Journal of Chromatography A, 1374 (2014) 134–144

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A sensitive quantification of the peptide apidaecin 1 isoforms in single bee tissues using a weak cation exchange pre-separation and nanocapillary liquid chromatography coupled with mass spectrometry

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ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form
10 November 2014
Accepted 14 November 2014
Available online 20 November 2014

Keywords:
Antimicrobial peptide
Apidaecin
Honey bee
Nanoflow liquid chromatography
Mass spectrometry
Quantification

ABSTRACT

Apidaecins represent an important group of antimicrobial peptides occurring in honey bee hemolymph, where they play an important role as key components of humoral immunity. The present study demonstrates the development of a highly sensitive assay for apidaecin 1 isoforms quantification in the hemolymph or body parts from honey bee individuals. The analytical protocol comprises apidaecins 1 purification and enrichment steps by weak cation-exchange chromatography (WCX) in laboratory-made WCX-Tip microcolumns combined with a desalting step on a reversed-phase sorbent (C8) carried in StageTips. Apidaecin-enriched fraction was analyzed by a reversed-phase based nanoliquid chromatography (C4) separation coupled with high-resolution mass spectrometry. The method performance was validated in its specificity, linearity (0–5 pmol), recovery (~45%), precision (<10% at 0.1 pmol), limit of detection (~50 fmol), limit of quantification (0.1 pmol) and sample stability. The method was successfully applied to analyze the content of apidaecin 1 isoforms in the following samples: hemolymph – 13.0 ng/μL (95% confidence interval of 7.5–18.6 ng/μL), thoraxes – 36.2 ng/unit (95% CI of 18.9–53.6 ng/unit) and heads – 12.9 ng/unit (95% CI of 9.1–16.7 ng/unit). Freshly emerged bees had apidaecin 1 isoforms levels below the limit of detection. Thus it was possible to use them as a competitive matrix for calibration standards to prevent losses of highly basic apidaecins. This new protocol for apidaecin 1 isoforms quantification represents a promising tool to study the role of apidaecins in honey bee immunity and can be considered as a proof-of-concept for the development of sensitive quantification methods for basic antimicrobial peptides in various organisms.

1. Introduction

Antimicrobial peptides play an important role in humoral immunity of all organisms [1]. They are responsible for the protection against bacteria, fungi and other microorganisms [2]. Their properties and functions in the immune system of many organisms such as plants [3], insects [2,4] and also humans [5] have been widely documented. Due to their recently described immunomodulatory properties, there is a large potential to use antimicrobial peptides also as therapeutics against inflammatory diseases [6] or viruses [7].

Honey bee immunity consists of three levels of defence: (1) physical barriers (the cuticle and the epithelium of the digestive tract), (2) cell immunity (hemocytes) and (3) humoral immunity (a phenol oxidase system, lectins, lysozyme and antimicrobial peptides) [8]. Apidaecins are antimicrobial peptides that were first isolated from the honey bee hemolymph at the beginning of the 1990s [9] (reviewed by Danihlík et al. [10]). Honey bee (*Apis mellifera*) genome analysis showed three types of apidaecin precursor sequences: apidaecins type 73 (Q06602, UniProtKB) encodes apidaecin, apidaecin 1A and apidaecin 1B; apidaecins type 22 (P35581,

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http://dx.doi.org/10.1016/j.chroma.2014.11.041
0021-9673/

UniProtKB) is cleaved into apidaecin 1A and apidaecin 1B and apidaecins type 14 (Q06601, UniProtKB) is composed of apidaecin 2, apidaecin 1A and apidaecin 1B [11]. Up to now, only three isoforms of apidaecin (1A, 1B and 2) have been detected on the peptide level in the honey bee hemolymph [9]. Another isoform named apidaecin 3 (Q06602 UniProtKB, gene name APID73) represents only a prediction from cDNA library [11], which has not yet been detected in honey bee samples. A concentration ratio of 1:20 between apidaecin 1A and 1B was assessed using chromatographic separation and sequence analysis of isolated peptides [9]. The sequences of all apidaecin isoforms [10] are summarized in Table 1S (Suplementary material).

The production of apidaecins is stimulated as a part of responses to pathogen challenge, e.g. bacterial infection. Gram-negative bacteria are more sensitive to apidaecins [11], however there are no significant differences in the antimicrobial activities among known apidaecins [9]. The total average concentration of apidaecin 1A, 1B and 2 in the bee hemolymph was previously reported at the level of 100 ng/μL [9,11].

Apidaecins are highly basic, heat stable and non-helical peptides, which belong to the proline-rich family of antimicrobial peptides [9]. The amino acid sequences of biologically active apidaecins consist of 18 amino acids, of which 16.6% and 33.3% represent arginine and proline, respectively, which contribute to their chemical stability. The presence of several basic amino acids increases the isoelectric point (p*I*) value of apidaecins up to 11.

Peptides are usually separated by reversed-phase high performance liquid chromatography (RP-HPLC) but the retention of arginine-rich peptides is sometimes problematic [12]. Polar arginine side chains interact with residual silanol groups of the column sorbent what results in peak tailing. Mass spectrometry (MS) is a modern and popular method for peptide identification and quantification. Collision-induced dissociation (CID) with a high collision energy is used for basic peptides to acquire fragmentation spectra [13] and thus the quantification of basic peptides by selected reaction monitoring (SRM) is complicated. It is possible to modify arginine side chains at the basic guanidium group to diminish their polarity. This modification helps to focus chromatographic peaks and allows decreasing the collision energy for CID fragmentation [12,14,15], however, such a chemical derivatization can result in multiple products not suitable for quantification [12].

So far, several different methods for the detection of bee antimicrobial peptides based on polyacrylamide gel electrophoresis [16,17], RP-HPLC [9,18,19] and MALDI-TOF MS [20,21] have been employed. Apidaecins were isolated under strong denaturing and acidic conditions from collected honey bee hemolymph samples. Alternatively, quantitative polymerase chain reaction (qPCR) is used for the quantification of expression of apidaecins genes in tissues [22] or whole bee bodies [23,24]. Although there have been studies interested in the expression level of apidaecin or other bee antimicrobial peptides, a reliable absolute quantification method for the whole bee body or separate tissues has been, until now, missing.

Here we present a new analytical approach for the isolation and quantification of apidaecin 1 isoforms (apidaecins 1) in the hemolymph or body parts of a single bee. As mentioned above, two apidaecin 1 isoforms 1A and 1B have been identified in a honey bee hemolymph [9], which differ only in one isobaric amino acid (Leu/Ile) at the peptide C-terminus. As the known biological effects [9,25,26] and described physico-chemical properties of both isoforms are identical, the isoform 1A with the amino acid sequence GNNRPVYIPQPRPPHPRI was selected for method development. The described procedure is based on a combination of reversed-phase weak cation exchange mixed-mode solid-phase extraction and nanocapillary liquid chromatography (nLC) coupled with an ultra-high resolution mass spectrometry (UHR-MS). An isotope dilution strategy with isotopically labelled synthetic standard of apidaecin 1A allowed precise and sensitive quantification of apidaecins 1 (as a sum of both isoforms 1A and 1B) in biological matrices in the low-femtomole to picomole range.

