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Liquid chromatography coupled to ion trap-tandem mass spectrometry to evaluate juvenile hormone III levels in bee hemolymph from *Nosema* spp. infected colonies

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ABSTRACT

It has been described a fast, simple and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to measure juvenile hormone III (JH III), which was used to study of the effects of *Nosema* spp. infection on JH III levels in bee hemolymph. Honey bee hemolymph was extracted by centrifugation and mixed with a solution of phenylthiourea in methanol. This mixture was then centrifuged and the supernatant removed and evaporated to dryness. The residue was reconstituted in methanol containing the internal standard (methoprene) and injected onto an LC–MS/MS (ion-trap) system coupled to electrospray ionization (ESI) in positive mode. Chromatography was performed on a Synergi Hydro-RP column (4 μm, 30 mm × 4.60 mm i.d.) using a mobile phase of 20 mM ammonium formate and methanol in binary gradient elution mode. The method was fully validated and it was found to be selective, linear from 15 to 14,562 pg/μL, precise and accurate, with %RSD values below 5%. The limits of detection and quantification were: LOD, 6 pg/μL; LOQ, 15 pg/μL. Finally, the proposed LC–MS/MS method was used to analyze JH III levels in the hemolymph of worker honey bees (*Apis mellifera iberiensis*) experimentally infected with different *Nosema* spp. (*Nosema apis*, Spanish and Dutch *Nosema ceranae* strains). The highest concentrations of JH III were detected in hemolymph from bees infected with Spanish *N. ceranae*.

1. Introduction

Juvenile hormones (JHs) are a class of sesquiterpenoid hormones found in the hemolymph of arthropods that are synthesized and released by the *corpora allata* [1]. These hormones play crucial roles in insect metamorphosis, development, reproduction and behavior, as well as in caste determination, flight, stimulation of migration, diapause regulation, stress resistance and aging [2,3]. Six JH subtypes have been identified and characterized in insects, the most common of which is JH III [4] (see structure in Fig. 1). In recent years, efforts have been centered on identifying the link between JH levels in honey bee hemolymph and behavioral alterations in worker bees infected by *Nosema* spp., which include accelerated behavioral maturation, a decreased tendency to feed the queen and an increased likelihood of foragers not returning to the colony [5]. While a link has been proposed between JH levels and early foraging behavior in *N. apis* infected bees [6], no such analysis has been performed for *N. ceranae*, the most prevalent microsporidium parasite of honey bees and the causal agent of the emergent illness known as nosemosis type C [5]. As in other insect species [7], increases in JH levels induced by miscrosporidia infection could explain the characteristic symptomatology of nosemosis type C observed in some areas of the world [8–10].

Several sensitive bioassays to measure JH have been described [11], of which radioimmunoassays (RIAs) are the most consistent and accurate technique to analyze insect hemolymph [12–15]. However, RIAs require specific antibodies for each JH subtype, and the results of these assays may not coincide with those of physicochemical analyses [16] due mainly to the cross-reactivity of the antiserum with other substances present in biological samples. Moreover, LC–MS/MS appears to be slightly more sensitive than RIA in determining JH titers [17] and as JH forms present in biological samples can be identified by LC–MS/MS, it may be more selective than RIA. However, as only one JH isoform (JH III) was analyzed here and in the previously mentioned study [17] the issue of sensitivity remains to be confirmed.

Gas chromatography coupled with mass spectrometry (GC–MS) is considered to be one of the most precise and accurate techniques

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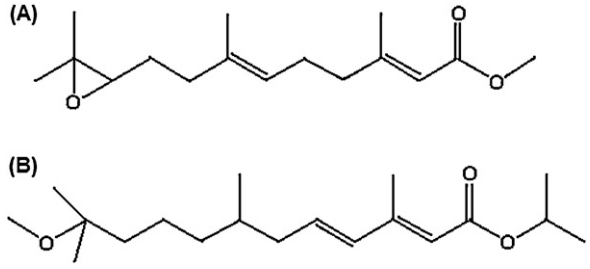

Fig. 1. Chemical structures of (A) JH III ($C_{16}H_{2603}$; MW 266.38) and (B) methoprene ($C_{19}H_{34}O_{33}$; MW 310.47).

to identify and quantify distinct JH homologs [18–20]. However, sample preparation for this type of analysis is often complex and tedious (involving sample purification prior to analysis by thin layer chromatography [TLC] or liquid chromatography [LC]), requiring large amounts of organic solvents and producing volatile derivatives, which affect the detection of JH degradation products.

Liquid chromatography has been used to identify JHs in several biological matrices [17,21–27] in combination with UV detection (due to the strong absorption of JH [21,22]) or more commonly MS, which can detect lower concentrations [17,23–27]. The coupling of LC to MS provides the same degree of accuracy as GC–MS but with the advantage of a shorter sample preparation time, and as no derivatization is required it is possible to simultaneously detect and quantify JH isomers. This technique was first used to measure JH III in the hemolymph of various insects, although not in bees [23], and it has since been used on several occasions to analyze JH in insect hemolymph [17,24,25]. However, the fragmentation of JH III in standards (*m*/*z* 235) generally differs from that observed in hemolymph (*m*/*z* 289, sodium adduct ion) [17,23–25].

