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Quantitative determination of juvenile hormone III and 20-hydroxyecdysone in queen larvae and drone pupae of *Apis mellifera* by ultrasonic-assisted extraction and liquid chromatography with electrospray ionization tandem mass spectrometry

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ABSTRACT

In this paper, a method for the rapid and sensitive analysis of juvenile hormone III (JH III) and 20-hydroxyecdysone (20E) in queen larvae and drone pupae samples was presented. Ultrasound-assisted extraction provided a significant shortening of the leaching time for the extraction of JH III and 20E and satisfactory sensitivity as compared to the conventional shake extraction procedure. After extraction, determination was carried out by liquid chromatography–tandem mass spectrometry (LC–MS/MS) operating in electrospray ionization positive ion mode via multiple reaction monitoring (MRM) without any clean-up step prior to analysis. A linear gradient consisting of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid, and a ZORBAX SB-Aq column (100 mm × 2.1 mm, 3.5 μm) were employed to obtain the best resolution of the target analytes. The method was validated for linearity, limit of quantification, recovery, matrix effects, precision and stability. Drone pupae samples were found to contain 20E at concentrations of 18.0 ± 0.1 ng/g (mean ± SD) and JH III was detected at concentrations of 0.20 ± 0.06 ng/g (mean ± SD) in queen larvae samples. This validated method provided some practical information for the actual content of JH III and 20E in queen larvae and drone pupae samples.

1. Introduction

Queen larvae and drone pupae have attracted an enormous upsurge of interest in recent years due to the presence of extensive nutritional value. Queen larvae develop from the fertilized egg of honeybee and have high nutritive value and health care function by feeding the fresh royal jelly due to its abundant compositions such as proteins, amino acids, vitamins, trace element, unsaturated fatty acids and biologically active substances possessing the special regulatory role including enzymes, hormones, nucleic acids, choline and flavonoids. There is evidence to suggest that queen larvae have several medicinal properties including immunomodulatory, anti-tumor, antioxidation, anti-fatigue, growth enhancement, improvement of blood circulation, digestive system and endocrine system [1–3]. Drone pupae are a kind of metamorphosis polypide hatched from unfertilized egg in the drone cell. It is believed to be a new ingredient for cooking different dishes and producing canned food because drone pupae have rich protein, vitamins, microelements, carbohydrates and hormones [4,5].

Juvenile hormone III (JH III), as a sesquiterpene hormone, is secreted from the corpora allata and is responsible for larval and pupal molting in the pre-imaginal discs stage and mediates the reproduction, polymorphism, sex pheromone production, immune function, morphogenesis and mating-related immuno-suppression [6–9]. 20-Hydroxyecdysone (20E), which is secreted from the prothoracic gland as a polyhydroxylated ecdysteroid hormone, has an anabolic effect to enhance the protein synthesis by assembling amino acids into the proteins and controls carbohydrate and lipid metabolism and cell immunity effects [10–12]. Both JH III and 20E jointly regulate the whole process of queen larvae and drone pupae development such as embryogenesis, molting, metamorphosis and reproduction and further affect the level of anabolic and catabolic

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hormones. The beneficial effects of JH III and 20E to the human health are present because they could make protein spirochete structure and amino acid sequence normalization and contribute to recover the cell structure destroyed by tumor.

Some analytical methods have been developed to quantify the presence of JH III or 20E at trace levels in complex biological samples such as insect or its hemolymph [13–21], helminth [22], animals used for meat production and calf urine [23,24] termites [25,26] based on bioassays, immunological analysis, gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry. Each method has specific advantages and drawbacks in the different historical stages of technology development. Bioassay method offers the greatest potential for measuring JH content in the early years without knowing more information about target compounds, but the chief disadvantage is low sensitivity and result variability [13]. Immunoassays are widely sought methods for quantification of hormones because of the virtues of high sensitivity and high sample throughput, but this approach usually possesses the poor precision and accuracy due to the presence of antibody cross-reactivity between target hormones and homologs arising from their similar structural features [14–16]. For quite a long time, gas chromatography coupled to mass spectrometry has been believed to offer a selective and sensitive method for the quantification of hormones in the selected ion monitoring mode, but its utility suffers from the tedious sample preparation and additional derivatization process owing to the low volatility and thermal instability of the hormones [17–20,22]. Recently, high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) has become a powerful combination for the JH III or 20E quantification from complex biological matrices at trace levels. It exhibits some advantages, such as satisfactory sensitivity and specificity because it provides further confirmation via molecular mass and specific structural fragmentation [21,23,24,27]. However, the validation of the method has not been performed for some studies such as linearity, precision, recovery, sensitivity and stability. Although some literatures and product websites have described that queen larvae and drone pupae are rich in JH III and 20E for controlling their biological characteristics in different development stages [11,28], until now there is no published method to simultaneously quantify JH III and 20E content in queen larvae and drone pupae samples of *Apis mellifera* as the different matrix relative to hemolymph of insect, helminth, termites.