2. Materials and methods

2.1. Chemicals

Standards of apidaecin 1A (purity > 99%) and isotopically [$^{13}C_6{}^{15}N_4$] labelled apidaecin 1A (at the C-terminus; purity > 98%) were synthesized by Clonestar (Brno, Czech Republic). The content of the synthetic peptide standards in solutions was quantified via amino acid analysis performed by the Protein Analysis Group, Functional Genomics Centre, Swiss Federal Institute of Technology, Zürich, Switzerland. The Oasis® WCX sorbent (30-μm particle size) was purchased from Waters (Milford, MA, USA) and the C8 reverse phase extraction discs were from 3M (St. Paul, MN, USA). Gel loader tips (0.5–20 μL), other pipette tips and microtubes were purchased from Eppendorf CZ (Prague, Czech Republic). Water, acetonitrile, methanol, 98% (v/v) formic acid (all of LC/MS grade quality) and trifluoroacetic acid p.a. grade were from Sigma–Aldrich (Steinheim, Germany). 2-Propanol for spectroscopy was from Merck (Darmstadt, Germany). If not otherwise stated, all other chemicals used were from Sigma–Aldrich in analytical purity grade.

2.2. Bee sampling and hemolymph collection

Honey bees (*Apis mellifera carnica*) were collected during the summer of 2012 in a private apiary in the Czech Republic. Freshly emerged bees and hive bees with an undefined age were sampled from the frames within the brood area of the hive. Colonies used as a source of bees had no clinical symptoms of infectious diseases (American and European foulbrood, *Nosema* sp., sackbrood virus and deformed wing virus) and were in a good condition. Bees were transported in cages to the laboratory within 2 h [27], frozen in liquid nitrogen and stored in a freezer at −80 °C until use. Hemolymph samples were collected immediately after bees had arrived to the laboratory.

2.3. Sample pretreatment

Freshly emerged bees were inserted into 2-mL microtubes with ceramic balls and 1 mL of 0.1% trifluoroacetic acid (TFA). Adult bees were dissected into heads, thoraxes and abdomens and the body parts were individually placed into 500 μL of 0.1% (v/v) TFA. Samples were homogenized in a FastPrep FP120 homogenizer (MP Biomedicals, Illkirch, France) at 5 m/s for 20 s. Peptides extraction was done using a modified procedure of Casteels et al. [9]. The homogenate was centrifuged at 15,000 × *g* at laboratory temperature for 10 min, then the supernatant was heated at 100 °C for 10 min, centrifuged again at 15,000 × *g* for 10 min. The clarified supernatant was finally lyophilized overnight (Lyovac GT-2, Leybold-Heraeaus, Germany). Highly basic antimicrobial peptides are very stable at low pH and high temperatures, therefore the heating of the sample was utilized to remove other peptides and proteins by their denaturation [9]. The lyophilizate was stored at −80 °C until next processing.

For freshly emerged bees, the lyophilized samples were reconstituted in 1 mL of 5% (v/v) formic acid (FA) prior to peptide purification, whereas the solid material from body parts was reconstituted in 100 μL of 5% (v/v) FA.

Hemolymph samples were collected from both freshly emerged bees and hive bees with undefined age. Approximately 2.5 μL of hemolymph were collected and dissolved in 100 μL of 0.1%

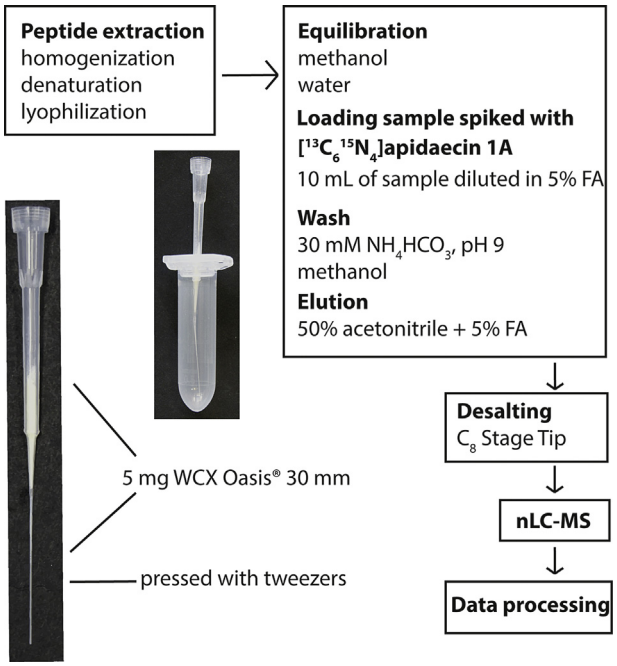

Fig. 1. Scheme of apidaecin 1 purification from honey bees or bee tissue extracts.

(v/v) TFA, processed identically to bee homogenates and resulting lyophilizates were redissolved in 100 μL of 5% (v/v) FA.

2.4. Enrichment of apidaecin 1 isoforms using mixed-mode weak cation-exchange chromatography

Apidaecins 1 from the bee hemolymph or body extracts was enriched using reversed-phase weak cation exchange mixed-mode chromatography on the Oasis® WCX 30 μm sorbent (Waters, USA) packed in laboratory-made tip microcolumns (further referred to as WCX-Tip microcolumns). They were prepared using 20-μL Gel-Loader Tips (Eppendorf) squeezed at the nozzle end by a tweezer. This modified tips were filled up with 5 mg of Oasis® WCX sorbent (see Fig. 1) and the resulting WCX-Tip microcolumns were inserted through holes poked in the tightly closed lids of 2-mL Eppendorf microtubes.

Repeated centrifugations at 1000 × *g* for 2 min were used to allow permeation of liquids through the Oasis® WCX sorbent in the WCX-Tip microcolumns. The sorbent was activated according to manufacturer's instruction [28]. Briefly, ready-to-use WCX-Tip microcolumns were centrifugally washed two times with 50 μL of methanol and equilibrated with 100 μL of water. Aliquots (10 μL) of the samples (hemolymph or bee body homogenate) were diluted in 50 μL of 5% FA and spiked with 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A standard. The complete mixtures of spiked samples were loaded onto prepared WCX-Tip microcolumns.

The bound compounds were eluted sequentially. First, the microcolumn was washed two times with 50 μL of 30 mM NH_4HCO_3 (pH 9, alkalized by conc. NH_4OH) to get rid of acidic compounds, and then two times with 50 μL of methanol to elute neutral compounds and molecules interacting with the reversed phase. Finally, peptides of interest were eluted by 2 volumes of 50 μL of 50% (v/v) acetonitrile containing 5% (v/v) FA. The eluted fractions were pooled and dried using a vacuum concentrator SpeedVac (Eppendorf). Samples for the subsequent nanoflow liquid chromatographic analysis were first desalted using a modified StageTip method [29] with C8 sorbent (3M Empore extraction disks). Briefly, two small C8 discs were cut out by a stainless steel needle with a blunt end and inserted into a 200-μL yellow pipette tip. The tips were then placed into microtubes through a hole poked in its closed lid. The following steps were achieved by centrifugation (1000 × *g*, 2 min): first, the discs were activated with 50 μL of isopropanol and equilibrated with two additions of 50 μL 5% (v/v) FA. The lyophilized eluates from WCX-Tip microcolumn purification were dissolved in 50 μL of 5% (v/v) FA and loaded onto the prepared C8-StageTip. The retained peptides were washed two times with 50 μL of 5% (v/v) FA and eluted by two additions of 50 μL of 50% (v/v) acetonitrile acidified with 5% (v/v) FA. Both eluates were pooled and dried using the vacuum evaporator at 45 °C.