It has been suggested that JH III sodium adduct ion (*m*/*z* 289) is detected as a base peak due to the high abundance of sodium ions in insect hemolymph [23], although metal ion traces in the analysis buffer have also been proposed as the source of sodium ions [17]. Several studies have described comparable fragmentation in both the standards and the matrix [26,27]. In the latter study, a newly validated LC–MS/MS method was used to analyze JH III and ecdysone in bees (queen larvae and drone pupae) [27], although no internal standards were used, and the matrices analyzed were bee larvae and pupae rather than hemolymph. Thus, it has been developed a new LC–MS/MS method to detect very low levels of a specific isoform of the juvenile hormone (JH III) in honey bee hemolymph, and optimized the treatment of the samples to ensure good recovery in a fast, simple and cost-efficient manner. The main goal of the present study was to validate this method and use it to analyze JH III levels in the hemolymph of uninfected *Apis mellifera iberiensis* worker bees and bees infected with *Nosema* spp. (*Nosema apis*, and Spanish and Dutch *Nosema ceranae*).

2. Materials and methods

2.1. Materials and chemicals

Juvenile hormone III (93%, JH III), phenylthiourea 99% (99%, PTC) ammonium formate (97%), and methoprene (internal standard; IS) were obtained from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). LC-grade methanol, isopropanol and dichloromethane were supplied by Lab-scan Ltd. (Dublin, Ireland). Syringe filters (17 mm Nylon, 0.45 μm) were purchased from Nalgene (Rochester, NY, USA) and deionized water was obtained from a Milipore Mili-RO plus system and a Mili-Q system (Bedford, MA, USA). A 5810R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany) and an oscillating Selecta Vibromatic stirrer (Barcelona, Spain) were also used. A six-port sample concentrator was purchased from Alltech Associates (Deerfield, IL, USA).

2.2. Preparation of standard solutions

Individual standard stock solutions (200,000 pg/μL, juvenile hormone and methoprene) were prepared in methanol, and an intermediate standard solution was prepared by combining aliquots of each standard stock solution, which were then diluted with methanol to produce a set of working standards. Matrix-matched standards were created by treating with 52 μL of hemolymph–methanol–PTC solution from uninfected worker bees with the sample. The dry extract was reconstituted in a methanol solution containing 1650 pg/μL of the IS (methoprene) and varying concentrations of JH III. To obtain a representative and homogenous pool of hemolymph samples with which to prepare the matrix-matched standards, bee hemolymph from 30 control bees was mixed and diluted with an adequate volume of methanol–PTC (100 pg/μL) solution. To perform matrix effect studies, the matrix-matched standards were used to construct an external standard calibration curve to compare the parameters of linearity with the standard solution calibration curves. All standards and stock solutions were stored in the freezer (−20 °C), where they were stable for over 4 weeks. Dichloromethane was used to remove methoprene from the recipient walls, as described previously [28].

2.3. LC–MS/MS system

For LC, an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC/MSD Trap XCT (G2446A) instrument was used in conjunction with electrospray ionization (ESI, G1948A) in positive ion mode. The LC instrument was equipped with a vacuum degasser (G1379A), a quaternary solvent pump (G1312A) and an autosampler (G1313A) with a thermostated column compartment (G1316A). The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and MSD Trap Control version 4.2. Data was analyzed using Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies (Palo Alto, CA, USA).

Chromatography was carried out on a Synergi Hydro-RP 4 μm, 80 Å column (30 mm × 4.60 mm i.d.), which was protected by a C_{18} guard column (4 mm × 2.0 mm i.d.), both from Phenomenex (Torrance, CA, USA). After the optimization study, the mobile phase components selected were ammonium formate (20 mM, pH 6.5; solvent A) and methanol (solvent B), applied at a flow rate of 0.5 mL/min in the following gradient: (i) 0 min (A–B, 35:65, v/v); (ii) 5 min (A–B, 15:85, v/v); (iii) 6 min (A–B, 5:95, v/v); (iv) 10 min (A–B, 5:95, v/v); (v) 12 min (A–B, 30:70, v/v); and (vi) 14 min (A–B, 35:65, v/v). A linear gradient was used between each of the segments, except for segments 3 (6 min) and 4 (10 min), where the mobile phase was maintained isocratic. The total run time was 14 min, with a 5-min equilibration step. The injection volume used was 20 μL and the column temperature 35 °C.

All ESI-MS/MS analyses were performed using multiple reaction monitoring (MRM) in ultra scan mass range mode, scanning from *m*/*z* 100 to 300 and *m*/*z* 100 to 350 for JH and IS, respectively. ESI-MS/MS detection conditions were optimized using segments for each compound. With the exception of some fragmentation parameters (Table 1), all the parameters studied had comparable effects on sensitivity. Optimal MS/MS conditions were set as follows: capillary voltage, +3500 V; drying gas (N_2) temperature, 250 °C; Nebulizer pressure, 30 psi; trap drive, 35; skimmer, 40 V: octopole RF amplitude, 130 V; capillary exit, 105.0 V; fragmentation scanning, 30–200%; isolation width (*m*/*z*), 4.0; fragmentation width (*m*/*z*), 10.0; max. accumulation time, 200 ms; ion charge

Table 1
ESI-MS/MS parameters in MRM mode.