In this study, a rapid and simple HPLC–MS/MS method was developed and validated for sensitivity, specificity, precision and stability in queen larvae and drone pupae samples of *A. mellifera* to simultaneously identify and quantitate JH III and 20E content via a simple extraction step without complicated clean-up steps followed by HPLC–MS/MS analysis in positive ion mode. In order to eliminate interferences of the matrix, the ion fragmentation was recorded by adjusting the diversion valve automatically controlled by software at different time segments. Additionally, this work provides information on the amounts of JH III and 20E in queen larvae and drone pupae, which is also very scarce in the literature. The validated method was successfully applied to quantify JH III and 20E content in queen larvae and drone pupae samples of *A. mellifera*.

2. Experimental

2.1. Chemicals and reagents

Juvenile hormone III (JH III, ≥65%) and 20-hydroxyecdysone (20E, ≥93%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, isooctane and formic acid were provided by Fisher Scientific Company (Pittsburg, PA, USA). Pure water was purified by a Milli-Q Plus device from Millipore (Bedford, MA, USA) and used throughout the study.

2.2. Preparation of standard solutions and calibration curves

Stock solutions of the JH III and 20E were prepared at concentrations of 0.5 μg/mL and 2 μg/mL in methanol, respectively, stored at −18 °C in the dark and used within 6 months. Individual working solutions of JH III (50 ng/mL) and 20E (400 ng/mL) were separately prepared by diluting the stock solutions with acetonitrile and stored at 4 °C in the dark for a maximum period of 3 months. Mixed calibration standards of 0.05, 0.1, 0.2, 0.5, 1, 10 and 20 ng/mL concentrations for JH III and 2, 5, 10, 20, 50, 100 and 200 ng/mL concentrations for 20E were prepared by diluting the individual working solutions further with acetonitrile. Calibration curves were constructed by plotting the peak area of JH III and 20E versus their concentrations in acetonitrile and the regression line were calculated using a weighted factor ($1/y$) least-squares linear regression mode.

2.3. Sample collection

Queen larvae incubated for 3 days were collected from the queen cell with a spoon and transferred into a sterile beaker filled with physiological saline (0.9% sodium chloride solution) to remove the adherent royal jelly. The whole body surface of queen larvae was dried with absorbent paper. Drone pupae of 18–21-day-old were collected from the capped drone cell. Queen larvae and drone pupae samples were separately ground in grinder and kept frozen at −20 °C in darkness until analysis.

2.4. Sample preparation based on ultrasonic-assisted extraction (UAE)

Aliquots (1.0 g) of queen larvae and drone pupae samples were weighed into a 10 mL centrifuge tube and spiked with mixed calibration standard solutions. After the addition of 5 mL methanol, 3 mL isooctane, the samples were sonicated for 10 min at 30 °C using an ultrasonic cleaning bath (model KQ100E, 100 W, 40 kHz, Kunshan Instruments, PR China). The mixed solution was centrifuged at 10,000 rpm for 5 min, and then the upper isooctane layer was transferred into the glass graduated test tube. Ultrasound-assisted extraction was repeated twice again with 3 mL isooctane. After the end of each sonication, isooctane solution was introduced in the glass graduated test tube. The remainder sample solution was centrifuged at 10,000 rpm for 5 min and the methanol phase was transferred into the same glass graduated test tube. The combined extracts were evaporated to dryness in a water bath at 40 °C under a stream of nitrogen. The residue was reconstituted in 1.0 mL of water (0.1% formic acid):acetonitrile (0.1% formic acid) (10:90, v/v), transferred to injection vials, and analyzed by LC–MS/MS.