2.5. MALDI-TOF MS analysis

For a fast inspection, MALDI-TOF MS of fractions eluted from WCX-Tip microcolumns was performed on a Microflex LRF20 instrument (Bruker Daltonik) equipped with a microScout ion source and a 337-nm nitrogen laser pulsing at a repetition rate of 60 Hz. α-Cyano-4-hydroxycinnamic acid was used as a matrix – 5 mg/mL in 60% (v/v) acetonitrile containing 0.1% (v/v) TFA. Each inspected fraction was desalted on a C8 StageTip, evaporated until dryness and then redissolved in 5 μL of 0.1% TFA. Then an aliquot (0.6 μL) was overlaid with 0.6 μL of the matrix solution on an MSP Anchor Chip™ 600/96 target plate and left for crystallization. Mass spectra were accumulated from 100 to 200 laser shots in the range of *m*/*z* 500–4000. Peptide calibration standard II (Bruker Daltonik) was used for the external calibration. Mass spectra were processed by flexAnalysis 2.4 (Bruker Daltonik).

2.6. Nanocapillary liquid chromatography and mass spectrometry (nLC–MS)

Identification and quantification of apidaecins 1 was performed on a system comprising a nanocapillary chromatograph (nanoEASY) coupled with an ultra-high resolution Q-TOF mass spectrometer (UHR-QTOF maXis) via nanoelectrospray ion source and controlled by CompassQTOF v 1.4 (all by Bruker Daltonik, Bremen, Germany). Separation of samples was carried out in a one-column setup (i.e. without any precolumn) with an analytical column (75 μm i.d. × 100 mm) ended with an IntegraFrit (New Objective, Inc., MA, USA) and packed with a C4 sorbent (ReproSil-Gold C4, 300 Å, 5 μm, Dr. Maisch; Ammerbuch-Entringen, Germany) in length of 5 cm. Chromatographic mobile phases were as follows: mobile phase A consisted of 0.4% (v/v) FA and mobile phase B consisted of 0.4% (v/v) FA in 90% (v/v) acetonitrile. Dried samples were dissolved in 30 μL of 0.1% TFA, shortly vortexed, sonicated for 5 min and transferred into total recovery glass vials (Waters, USA). The sample (5 μL) was loaded on the column with the intelligent flow control at a pressure of 200 bar and the retained peptides were washed with next 15 μL of mobile phase A under the same conditions. Peptide elution was done with 25-min fast gradient elution (all mobile phase changes were linear, 0 min, 2% B, 3 min, 8% B, 15 min, 65% B, 16 min, 85% B, 21 min, 85% B, 22 min, 2% B, 25 min, 2% B) at a flow rate of 400 nL/min. Each sample was injected twice to check the reproducibility of response during the process of nanoelectrospray ionization.

Full MS spectra were acquired in the positive MS mode in the range of *m*/*z* 350–2200 with a frequency of 0.5 s per MS. Prior to analysis, the mass spectrometer was always calibrated in the *m*/*z* range of 118–2722 (ESI-L TuneMix, Agilent Technologies Part. No: G1969-85000) and tuned for a minimal resolution of 25,000 full width at half maximum (FWHM) using a calibration ion at *m*/*z* 922.0098. Other settings were as follows: Source (capillary voltage 4500 V, desolvation gas 0.4 bar, dry gas 4 L/min, dry temperature 180 °C). All raw data were processed using DataAnalysis software v 4.0 SP5 (Bruker Daltonik). Targeted analytes (apidaecins 1 and the internal standard [$^{13}C_6{}^{15}N_4$]apidaecin 1A) were checked in

a defined retention window of ± 0.5 min. Data analyses for analytes were carried out by extracting high-resolution accurate mass traces (±0.002 Da) concerning the most dominating protonated ion charge state.

2.7. External calibration

To quantify the apidaecins 1 level in the hemolymph or bee body samples, a calibration curve was generated using the standards of apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A diluted in a purified homogenate from freshly emerged bees. Prior to standard addition, the homogenate (5 μL) was diluted in 50 μL of 5% (v/v) FA and loaded onto a WCX-Tip microcolumn. The purification process was identical to that described above for real hemolymph or bee body samples.

A calibration series was prepared using 0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5 and 5 pmol of apidaecin 1A standard. All calibration points were spiked with 1 pmol of [$^{13}C_6{}^{15}N_4$]apidaecin 1A standard in a final volume of 30 μL of 0.1% (v/v) TFA. In addition, all calibration points were prepared in duplicates and each point was measured in three technical replicates. High-resolution extracted ion chromatograms (HR-EIC) were generated as a sum of the two most abundant isotopic peaks ([M+4H]$^{4+}$) of apidaecin 1A at m/z 527.796 plus 528.048 and of [$^{13}C_6{}^{15}N_4$]apidaecin 1A at m/z 530.298 plus 530.549. Areas under extracted ion chromatographic peaks were integrated to get total peak areas. Ratios of peak areas of apidaecin 1A vs [$^{13}C_6{}^{15}N_4$]apidaecin 1A were plotted against spiked amounts of apidaecin 1A (in pmol).

2.8. Quantification of apidaecin 1 isoforms in bee samples

Apidaecins 1 were enriched by the protocol using WCX-Tip microcolumns as mentioned before and analyzed by nLC–MS. Raw data were processed in the same way as for the calibration series and the ratio of peak areas of apidaecins 1 vs [$^{13}C_6{}^{15}N_4$]apidaecin 1A was calculated. The content of apidaecins 1 in the injected sample was estimated from the calibration curve and recalculated to the original bee sample using equations described below. The diluting factor for apidaecins 1 content in homogenates and in hemolymph was 10 because 10 μL from total 100 μL were loaded onto the WCX-Tip microcolumn. In case of the hemolymph, the final concentrations were expressed relative per 1 μL, thus the calculated concentrations need to be divided by the sample volume (2.5 μL). Apidaecins 1 content in homogenates made from bee body parts was calculated according to the following equation: apidaecins 1 (ng) = quantity of apidaecin (pmol) × 10 (diluting factor) × 2107 (molecular weight of apidaecin 1) × 0.001. Apidaecins 1 concentration in bee hemolymph was calculated according to following equation: apidaecins 1 (ng) = [quantity of apidaecin (pmol) × 10 (diluting factor) × 2107 (molecular weight of apidaecin 1) × 0.001]/2.5.

2.9. WCX-Tip microcolumn capacity

The WCX-Tip microcolumn capacity was calculated as the peak ratio of the standards of apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A. The capacity values for bee hemolymph were plotted against the homogenate volume loaded on the column (5, 10, 15, 20, 25, 30 and 40 μL). All homogenate volumes were spiked with the standards at the concentration of 5 pmol of apidaecin 1A and 1 pmol of [$^{13}C_6{}^{15}N_4$]apidaecin 1A.

WCX-Tip microcolumn capacity was also tested for loading the standard of apidaecin 1A on the sorbent. To achieve that, hemolymph samples (10 μL) were spiked with a constant amount of [$^{13}C_6{}^{15}N_4$]apidaecin 1A (1 pmol) and with an increasing amount of the standard of apidaecin 1A (0, 5, 10, 15, 20, 25, 30, 35 and 40 pmol).