Compound	Quantification transition	Confirmation transitions	Drying gas flow (N_2, L/min)	Fragmentation cut off	Fragmentation amplitude (V)
JH III	267.1 $[M+H]^+ > 235.0$ $[M+H-CH_3OH]^+$	267.1 $[M+H]^+ > 217.1$ $[M+H-CH_3OH-H_2O]^+$ 267.1 $[M+H]^+ > 249.1$ $[M+H-H_2O]^+$	12	68	0.8
Methoprene (IS)	279.0 $[M+H-CH_3OH]^+ > 237.0$ $[M+H+CH_3-C-CH_3]^+$	279.0 $[M+H-CH_3OH]^+ > 191.1$ $[M+H+(CH_3)_2C-H_2O-CO]^+$	7	72	1.0

control (ICC), 200,000; delay, 5 ms. To minimize potential interference of the matrix, the MSD Trap system automatically controlled the run at different times: at $t_{0–2.5\,min}$ to waste, $t_{2.5–9.8\,min}$ to mass and $t_{9.8–14\,min}$ to waste. The MS/MS transitions monitored for each compound are summarized in Table 1. Standard calibration curves obtained with an internal standard were used for quantification, and the relative response factors for the most intense MS/MS JH transitions were compared with those of methoprene. These calibration curves were constructed by plotting the ratio of the JH signal (areas) to the IS in the *y*-axis, against the concentration values in the *x*-axis. A weighting factor of $1/x^2$ was applied to the linear regression analysis and a second MRM transition monitored for confirmatory purposes. During integration a Gauss function was used as a smoothing algorithm.

2.4. Honey bee hemolymph samples

Several methods were used to extract hemolymph from insects [26,29–31]: from the whole body [26] via puncture between the second and third abdominal segment [29], from the *corpora allata* [30], or following dissection of the insect wings or antennae [31]. After testing each of these techniques, the latter was chosen [31] for its simplicity and good results. Briefly, bees were frozen and their mouth parts were glued shut to prevent any possible contamination. Next, the distal ends of the antennae were clipped with scissors and the internal organs removed to determine their infection status. Each bee was placed upside down in a centrifuge tube and spun at 10,000 rpm for 30 s, inducing the exudation of hemolymph from the cut ends of the antennae. Subsequently, 2 μL of hemolymph was diluted with 50 μL of a methanol–PTC (100 pg/μL) solution, a step not included in the protocol of Mayack and Naug [31], and the samples were finally stored at −20 °C.

2.5. Experimental Nosema infection

Honey bees were experimentally infected with *Nosema* spp. as described previously [32]. To collect *Nosema* spores for experimental infection, adult honey bee workers were gathered from naturally infected colonies located at the Centro Apícola Regional of Marchamalo (CAR), Central Spain (Spanish *N. ceranae* strain), or they were kindly donated by Dr. Van der Zee from the Netherlands Centre for Bee Research (Dutch *N. ceranae* strain and *N. apis*). Nosema infection was confirmed by PCR as described previously [33] and *Nosema*-free bees, confirmed by PCR, were obtained from three uninfected colonies of *Apis mellifera iberiensis*. Frames of capped brood were kept in an incubator (Memmert, Germany) at 34 ± 1 °C. Emerging worker bees were removed randomly and groups of 25 bees were confined to 4 different cages that were kept in the incubator for 5 days at 33 ± 1 °C. The bees were fed ad libitum with a sucrose solution (50% (w/w) in distilled water) combined with 2% Promotor L (Laboratorios CALIER, Barcelona, Spain), a commercial mixture of amino acids and vitamins [33]. Experimental infection of 5-day-old *Nosema*-free bees was carried out as described previously [34]. Briefly, bees were starved for 2 h prior to infection and then fed individually with 2 μl of 50% sucrose solution containing 50,000 fresh spores/μl of *N. ceranae* (Spanish or Dutch strain) or *N. apis* (1 cage of 25 bees per strain). To administer the correct dosage, honey bees were anesthetized with CO_2 to facilitate handling. Upon regaining consciousness, a droplet of 50% sucrose solution mixed with the spores was administered to each bee by placing the micropipette against the bee's mouthparts until the entire droplet was consumed. Uninfected control bees were fed with 2 μl of the 50% sucrose solution (1 cage of 25 bees). Hemolymph was extracted from 5 bees per cage on four consecutive days after infection, using the extraction procedure described above.

2.6. Sample preparation

Hemolymph extraction conditions were determined after conducting the optimization studies described below. Briefly, solutions containing 2 μL of worker bee hemolymph diluted in 50 μL of the selected solvent mixture (methanol–PTC, 100 pg/μL) were thawed at room temperature and transferred to an eppendorf vial. The mixture was centrifuged for 10 min at 8500 rpm and 10 °C, and the supernatant was removed and dried under a stream of N_2. The extract was then reconstituted with 50 μL of a solution containing 1650 pg/μL of methoprene (IS) in methanol and injected onto the LC–MS/MS system.

