. Such subsequent analyses are beyond the scope of peptide barcoding, more time-consuming and can alternatively be performed by research groups mainly interested in the function of the venom peptides. A major goal of these analyses is the identification of new peptides, which can then be tested for e.g. specific ion channel activity. Here we show few approaches

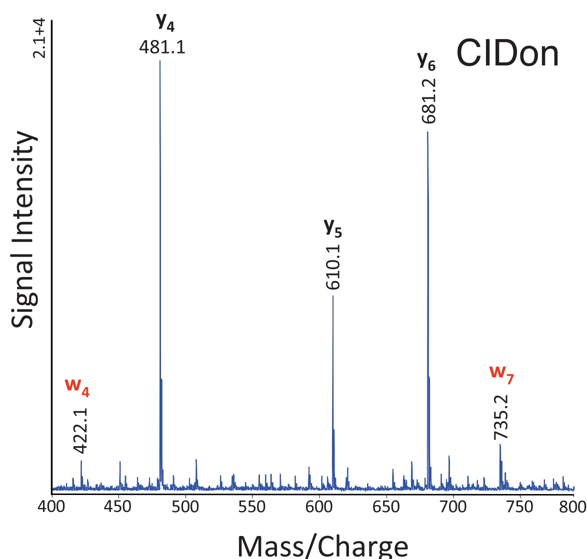

Fig. 9 – MS/MS fragment spectrum (*m/z* 400–800) of SPITC-derivatized peptide at *m/z* 1490.9 under condition of high gas pressure CID. Side chain fragments (*w*-fragments) typical of Leu allowed the assignment of Leu at positions 7 and 10 from the N-terminus.

that do not necessitate HPLC-purification. First of all, MS/MS fragmentation of selected peptides can easily confirm peptide identity in case of a mass match with already known venom peptides (Fig. 7). These confirmations can be performed using the same sample spots that have been used for MFPs already. For *de-novo* sequencing of novel peptides, however, we recommend SPITC-derivatization [30] of the peptides prior to MS/MS fragmentation, which supports the presence of y-fragment series and thus facilitates interpretation of MS/MS data (Fig. 8). Assignment of isobaric AAs (L/I, K/Q) is generally possible. First, acetylation of peptides directly on the sample spot leads to esterification of free amino groups, which affects the unblocked amino groups at the N-terminal residue but also the side-chain of K-residues (not shown; for venom peptides [31]). Secondly, generation of side-chain fragments such as *w*-fragment ions supports the differentiation of L/I-residues [32] (Fig. 9). The presence of multiple disulfide bridges is typical of a large number of venom peptides and disulfide bonds can be detected by reduction/alkylation experiments [33] or the use of 1,5-DAN matrix, which causes reduction of cysteine [23,34]. These experimental steps might result in the identification of many of the abundant venom peptides that do not exceed 35–40 AAs. Only if the description of the complete venom profile is the main topic, more time-consuming analysis of HPLC-separated peptide fractions [19,35] or transcriptome analysis of the venom gland [25,36] will become necessary.

In summary, the manuscript describes a very simple protocol to use venom samples of scorpions for species identification, which also considers recent developments in MALDI-TOF mass spectrometry. The protocol was developed with specimens of *Heterometrus cyaneus* that were kept in captivity but later tested under field conditions with scorpions caught in Southern Africa (not shown). In Southern Africa, venom samples from up to 20 specimens per h have been prepared for MFPs, and subsequent analyses of peptide sequences.

Conflict of Interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.017.

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