2.10. Recovery

To estimate the recovery of the introduced method, a series of differentially spiked samples was prepared. The freshly emerged young bees, which had the endogenous level of apidaecins 1 below the limit of detection, were processed according to the described extraction and purification method. The lyophilizates were dissolved in 1 mL of 5% (v/v) FA. Samples (5 μL each) were spiked with a defined amount of the standards of apidaecin 1A (0.5, 1.0 and 2.5 pmol) and [$^{13}C_6{}^{15}N_4$]apidaecin 1A (0.5, 1.0 and 2.5 pmol). In total, 9 samples with varying amounts of apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A were prepared and each of them was made in four replicates. Additionally, all analyses were performed also in two technical replicates. All these samples were processed using the introduced protocol (WCX-Tip microcolumn plus C8-StageTip chromatography) and analyzed by nLC–MS. To define a 100% peptide recovery, a set of control samples was arranged by a direct dilution of the same amount of the standards of apidaecin 1A (0.5, 1.0 and 2.5 pmol) and [$^{13}C_6{}^{15}N_4$]apidaecin 1A (0.5, 1.0 and 2.5 pmol) into a purified homogenate just before nLC–MS analysis.

2.11. Method validation

A validation for quantitative method parameters was performed under consideration of the following points: precision, accuracy, linearity, sample stability, limit of detection (LOD) and lower limit of quantification (LLOQ).

Samples were processed by sample pretreatment procedure as described in Section 3.3. The lyophilizates of the bee hemolymph and bee body extracts were finally reconstituted in 5% (v/v) FA. The first set of samples was spiked only with 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A standard to estimate the endogenous level of apidaecins 1. The second set of samples was spiked with the synthetic standards of apidaecin 1A (0.5, 1 and 2 pmol) and 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A. Both sets were processed using the introduced protocol and analyzed by nLC–MS. The content of apidaecins 1 was determined by a standard isotope dilution method and used for the method validation. Each sample was analyzed in triplicate.

2.11.1. Precision and accuracy

The precision and accuracy were determined with four independent hemolymph samples and four whole bee homogenates, which were analyzed in two replicates. Both parameters were determined for three apidaecin 1A additions (0.5, 1 and 2 pmol) into the sample and 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A addition. They were also evaluated for bee body homogenates spiked only with the standards of 1 pmol apidaecin 1A and 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A.

2.11.2. Linearity

Four different purified hemolymph samples were chosen for a linearity evaluation within the working concentration range. The samples were spiked with an increasing amount of the standard of apidaecin 1A (0, 0.5, 1 or 2 pmol) and constant amount of 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A. The ratio of apidaecin 1A vs [$^{13}C_6{}^{15}N_4$]apidaecin 1A was plotted against the spiked apidaecin 1A amount (in pmol).

2.11.3. Stability

Pre-treated and lyophilized samples were stored for several weeks at −80 °C until apidaecins 1 purification and analysis. The stability of samples reconstituted in 5% (v/v) FA was tested after the storage at −80 °C for one and two weeks.

2.12. Quantification of proteins

Bradford assay was used for protein quantification in samples [30]. Bovine serum albumin served as a standard.

3. Results and discussion

3.1. Isolation of apidaecin 1 isoforms from a complex biological matrix

The main goal of this work was to establish a highly sensitive assay for the quantification of apidaecins 1 in a single bee tissue. Based on physicochemical properties of apidaecins, a new approach has been developed which combines a solid-phase microextraction of apidaecins 1 using weak cation-exchange chromatography and a reversed-phase purification of the enriched material followed by nLC–MS including a quantification of the desired peptide via stable isotope dilution method (Fig. 1).

Antimicrobial peptides are usually strongly cationic and heat-stable molecules [2]. Apidaecins 1 show a theoretical p*I* value of 11.7 and they strongly bind to a cation-exchange sorbent with negatively charged groups. Ion-exchange chromatography has been utilized over a long time for the separation of various charged molecules including peptides and proteins [31]. Strong and weak cation exchangers (SCX and WCX, respectively) are available, which differ with regard to the pH dependence of the ionization state of their functional groups. SCXs have a constant charge density on their surfaces over a broad pH range, whereas the charge density of WCXs changes with pH.

Mixed-mode reversed-phase/weak cation-exchange chromatography has been successfully introduced for the purification of positively charged proteins or peptides as lactoferrin [32], endostatin [33], vasopressin and desmopressin [34] as well as breast cancer biomarker peptides including the highly basic bradykinin [35]. At the first attempt in this study, a strong cation-exchange sorbent covered with benzenesulfonic acid functional groups (Cation-SR, 3M Empore High Performance Extraction Disks) was applied for the isolation of apidaecin 1A standard. The recovery of apidaecin 1A standard from this sorbent was low and irreproducible because the peptide was strongly retained. Highly basic solutions (0.1 M sodium hydroxide or 0.1% tert-butyl ammonium hydroxide) were tested to increase the recovery but without any success (data not shown).

In further experiments, the mixed-mode sorbent OASIS® WCX (Waters) was found optimal for the enrichment of apidaecins 1. This sorbent contains carboxylic functional groups combined with reverse phase. Apidaecins 1 and other basic substances from acidified sample were successfully purified. Two consecutive washing steps comprising ammonium bicarbonate buffer (pH 9) and methanol were used to remove polar compounds (p$I \leq 9$) and also non-polar compounds. At pH 9, highly basic peptides with p*I* over 11 keep a strong retention on a WCX sorbent [36]. Elution of desired analyte was performed by 50% acetonitrile acidified with 5% FA. Both retention and release of apidaecin 1A standard from the WCX-Tip microcolumn was checked by MALDI-TOF MS and subsequently also by nLC–MS analysis. The standards of apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A were detected only in the eluate fraction (see Fig. 2).

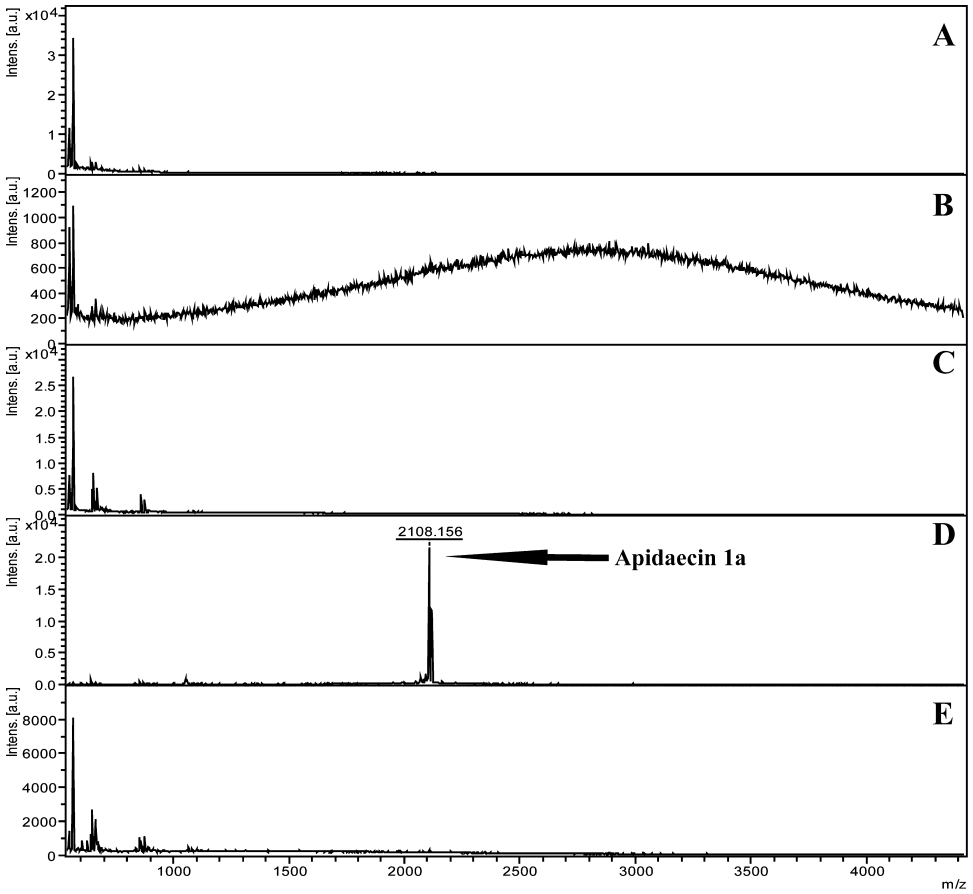

Fig. 2. MALDI-TOF MS spectra show the retention and release of apidaecin 1A from the WCX-Tip microcolumn. The spectra are ordered according to the purification procedure: A – sample loading, B – wash with 30 mM NH_4HCO_3 (pH 9), C – wash with methanol, D – first elution with 50% acetonitrile acidified with 5% FA, E – second elution with 50% acetonitrile acidified with 5% FA. The MALDI-TOF spectra were recorded as described in Section 2.