2.7. Method validation

Validation was carried out according to the U.S. Food and Drug Administration (FDA) Guidance for Industry (Validation of Bioanalytical Methods) [35] and the International Conference on Harmonization (ICH [36]), determining selectivity, ruggedness, the limits of quantification and detection, as well as linearity, precision and trueness.

To determine the selectivity of the proposed method, a set of non-spiked worker bee hemolymph samples ($n = 6$) were injected onto the LC–MS/MS system, and the results compared with those obtained from standard JH III solutions.

Ruggedness tests were performed to determine the effects of minor variations in the extraction procedure, which are expected when preparing fresh solvents and mobile phases. We also studied the effect of the amount of PTC added to the dilution solution on the JH III concentration. Finally, we assessed the stability of JH III in standard solutions over 4 weeks at the different temperatures (−20 °C, 4 °C, 25 °C and 35 °C) and light (light or darkness) used during the method's development.

As matrix and sample treatment did not significantly affect the analyte signal, the detection (LOD) and quantification limits (LOQ) were determined experimentally by measuring the magnitude of the background analytical response to quantify the transition at the retention time of JH III of methanol and JH III solutions in methanol. The LOD and LOQ were estimated as 3 and 10 times the signal-to-noise ratio, respectively. It should be noted that these limits were determined in real samples by making progressive dilutions of hemolymph samples extracted from uninfected bees and determining the concentrations via calibration curves.

Standard calibration curves were used to quantify JH III levels in worker bee hemolymph, since the matrix and sample treatment did not affect the analyte signal. The standard solutions

used to construct the calibration curve were made by preparing different methanol solutions containing the equal amounts of IS (1650 pg/μL) and variable amounts of JH III over a concentration range of 15–14,562 pg/μL (calibration levels of 15, 40, 108, 288, 768, 2048, 5461, 14,562 pg/μL), maintaining a final volume of 50 μL. These solutions were used to reconstitute the dry honey bee hemolymph extracts obtained after treating the selected samples, and finally, a 20 μL aliquot of these solutions was injected onto the LC–MS/MS system. Standard calibration curves were constructed by plotting the concentration against the ratio of the JH III to IS peak areas.

Reproducibility and intermediate precision were assessed by repeated sample analysis, using hemolymph samples obtained on the same day ($n = 5$). A calibration curve was generated for each run, and 5 replicates were analyzed for each of the 4 spiking levels (40, 650, 5000 and 14,000 pg/μL). JH III recovery was determined in 5 replicates at 4 concentrations (40, 650, 5000 and 14,000 pg/μL).

For reproducibility, precision and matrix effect studies bee hemolymph samples were prepared as described for the calibration samples. Hemolymph samples for recovery studies were prepared as described above and spiked after treatment, while the samples used to study the influence of sample treatment were spiked before treatment. Briefly, 50 μL methanol solutions containing different amounts of JH III were prepared and used to spike bee hemolymph solutions containing 2 μL of worker bee hemolymph diluted in 50 μL of the selected solvent mixture (methanol–PTC, 100 pg/μL). Next, these hemolymph mixtures were treated and the dry extracts reconstituted with 50 μL of a solution containing 1650 pg/μL of methoprene (IS) in methanol. The amounts of JH III added to the initial spiking solutions were calculated taking into account the volume used to reconstitute the dry extracts in order to obtain the same final concentrations of JH III (40, 650, 5000 and 14,000 pg/μL).

3. Results and discussion

3.1. Sample preparation

To date, chromatographic analyses of honey bee JH have predominantly used a methanol–isooctane mixture [16,18–20], which allows the complete precipitation and subsequent separation of proteins. Following this method, 2 μL of honey bee hemolymph was diluted with 50 μL of an isooctane–methanol mixture (1:1, v/v), and the mixture was then vigorously and mechanically stirred for 30 s, and allowed to stand at room temperature for 30 min. After centrifuging the mixture for 30 min at 8500 rpm, the upper phase containing the isooctane was separated and transferred to a new vial. The methanol phase was stirred and centrifuged (10,000 rpm for 30 min) and the upper phase was combined with the initial isooctane phase. This isooctane solution was concentrated to dryness under a stream of N_2 and the residue was reconstituted with 50 μL of methoprene (1650 pg/μL) in methanol, and then transferred to a vial for immediate injection onto the LC–MS/MS system.

As the initial chromatograms were not sufficiently clean and the sample processing time was excessively long, trials were performed to optimize the protocol by varying the centrifugation and stabilization times, and the extraction solvent used. Comparable results were obtained after decreasing the stabilization and centrifugation times to 5 and 10 min, respectively, thereby decreasing the overall sample treatment time. The same procedure was then performed without repeating the phase separation, resulting in a higher recovery due probably to the shorter treatment time and reduced sample loss.