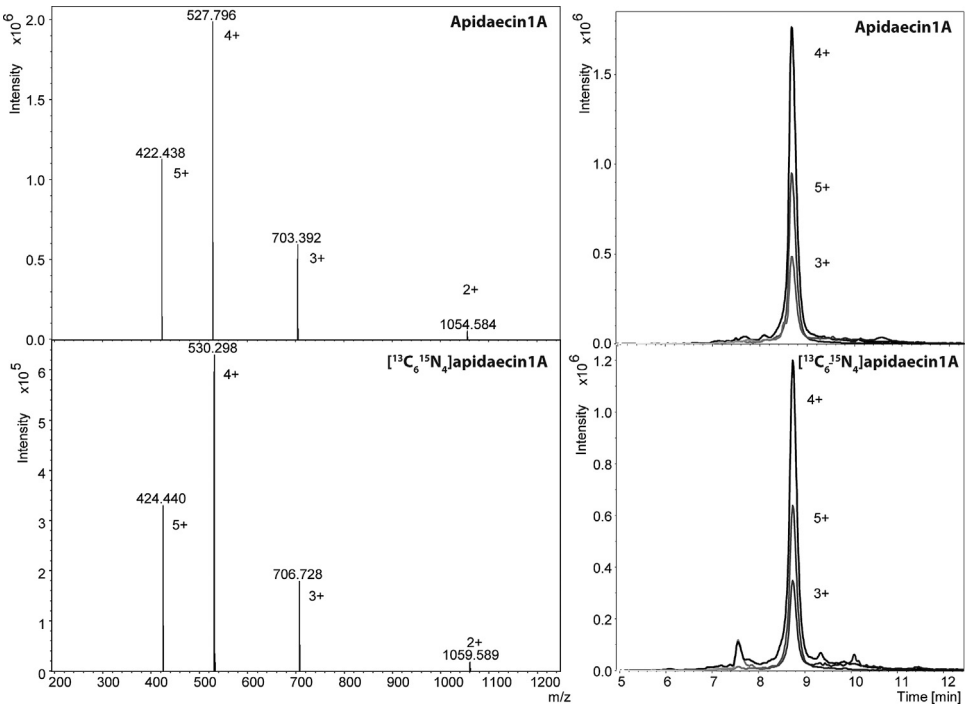

Fig. 3. ESI+ mass spectra of apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A and overlaid extracted ion chromatograms for the most intense charged states: 3+, 4+ and 5+.

3.2. Separation and detection of apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A by nLC–MS

Reversed-phase (RP) liquid chromatography has become a common choice for the separation of peptides and proteins, especially when it is directly coupled to mass spectrometry for the analyte detection. The mechanism of binding of a peptide to RP surface differs from that of small molecules which are subjected to a continuous partitioning between the mobile and stationary phases. Peptides, which are too large to partition into the hydrophobic phase, directly adsorb to the RP surface and remain adsorbed until the concentration of organic solvent reaches the critical concentration level necessary to cause desorption [37].

The separation of the enriched highly basic fraction of peptides was tested on several reversed-phase sorbents differing in the length of the hydrocarbon chain (C18, C8 or C4) and the size of pores (90 or 300 Å). The best resolution and peak shape was achieved using a capillary column packed with the C4 sorbent with large 300-Å internal pores (data not shown).

Apidaecins 1 are highly basic peptides containing three arginines and one histidine. Based on this characteristic we anticipated that a mass spectrum of apidaecin 1A standard after electrospray ionization would contain multiply charged pseudomolecular ions. As expected, such ions were detected in the range from 2+ to 5+. The most intense ions of apidaecin 1A were observed at m/z 703.392 ($[M+3H]^{3+}$), 527.796 ($[M+4H]^{4+}$) and 422.438 ($[M+5H]^{5+}$). A similar distribution of ions was detected also for [$^{13}C_6{}^{15}N_4$]apidaecin 1A at m/z 706.728 ($[M+3H]^{3+}$), 530.298 ($[M+4H]^{4+}$) and 424.440 ($[M+5H]^{5+}$); (see Fig. 3).

Nowadays, SRM has been proved as a very useful, robust and accurate technique for protein and peptide quantification using tandem mass spectrometry (MS/MS) on triple quadrupole-like instruments [38]. This approach is based on the assumption that a pair of a precursor ion and one or more of its most intense and specific fragment ions (which are formed from the precursor ion after the fragmentation process takes place) can be selected for peptide quantification. A new targeted proteomics approach called parallel reaction monitoring (PRM) has been also described as a relevant tool for protein and peptide quantification using quadrupole-equipped high resolution and accurate mass instruments containing Orbitrap or TOF mass analyzers [39]. During PRM, the third analyser of tandem mass spectrometer permits the parallel detection of all target fragment ions in a single high resolution mass analysis. We selected the 4+ and 3+ ions to test the fragmentation of apidaecin 1A standard. As predicted, both the composition (3× arginine, 1× histidine) and high intra-molecular stability of apidaecin 1A limit the fragmentation, especially the $[M+3H]^{3+}$ precursor ion (see Supplementary material Fig. 1S) provided no fragmentation. This might be explained by the sequestration of all incorporated protons by the arginine residues [40]. On the other hand, the $[M+4H]^{4+}$ ion (see Supplementary material Fig. 2S) could be fragmented to some extent, probably due to the proton bound at the histidine residue, which was possibly activated by applying a proper collision energy. This proton "mobilization" was followed by peptide fragmentation [40]. Although the $[M+4H]^{4+}$ could be fragmented, the product ions observed had weak and variable intensities and thus it was difficult to generate a stable single product.

In this work, the high resolution performance of a UHR-Q-TOF maXis mass spectrometer (Bruker Daltonik) was employed. The instrument is able to collect highly resolved full mass spectra in a MS and also MS/MS mode (PRM mode) for checking the isotopic patterns of multiply charged ions and also to extract chromatograms with a very narrow window of m/z 0.01 or less. We tested both approaches for the requested analysis of apidaecins 1 and finally the MS mode was chosen for the protocol development. There were two main reasons for this approach. First, the sensitivity in the MS mode was higher when compared to the PRM approach. Secondly we were always able to confirm the specificity for apidaecin 1A by extracting chromatograms for at least three detected charge states from the series of 2+, 3+, 4+ and 5+ (see Fig. 2). For the quantitative analysis, extracted ion chromatograms for a sum of the two most intense isotopic peaks of the quadruply charged ions of apidaecin 1A (m/z 527.796, 528.047) and [$^{13}C_6{}^{15}N_4$]apidaecin 1A

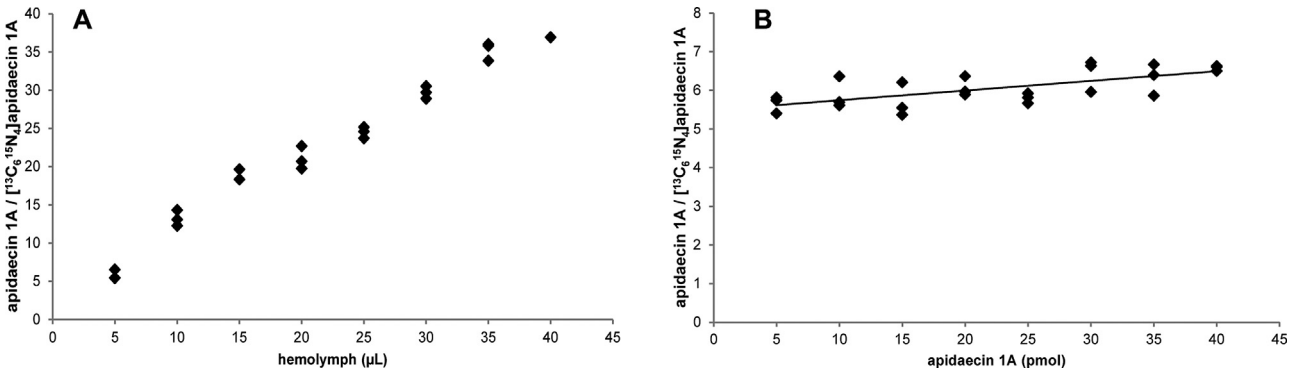

Fig. 4. Determination of column capacity. Increasing pre-treated biological sample (hemolymph) volume was constantly spiked with the synthetic standards of 5 pmol (10.5 ng) apidaecin 1A and 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A (A). 10 μl bee hemolymph was spiked with raising amount of apidaecin 1A (in range 5–40 pmol; 10.5–84.3 ng, respectively) and constant addition of 1 pmol [$^{13}C_6{}^{15}N_4$]apidaecin 1A (B).

(*m*/*z* 530.298, 530.549) were utilized. The accurate quantification of apidaecin 1A was based on applying the appropriate internal standard [$^{13}C_6{}^{15}N_4$]apidaecin 1A, which also allowed to check the recovery of the whole purification process.

3.3. External calibration

Apidaecin 1A standard was found to adsorb very fast on various surfaces. This was particularly inconvenient when working with solutions containing its low concentrations. Thus it was necessary to search for another way how to store samples and to determine the calibration curve for low apidaecins 1 concentration in real samples. The addition of different compounds (free arginine or a tryptic digest of bovine serum albumin) was attempted to stabilize apidaecin 1A in solution but without any success (data not shown). During the development and optimization of this assay, we realized that the freshly emerged bees, processed to homogenates either from the whole bodies or from hemolymph samples, contained endogenous apidaecins 1 at concentration levels below the limit of detection. Thus we anticipated that the biological material could represent a suitable matrix for the dilution of synthetic apidaecins to stabilize their low-concentration working solutions needed to prepare the calibration curve and evaluate the linearity of the method. The final calibration set contained increasing amounts of the synthetic standards of apidaecin 1A (0–5 pmol) in the presence of a constant amount (1 pmol) of [$^{13}C_6{}^{15}N_4$]apidaecin1A. As a result of nLC–MS analyses, the ratio of apidaecin 1A vs [$^{13}C_6{}^{15}N_4$]apidaecin 1A was plotted against the apidaecin 1A amount. The obtained experimental data could be fitted with a linear regression model, with the respective equation of $y = 1.0691x + 0.004$ ($R^2 = 0.9986$).

3.4. Column capacity

The WCX-Tip microcolumns were adapted for the isolation and purification of the desired peptide. This approach called Stop and Go Extraction Tips (StageTips) was originally developed and described by Rappsilber and coworkers [41] and is commonly used for the purification of peptides and proteins in proteomics. The capacity of the WCX-Tip microcolumn was evaluated in two independent steps. First, the spiked biological material (comprising constantly 5 pmol of apidaecin 1A standard and 1 pmol of [$^{13}C_6{}^{15}N_4$]apidaecin 1A standard) was loaded onto the WCX-Tip microcolumn containing 5 mg of Oasis® WCX sorbent in different volume amounts and the results were evaluated. As depicted in Fig. 4A, increasing hemolymph volume loaded onto the WCX-Tip microcolumn did not result in overloading the sorbent capacity. The increasing ratio of apidaecin 1A/[$^{13}C_6{}^{15}N_4$]apidaecin 1A represented increasing endogenous concentrations of apidaecin 1A in loaded sample. Secondly, the WCX-Tip microcolumn capacity was examined for an increasing amount of apidaecin 1A standard spiked to the original bee hemolymph which defined the protein/peptide background. The estimated capacity of 5 mg of Oasis® WCX sorbent was higher than 40 pmol (84.3 ng) of apidaecin 1A standard as it is shown in Fig. 4B. The optimized amount of 10 μL of bee hemolymph was below this capacity limit, which prevented from loses of apidaecins 1 or [$^{13}C_6{}^{15}N_4$]apidaecin 1A standard during the purification process. This setup enables analysis of samples with a potentially high natural apidaecins 1 concentration.

3.5. Recovery

The recovery was calculated as the ratio of peak areas of purified apidaecin 1A standard in the control sample. The recovery of apidaecin 1A standard ranged from 40 ± 3.9% to 55 ± 6.6% (Table 1). A similar recovery in the range from 40 ± 2.5% to 48 ± 4.6% was achieved also for the internal labelled standard [$^{13}C_6{}^{15}N_4$]apidaecin 1A (Table 2S). The recovery values of analytes purified by cation-exchange chromatography from a complex matrix such as human plasma appear between 80% and 100% but may be reduced also to only 30% [42]. On the other hand, the relatively low recovery determined for apidaecin 1A standard was reproducible and independent of its concentration in the sample.

Isotope-labelled standards are used to eliminate systemic and random mistakes which can occur during purification steps [43]. Here the use of [$^{13}C_6{}^{15}N_4$]apidaecin 1A allowed to determine the analyte recovery based on the peak area ratios of the standards of

Table 1
Recovery of apidaecin 1A purification calculated from peak areas and recovery recalculated on internal standard [$^{13}C_6{}^{15}N_4$]apidaecin 1A.[a]

IS (pmol)	Apidaecin 1A (pmol)	*n*	Recovery		Recovery recalculated on IS	
			Average (%)	SD	Average (%)	SD
0.5	0.5	4	54	5.9	85	5.7
	1	4	43	6.0	94	5.6
	2.5	4	55	6.6	90	3.7
1	0.5	4	47	5.1	87	6.5
	1	4	48	8.3	97	4.7
	2.5	4	49	5.1	88	6.6
2.5	0.5	4	42	3.1	100	7.1
	1	4	40	3.9	100	4.2
	2.5	4	50	4.9	91	2.0

[a] IS is synthetic standard [$^{13}C_6{}^{15}N_4$]apidaecin 1A. SD is standard deviation calculated by in-build function of MS excel 2010.