To identify the best solvent for hemolymph dilution, we tested methanol, methanol:isooctane (1:1, v/v) and methanol–PTC

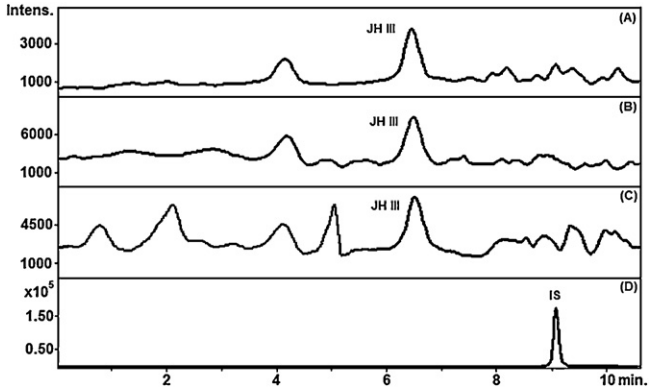

Fig. 2. MRM chromatograms from uninfected honey bee hemolymph samples dissolved in (A) methanol, (B) PTC in methanol (100 pg/μL) and (C) methanol:isooctane (1:1, v/v). (D) MRM chromatogram showing the signal obtained for the IS (methoprene, 1650 pg/μL). The LC–MS/MS conditions are described in Section 2.3 and Table 1.

(100 pg/μL), the latter of which has been proposed to favor the stabilization of JH III in hemolymph [37]. In all hemolymph samples diluted with methanol:isooctane, a precipitate was observed following the centrifugation step, which had to be removed before continuing to treat the sample. This precipitate was found in only some samples (30%) diluted with the other two solvents, and no further differences were observed with respect to the initial protocol. After injection of the extracts onto the LC–MS/MS system, a JH III peak was detected at 6.5 min in all cases (Fig. 2), evident in the MRM chromatograms obtained for hemolymph samples from uninfected worker bees diluted with each of the three solvents. The strongest signals were obtained for hemolymph samples diluted in methanol–PTC. This solvent provided a slightly stronger signal, a cleaner MRM chromatogram and results that were more concordant with the levels of JH III expected by technicians at the CAR, when compared with methanol:isooctane. An added advantage of PTC was its stabilizing effect, which favored JH III measurement. Based on these observations the methanol–PTC solution was chosen as the dilution solvent.

To assess the efficiency of the sample treatment proposed, we compared the results obtained for bee hemolymph samples spiked with four different JHIII concentrations, either before or after sample treatment, with those obtained for the same JH III concentrations diluted in methanol. The resulting recovery ranged from 94% to 103% in all cases, indicating that the sample treatment procedure selected was adequate (Table 2). In the chromatograms, the JH III peak was resolved at the baseline and had a good shape, as did the IS peak (Figs. 2 and 3).

3.2. LC conditions

The first trials were devoted to the selection of the most suitable stationary phase to analyze JH III. In previous studies [17,24–26], separation was usually carried out in reverse phase mode using C_8 and C_{18} columns, with a water/methanol mobile phase. A series of experiments were performed with these stationary phases using individual and mixed standards of JH III and methoprene. Methoprene was selected as the IS to improve MS/MS reproducibility based on several previous studies [17,23,24,26]. Moreover, a salt (ammonium formate) was used instead of water in the mobile phase to improve chemical ionization and thus, to facilitate mass spectrometry detection.

No significant differences between stationary phases were observed in terms of retention or resolution. However, the Synergi Hydro-RP analytical column was selected as the stationary phase

Table 2
Precision and recovery data obtained for uninfected bee hemolymph samples spiked: (A) after or (B) before, the treatment with the procedure described in Sections 2.5, 2.6 and 3.1.

Compound	Spiking level (pg/μL)	Precision studies (n = 5)				Recovery studies (n = 5)					
		Repeatability		Intermediate precision		Evaluation of the matrix effect (A)			Evaluation of the sample treatment effect (B)		
		Measured concentration (pg/μL)	RSD (%)	Measured concentration (pg/μL)	RSD (%)	Measured concentration (pg/μL)	Recovery (%)	RSD (%)	Measured concentration (pg/μL)	Recovery (%)	RSD (%)
JH III	40	40	5	41	4	41	102	5	39	98	5
	650	670	4	665	4	675	104	4	661	102	4
	5000	4800	5	4730	4	4750	95	4	4700	94	5
	14,000	14,200	4	14,180	4	14,150	101	5	14,400	103	5

of choice based on the shorter analysis time under optimal conditions. The mobile phase flow rate was set at 0.5 mL/min as higher flow rates were not suitable for MS/MS. Acetonitrile was also tested as an organic solvent but it gave in a poorer shape and signal for JH III and IS peaks than methanol. A gradient elution was selected in order to decrease the chromatography run time and to obtain the best resolution between peaks (see Section 2.3).

Other parameters such as ionic strength and pH were evaluated to determine the most appropriate mobile phase composition. When varying concentrations of ammonium formate were analyzed (10, 20 and 30 mM), the salt concentration did not affect peak shape or retention time, although concentrations of 20 mM produced the most intense MS signal. The influence of pH on analyte retention was also studied by adding acetic acid or ammonium hydroxide to the mobile phase. The pH range studied was 1.5–7.0, which corresponded to the stable pH range of the column. As no effect of pH on retention was observed, the working pH chosen was that of 20 mM ammonium formate (pH 6.5).