Table 2
Main validation results for apidaecin 1A.*

	Precision (%)			Accuracy (%bias)			Linearity		LOD[c]	LLOQ (%)[d]
	At 0.5 pmol	At 1 pmol	At 2 pmol	At 0.5 pmol	At 1 pmol	At 2 pmol	Slope	Coeff. of corr.		At 0.1 pmol
Hemolymph[a]	7.9	1.5	4.3	−3.0	3.4	−3.6	1.05 ± 0.05	0.99 ± 0.01	50 pg/μL	8.8 (1.6)
Homogenate[b]		8.0			−3.3					

* Apidaecin 1A standard was spiked in a hemolymph at three different concentration and in a complete bee homogenate at single concentration and these samples were used for validation the presented method.
[a] Number of processed hemolymph samples were 4 × 3, total 12.
[b] Number of processed of complete bee homogenates were 15.
[c] LOD represents the limit of detection of the method.
[d] LLOQ represents the lower limit of quantification of the method. The accuracy of established LLOQ at 0.1 pmol level with the precision stated in the bracket.

apidaecin 1A and [$^{13}C_6{}^{15}N_4$]apidaecin 1A. The recalculated recovery values were from 85 ± 5.7% to 100 ± 7.1% compared to the control set of samples.

3.6. Method validation

Validation of the method for quantitative analysis was performed with the internal standard [$^{13}C_6{}^{15}N_4$]apidaecin 1A. The determined analytical parameters for apidaecin 1A quantification are summarized in Table 2. It is evident that the newly introduced procedure of quantification of apidaecins 1 in bee samples is highly accurate and efficient. The method shows excellent specificity as there were no interfering signals in the monitored ion traces in the retention window of the analyzed freshly emerged bees where the concentration of apidaecins 1 appeared below the LOD.

3.6.1. Precision and accuracy

Precision and accuracy were estimated using 4 samples of hemolymph samples containing non-zero endogenous concentrations of apidaecins 1 (3.5, 7.4, 5.5 and 18.4 ng/μL) which were spiked with three amounts of the standard of apidaecin 1A (0.5, 1 and 2 pmol; 1.1, 2.1 and 4.2 ng, respectively). The respective precision values were 7.9, 1.5 and 4.3%. The accuracy was assessed at −3.0%, 3.4% and −3.6% bias for 0.5, 1 and 2 pmol of apidaecin 1A, respectively. Both parameters were also evaluated for 15 whole-bee homogenates where the precision was 8.0% and accuracy −3.3% bias for the addition of 1 pmol of apidaecin 1A standard.

3.6.2. Linearity

The developed method showed excellent linearity, as the experimental data of measured concentrations of apidaecins 1 could be fitted into a linear regression model within the entire working range from 0 to 5 pmol of peptide amount in analyzed samples (which corresponds to 0–42 ng/μL of apidaecin 1A in hemolymph or 0–105 ng per thorax or head, respectively), with a corresponding correlation coefficient (R^2) of 0.99 ± 0.01.

3.6.3. Stability

The high p*I* of apidaecins complicates handling of sample extracts and their purification and analysis. In addition, basic peptides have a high affinity to plastics and glass surfaces [44]. As determined in preliminary studies, it is necessary to avoid loses of the analyte during sample processing and storage before analysis. This has included the selection of the optimal plasticware and glassware for processing samples until they are ready for analytical detection [45]. Pretreated and lyophilized samples can be stored at −80 °C for months. On the other hand, samples reconstituted in 5% FA had to be processed on the same day because of the fast disappearance of target analyte. Lyophilized samples after the C8 desalting step and vacuum concentration were stored at −80 °C for weeks in standard plastic microtubes and it was essential to dissolve them in 0.1% TFA before loading to nLC to avoid further loses of the analyte.

3.6.4. LOD and LLOQ

The LOD concentration was estimated by signal-to-noise ratio of the quadruply charged ion at *m*/*z* 527.796 ([M+4H]$^{4+}$) in defined retention time window. Each signal with S/N of at least 3 considering the dynamic range of the detector (signals < 1 × 10^3 were not considered) was used. In consequence, LOD was established at the concentration level of 50 pg/μL, 24 fmol/μL, respectively.

LLOQ was determined as the lowest concentration which is still possible to quantify with an acceptable precision. At the same time, at least two different charge states of the monitored analyte should be detected (see Supplementary material Fig. 3S). LLOQ was calculated from samples spiked with 0.1 and 0.2 pmol of apidaecin 1A. Each concentration was measured two times in six replicates (n = 6 + 6). LLOQ was established at 0.1 pmol of the standard of apidaecin 1A (see Supplementary material Fig. 4S). The calculated accuracy and precision at this concentration level is 8.8% and 1.6%, respectively.

3.7. Quantification of the natural content of apidaecin 1 isoforms in bee hemolymph and tissue samples using [$^{13}C_6{}^{15}N_4$]apidaecin 1A

Honey bee hemolymph is a complex solution of sugars [46], amino acids [47], lipids [48] and proteins and other biomolecules essential for honey bee physiology. In addition, it contains hemocytes responsible for many vital functions, namely as a cellular component of bee immunity responses [49]. There have been a few papers published describing detection of apidaecins [9] and other antimicrobial peptides in bee hemolymph [18,19]. The first report providing a view on the level of apidaecins in this tissue was described by Casteels et al. [9]. Their method was based on a reversed-phase liquid chromatography with UV–vis detection. Due to the low sensitivity of this method, it was necessary to process hundreds of bee individuals to detect low-abundance antimicrobial peptides apidaecins in bee hemolymph where the determined concentration of apidaecins was about 100 ng per μL of hemolymph [9,18,19].

Several bee pathogens have been reported to influence apidaecin gene expression. *Nosema ceranae* infection induced a down-regulation of the expression of apidaecin and also other bee antimicrobial peptides hymenoptaecin, abaecin and defensin in bee abdomens determined 3 and 6 days post infection [23]. On the other hand, the apidaecin expression was increased in the midgut tissue from *Nosema ceranae*-positive bees [22]. Siede et al. [24] followed the induction of gene expression for four bee antimicrobial peptides abaecin, apidaecins, defensin1 and hymenoptaecin caused by injections of bacterial immunomodulators (lipopolysaccharides, peptidoglycan, lipoteichoic acid) or *Paenibacillus larvae* cells and compared it with that in a control non-treated group.

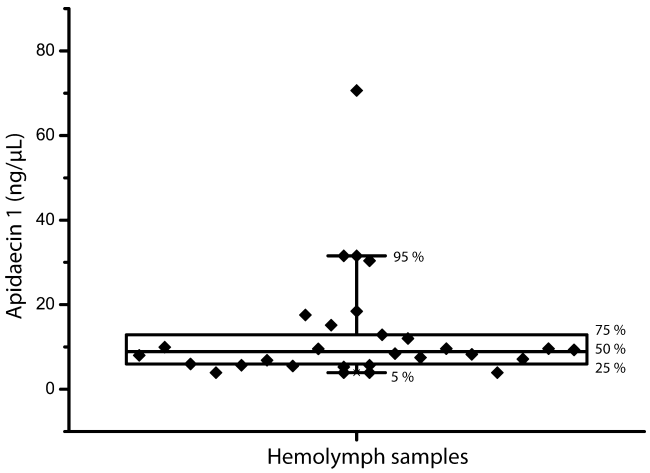

Fig. 5. Apidaecin 1 concentrations in hive bees. The box defines confidence intervals of 25 and 75%; the error bar indicates confidence interval of 5 and 95%.