Once the mobile phase composition had been optimized, the influence of the injection volume and temperature was assessed. Analysis of injection volumes from 10 to 30 μL revealed no significant improvement in the signal to noise ratio as compared with that obtained with 20 μL, but a loss of symmetry and peak shape was observed. Over the range of temperatures tested (25–55 °C), a slight decrease in retention time was observed with increasing temperature. However, at temperatures above 35 °C, the decrease in retention time did not compensate for the loss of peak symmetry.

Using the chromatography conditions described above, the overall run time was shorter (5–15 min) than that described in most methods previously used to analyze JH III in insect hemolymph and related matrices [17,23–26]. While one study reported a slightly shorter retention time for JH III from queen bee larvae and drone pupae samples (≈1 min) [27], no internal standard was included and the matrix analyzed was different to that used in the present study. As evident in the MRM chromatograms obtained using optimized LC conditions (Figs. 2 and 3), the peaks were perfectly resolved and exhibited good symmetry.

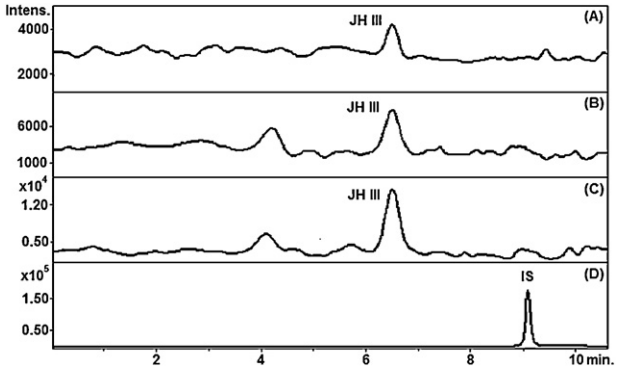

Fig. 3. MRM chromatograms obtained from (A) JH III solution in methanol prepared at LOQ level (15 pg/μL), (B) an uninfected honey bee hemolymph sample (70 pg/μL) and (C) a hemolymph sample from a honey bee infected with *Nosema ceranae* (Spanish strain; 130 pg/μL). Both hemolymph samples were collected on the first extraction day. (D) MRM chromatogram showing the signal obtained for the IS (methoprene, 1650 pg/μL). The LC–MS/MS conditions are described in Section 2.3 and Table 1.

3.3. MS/MS optimization

To define the optimal MS/MS conditions for JH III and IS, individual standard solutions of both analytes in methanol (1000 pg/μL) were introduced directly into the MS/MS in infusion mode, using a syringe infusion pump at a flow-rate of 5 μL/min and ESI in positive mode. In ESI-MS, JH III exhibited the most intense $[M+H]^+$ (m/z 267.1), while $[M+H–CH_3OH]^+$ was most intense for the IS (m/z 279.1). Thus, these ions were selected as precursor ions to generate the product ions in MRM mode (Table 1).

A flow injection method was employed to ensure maximum sensitivity for JH III. Accordingly, 20 μL of a mixed standard solution of JH III and IS (1000 pg/μL) was introduced into the mass spectrometer in the initial chromatography mobile phase at 0.5 mL/min, and optimizing the following parameters: drying gas temperature, drying gas flow, nebulizer gas pressure, trap drive, capillary exit, capillary voltage, skimmer, delay time, ion charge control (ICC), fragmentation amplitude and isolation width (the selected optimal conditions are indicated in Section 2.3).

The proposed regulations (U.S. FDA and E.U.) using the MRM mode as the analytical method for confirmatory LC–MS/MS state that the accuracy determined for an analyte using one ratio must be within 10% of the absolute value produced by an authentic standard. However, if two ratios are used, as in the present study, this margin increases to 20%. To confirm the identity of the JH III, the ratios of the ion transitions relative to the quantification transitions in standard solutions, and in uninfected honey bee hemolymph samples at four different spiking levels were defined (Table 3). In all cases, the transition intensity ratios for the hemolymph samples were in close agreement with the ratios generated by JH II standard solutions. For all samples listed in Table 3, the accuracy between standard and hemolymph samples was between 2% and 6%. This confirmatory method was robust, as similar ratios and degrees of accuracy were obtained at the different levels of spiking assayed (Table 3). The mass spectra of the most intense MRM transition for JH III in the standards and hemolymph samples were also determined to compare these with previous studies [17,23–27]. The resulting mass spectra (Fig. 4) confirmed that the major ions obtained were the same in both cases, although some differences in the intensity of minor ions such as m/z 211, 251 and 252 were observed, possibly due to the influence of certain matrix

Table 3
Confirmation of JH III in honey bee hemolymph samples ($n = 5$). The response for JH III using the three MRM transitions shown in Table 1 were acquired, and the ratios of peak areas relative to that for the quantification transition (m/z 267.1 > 235.0) are shown for (A) uninfected honey bee hemolymph samples and (B) JH III solutions in methanol at different spiking levels. Prior to calculate the ion transition ratios all the areas were divided the area of the IS. The values shown are means ± SD with (%RSD).

Spiking level (pg/μL)		Transition 1 267.1 > 235.0	Transition 2 267.1 > 217.1	Transition 3 267.1 > 249.1
40	A	1	0.24 ± 0.02 (2.1)	0.45 ± 0.01 (1.8)
	B	1	0.23 ± 0.02 (1.7)	0.44 ± 0.01 (2.2)
650	A	1	0.25 ± 0.03 (2.7)	0.46 ± 0.02 (2.3)
	B	1	0.24 ± 0.01 (2.0)	0.44 ± 0.02 (1.5)
5000	A	1	0.25 ± 0.02 (1.9)	0.47 ± 0.03 (2.8)
	B	1	0.26 ± 0.01 (2.2)	0.45 ± 0.01 (1.8)
14,000	A	1	0.24 ± 0.04 (2.5)	0.46 ± 0.03 (1.9)
	B	1	0.25 ± 0.02 (1.7)	0.45 ± 0.02 (2.1)

components. Importantly, the JH III sodium adduct ion (m/z 289) was not observed in any of the mass spectra.

3.4. Validation of the LC–MS/MS method

To determine the selectivity of the proposed method, a set of non-spiked hemolymph samples was injected onto the LC–MS/MS system. No interference of the matrix compounds was detected on the time of analyte elution. As it is impossible to obtain bee hemolymph without JH III, the mass spectra of the JH III peak in the standard and bee hemolymph solutions were compared (Fig. 4). Both mass spectra were quite similar, although as previously reported in Section 3.3, some minor differences in ion intensity were observed and certain ions only appeared in the JH III spectrum. Moreover, the ratios of the selected ion transitions were successfully compared (Table 3).

Several tests were conducted to assess the ruggedness of the proposed LC–MS/MS method. First, the influence of slight variations in mobile phase components was studied. The chromatographic peak parameters (area, width and retention time) for JH III were analyzed when using fresh and old solvents, pre-prepared and recently prepared ammonium formate, and ammonium formate with minor variations in pH and concentration. In all cases, no significant variations in any of the JH III peak parameters were observed. The influence of PTC concentration on the JH III signal was also studied yet no differences in the analyte signal were detected in solutions with PTC concentrations ranging from 95 to 105 pg/μL. The stability of JH III was also tested in standard solutions over 4 weeks at both low (40 pg/μL) and high (14,000 pg/μL) concentrations, and in the different temperatures (−20 °C, 4 °C, 25 °C and 35 °C) and light (light or darkness) conditions employed during the development of the method. The ratio of areas between the JH III and the IS for pre-prepared and freshly prepared solutions stored as described above were compared. Only a slight variation was observed between the areas at the two different concentrations, and in all cases the ratios were close to 100%.

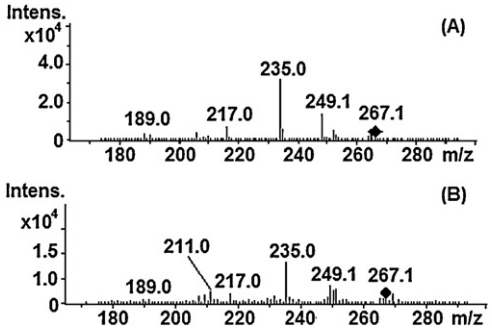

Fig. 4. Full scan ESI-MS/MS spectra of the JH III peak in (A) a standard solution and (B) a honey bee hemolymph sample. The ESI-MS/MS conditions are described in Section 2.3 and Table 1.

The limits of detection (LOD) and quantification (LOQ) of the method were calculated as described in Section 2.7 (Table 4) and for JH III the LOD was 5 pg/μL and the LOQ 15 pg/μL. To the best of our knowledge this is the first time that JH III levels have been measured experimentally in bee hemolymph using LC–MS/MS. The LOD and LOQ values obtained with the proposed method were compared with those calculated for other insect hemolymph samples using LC–MS/MS [17,23,26], and found that our they were slightly better than those reported previously, which ranged from 6 to 8 pg/μL and 20 to 25 pg/μL for the LOD and LOQ, respectively. Moreover, comparative evaluation of the results obtained with the described method and the LOD values reported using a reference technique such as RIA revealed values that were slightly better (1.9 pg/μL) [15] or worse (14 pg) [17], depending on the study. In both cases the LOD values were quite similar, although the matrices analyzed were not honey bee hemolymph samples.

Several tests were carried out to analyze matrix effects on the MS/MS determination of JH III. First, the results obtained using standard and matrix-matched samples spiked with four different JH III concentrations (Table 2) were compared, measuring the JH III signal of the spiked hemolymph samples and the standard solutions. Honey bee hemolymph samples contain endogenous levels of JH III, which must be taken into account when comparing the results. Thus, to calculate the signals for the honey bee hemolymph samples, the JH III areas corresponding to endogenous JH III levels must be also determined. Those areas were subtracted from the total area obtained for the spiked honey bee hemolymph samples and the resulting areas were compared with the standard solutions. In all cases these areas were divided by the IS area to obtain the corresponding ratio of areas. To correct for the possible influence of sample treatment, matrix-matched samples were spiked after treatment of the bee hemolymph samples. Similar results were obtained at all the concentrations assayed (with recovery percentages of 95–104%). Hence, neither ion suppression nor enhancement appeared to occur during the analysis. The slopes of the standard and matrix-matched calibration curves were also compared (Table 4), revealing an overlap of the confidence intervals and indicating that the matrix had no effect on JH III signal. Thus, samples were quantified using the standard solution calibration curves and the graphs obtained were straight lines with an intercept that did not significantly differ from zero ($p < 0.05$). Indeed, the results obtained were linear across the range studied, confirming a lack of bias (Table 4).