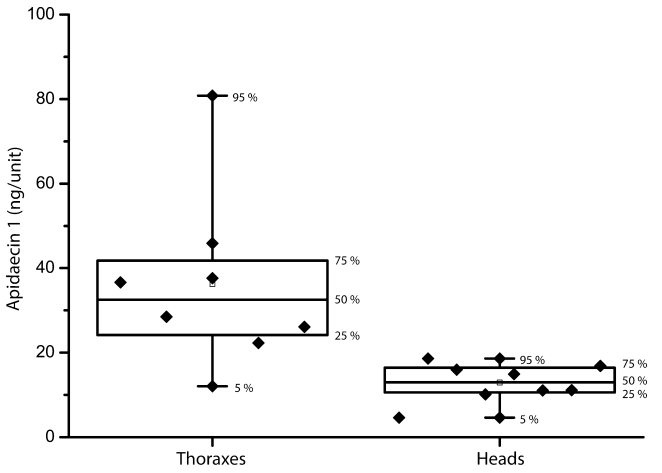

Fig. 6. Apidaecin 1 amount in thoraxes and heads of hive bees. The box defines confidence intervals of 25 and 75%; the error bar indicates confidence intervals of 5 and 95%.

There were no statistical differences observed between the two groups. Surprisingly, an expression of antimicrobial peptides was induced only by a manual manipulation with bees in the laboratory. The antimicrobial activity of hemolymph decreases with the age [50]. Interestingly, Jefferson et al. [22] did not observe any change in expression of apidaecin, hymenoptaecin, defensin 1, defensin 2 and apisimin in fat body tissue of nurse and forager abdomens. The only statistical decrease was reported for the peptide abaecin [22]. The widespread *Varroa* mite (*Varroa destructor*) is a vector of the deformed wing virus (DWV). Its high infestation produces a high virus level in colony [51]. The high titre of DWV then strongly down-regulates the transcription of NF-κB [52], a gene family with an important role in insect immune pathways and antimicrobial peptides expression regulation [53].

To determine the ratio of apidaecins 1 level to total protein level, protein concentration was quantified in honey bee hemolymph samples. The concentration was 11.3 ± 4.72 μg/μL ($n = 28$) for hive bees collected from brood area and 10.0 ± 2.82 μg/μL ($n = 23$) for freshly emerged bees, respectively. Homogenates prepared from freshly emerged bee bodies and body parts were also analyzed for protein concentration. Freshly emerged bee homogenates (total volume 1000 μL) contained 1.14 ± 0.17 μg/μL ($n = 10$), thorax homogenates (total volume 100 μL) 8.4 ± 2.58 μg/μL ($n = 15$) and head homogenates (total volume 100 μL) 6.5 ± 1.3 μg/μL ($n = 17$) of protein.

We have developed a method, which is suitable for quantification of apidaecins 1 in honey bee tissues. This method was validated and then used for a study with different honey bee samples. Freshly emerged bees were sampled either directly from the frame, or hemolymph samples were collected from freshly emerged bees. These samples ($n = 12$) had a very low endogenous concentrations of apidaecins 1, which were below the limit of quantification. However, hive and forager bees ($n = 25$) collected randomly from the brood area in the hive had a quantifiable apidaecins 1 concentration in their hemolymph (95% confidence interval) from 7.49 to 18.55 ng/μL (Fig. 5). The apidaecins 1 amount was 0.12% (mean) and 0.07% (median) from the total protein content in hemolymph. The exact age of the tested adult bees was not determined. For comparison, Nagata et al. [54] quantified peptide HemaP in *Bombix mori* at hundreds of nanograms per mL of hemolymph by ELISA assay. A method based on HPLC-diode array detector – tandem mass spectrometry was optimized for melittin and apamin quantification in bee venom (both are basic peptides with calculated p*I* 12 and 8.55, respectively) [55]. The expression of genes coding for apidaecins is known to be induced in response to microbial infections. In consequence of the multi-copy gene structure, it results in the formation of pre-propeptide molecules which are later processed by a specific proteolysis to various active antimicrobial peptides in the hemolymph – apidaecins 1A, 1B and 2 [9,11]. In contrast to mature bee, the analyzed freshly emerged bees apparently had no direct contact with the external environment, which might explain their non-detectable apidaecins 1 concentrations in the hemolymph. If this results from low apidaecin gene expression or low rate of the pre-propeptide processing to apidaecins needs to be addressed in future studies.

The low level of apidaecins 1 in the hemolymph of freshly emerged bees might be connected with lower immunocompetence of young bees compared to older bees [56]. On the other hand, the quantification of apidaecins 1 in hemolymph samples of hive bees sampled within a brood area demonstrated surprisingly the occurrence of outliers with very high concentrations. Although the exact age of the analyzed bees could not be determined, this result might suggest that those particular bees had potentially been during their life attacked by a microbial pathogen. Another explanation, which has not been investigated until now, might be that concentration of apidaecins 1 increases with the age of bees either as a physiological mechanism or as a result of repeated exposures to pathogen challenges. Nurses are usually more abundant in the centre of the brood area of the hive compared to foragers which perform jobs outside of hives, reviewed by [27]. This phenomenon surely deserves further investigation in studies combining both the assessment of apidaecin gene expression and peptide level during the whole honey bee life span. Apidaecins 1 content was additionally analyzed in bee heads ($n = 8$) and thoraxes ($n = 8$), Fig. 6. The amount of apidaecins 1 was in thoraxes and heads (95% confidence interval) from 18.86 to 53.61 ng per unit and 9.11 to 16.71 ng per unit, respectively. The average weight of thorax and head was determined to 40.2 ± 4.35 mg ($n = 41$) and 12.0 ± 1.81 mg ($n = 41$). Here the results of quantification clearly show both usefulness and universal applicability of the newly developed method regardless of the analyzed tissue type.

Our data with levels of apidaecins 1 in bee tissues provide the first insight into the production of antimicrobial peptides in bees which are bred under natural bee hive conditions. The optimized method, which is now available, can be useful for a future research on humoral immunity of bees. This contribution also offers a possible strategy to design optimized protocols for a convenient analysis of strongly basic peptides occurring in different types of organisms.

4. Conclusion

A complete procedure for the quantification of antimicrobial peptides apidaecin 1 isoforms has been developed and validated. The method comprises processing of bee samples such as whole bodies, body parts or hemolymph including enrichment of basic peptides using weak cation exchange mixed-mode chromatography and a final separation and analysis of apidaecins 1 by nanocapillary chromatography coupled with a high-resolution quadrupole-time-of-flight MS and MS/MS. This approach offers high sensitivity and selectivity and provides the opportunity to trace this peptide in hemolymph or other selected part of a single bee. Sum of apidaecins 1 can be precisely quantified by isotope dilution strategy in the range of 0.1–5 pmol (0–10.5 ng) and the specificity can be achieved by checking at least 2 or 3 different charge states of the analyte. To facilitate absolute peptide quantification, the whole purification and enrichment steps include the use of isotopically labelled synthetic [$^{13}C_6{}^{15}N_4$]apidaecin 1A as an internal standard. The presented protocol allows quantifying apidaecins 1 at the level of tens nanogram per μL of hemolymph or of milligrams of tissue. The first tests with biological samples showed differences in concentrations of apidaecins 1 between freshly emerged bees and hive bees with undefined age. This new method represents a valuable tool for future studies of the role of the humoral components of the immune system of honey bees. This analytical approach can be considered a proof-of-concept for the development of other methods to analyze highly basic antimicrobial peptides.

Conflicts of interest

All authors declare no financial/commercial conflicts of interest.

Acknowledgment

This work was supported by the grant LO1204 from the National Program of Sustainability I by the Ministry of Education, Youth and Sports, Czech Republic.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.11.041.

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