The precision (repeatability and intermediate precision) of the proposed method was determined by analyzing hemolymph obtained from uninfected samples spiked with four different JH III concentrations (Table 2). As is usual in experiments using MS detection, an IS (methoprene) was used to ensure greater precision. The precision of the method is witnessed by the %RSD

Table 4
LOD, LOQ and calibration data values ($n = 5$) obtained for JH III.

Compound	LOD (pg/μL)	LOQ (pg/μL)	Calibration curve type	Concentration range (pg/μL)	b	s_b	a	s_a	R^2	$s_{y/x}$
JH III	5	15	Standard solution	15–14,562	6.9×10^{-5}	0.5×10^{-6}	−0.0020	0.0028	0.9996	0.0069
			Matrix matched	15–14,562	7.1×10^{-5}	0.5×10^{-6}	−0.0007	0.0031	0.9996	0.0075

a, intercept with the y-axis; s_a, standard derivation of the intercept; b, slope; s_b, standard derivation of the slope; $s_{y/x}$, standard derivation of the vertical distances of the points from the line.

Table 5
Concentration (pg/μL) of JH III in workers honey bee hemolymph samples (five samples per day and type of infestation).

Extraction day	JH III (pg/μL)											
	Uninfected			*Nosema apis*			Spanish *Nosema ceranae*			Dutch *Nosema ceranae*		
	Min.–Max. values	Mean	RSD (%)	Max.–Min. values	Mean	RSD (%)	Max.–Min. values	Mean	RSD (%)	Max.–Min. values	Mean	RSD (%)
1	68–85	77	8	106–114	109	3	125–135	130	3	120–126	124	2
2	72–76	74	2	104–109	107	2	127–135	131	2	120–125	122	2
3	100–110	106	4	137–145	143	2	159–166	163	2	152–157	155	1
4	102–110	107	3	138–147	141	2	163–169	166	2	155–159	157	1

values obtained, which were below 5% for both relevant parameters (Table 2). Finally, recovery and trueness were evaluated using bee hemolymph samples spiked with four different JH III concentrations, added either before or after the sample treatment. Good recovery was observed and the %RSD values were below 5% for the concentrations assayed (Table 2). The areas for JH III in standard and matrix-matched samples were compared by calculating the area corresponding to the spiking level in matrix-matched samples, as described for the matrix effect studies.

3.5. Method application

Hemolymph samples obtained from four different groups of bees were analyzed: healthy controls; bees infected with Spanish or Dutch *N. ceranae* strains; and bees infected with *N. apis*. All samples were analyzed using the LC–MS/MS procedure described and validated above, the results of which are summarized in Table 5. Hemolymph samples from uninfected bees contained lower JH III concentrations than those infected by *Nosema* spp. The highest concentrations were detected in hemolymph from bees infected with *N. ceranae* (Spanish and Dutch strains), which were slightly higher than in *N. apis* infected bees. MRM chromatograms obtained from a 15 pg/μL (LOQ level) JH III solution and from hemolymph samples from uninfected and Spanish *N. ceranae* infected honey bees are shown in Fig. 3. The JH III signal from the hemolymph of infected honey bees presented the strongest signal, which was approximately 2 times stronger than that detected in uninfected honey bees or 9 times that in a standard solution of JH III at the LOQ.

These results are consistent with those of a previous chiral-specific radioimmunoassay that demonstrated higher JH titers in *N. apis* infected preforaging workers than in control bees [6,12,13]. These *N. apis* infected bees foraged at an earlier age than corresponding healthy controls. In our experimental conditions, bees infected with *N. ceranae* had the highest hemolymph concentrations of JH III, suggesting that foraging is induced earlier than in uninfected bees or those infected with *N. apis*. However, further field studies will be necessary to determine whether infected worker honey bees do indeed engage in premature foraging and other risky behaviors [38]. The division of labor among worker bees is age-dependent, whereby young adults work in the hive, performing tasks related to colony maintenance and brood care. Thus, premature foraging is likely to disturb the social interactions in the colony and consequently, affect the longevity of individual bees [39].

4. Conclusions

It has been developed a simple and sensitive LC–MS/MS method to measure JH III levels in *Apis mellifera iberiensis* hemolymph, with notable advantages over previous approaches in terms of analysis time and sensitivity. This method was consistent and reliable, it was not influenced by the matrix, and it produced good recovery and clean chromatograms with the optimized sample treatment. Using this method, hemolymph levels were analyzed in worker honey bees infected with different strains of *Nosema* spp. to study the link between specific microsporidia strains and JH III levels. The highest JH III concentrations were found in the hemolymph of infected bees, particularly those infected with *N. ceranae*. Moreover, those higher JH III levels found in infected bees were in agreement with previous studies of naturally infected bees.

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