2.5. Shake-flask extraction

The queen larvae and drone pupae samples were treated with the extraction solution by mechanical shaking using oscillator for 2 h instead of ultrasonically assisted extraction. The other procedures are as described for ultrasonic-assisted extraction section.

2.6. LC–MS/MS analysis

The LC–MS/MS system comprised the LC instrument 1200 from Agilent Technologies (Agilent, Waldbronn, Germany) with a vacuum degasser (G1322A), a binary pump (G1312B), an auto sampler (G1367D), a column compartment (G1316B) and system controller.

Table 1
Experimental LC–MS/MS parameters used for the determination of JH III and 20E by MRM in the positive ion mode.

Compound	Transitions (*m*/*z*)	Proposed product ion	CV (V)[a]	CE (eV)[b]	DT (ms)[c]
JH III	267.1 → 235	$[M-CH_3OH+H]^+$	80	1	60
	267.1 → 217.1	$[M-CH_3OH-H_2O+H]^+$	80	2	60
20E	481.1 → 445	$[M-2H_2O+H]^+$	120	8	60
	481.1 → 371	$[M-2H_2O-C(CH_3)_3OH+H]^+$	120	10	60

[a] Capillary voltage (V).
[b] Collision energy (eV).
[c] Dwell time (ms).

An Agilent 6460 triple quadruple tandem mass spectrometer coupled to electrospray ionization (ESI) interface and Agilent Jet Stream Ion Focusing (Agilent Technologies, Palo Alto, USA) was used for mass analysis and quantification of target analytes. The system operation, data acquisition and analysis were controlled and processed by the MassHunter software.

The chromatographic separation was carried out using a Zorbax SB-Aq column (100 mm × 2.1 mm, 3.5 μm) (Agilent, Wilmington, DE). JH III and 20E were separated using binary gradient elution. Mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient started with 20% of component B: acetonitrile (0.1% formic acid) for 0.5 min and then increased to 95% within 0.1 min. This composition was kept till 4.2 min, and then decreased to 20% of component B within 0.1 min. The total run time was 10 min, and an equilibration step of 5.7 min was included. The flow rate of the mobile phase and the column temperature were set at 0.3 mL/min (injection volume: 5 μL) and 30 °C, respectively. In order to avoid carryover, the auto sampler needle was rinsed automatically with the mixed solution (80% component A and 20% component B) for 3 s among a series of calibration standards, control samples and spiked samples.

The mass spectrometer was operated in the positive ion mode. The tuning parameters were optimized for JH III and 20E: gas temperature 350 °C, drying gas flow 6 L/min, nebulizer pressure 35 psi, V_{cap} voltage: 3500 V, sheath gas temperature: 350 °C, sheath gas flow: 9 L/min, Nozzle voltage: 1000 V. The full-scan MS spectra were acquired by scanning the mass spectrometer in the *m*/*z* range of 200–2000 at a unit mass resolution. The mass spectrometry parameters applied for JH III and 20E with regard to the transitions from precursor to product ions were shown in Table 1. In order to eliminate interferences of the matrix including salts and other impurities, the ion fragmentation was recorded by adjusting the diversion valve automatically controlled by MassHunter software at $t_{0-2.1\,min}$ to waste, $t_{2.1-2.6\,min}$ to mass, $t_{2.6-5.1\,min}$ to waste, $t_{5.6-5.8\,min}$ to mass and $t_{5.8-10\,min}$ to waste.

3. Results and discussion

3.1. Optimization of LC–MS/MS conditions

In order to improve separation efficiency and obtain the optimal response for two analytes, different mobile phase compositions were evaluated in the LC–MS/MS analysis. Methanol and acetonitrile, as the most relevant organic solvents in reversed-phase chromatography, were utilised to increase the sensitivity of target analytes and obtain appropriate retention times. We used the mobile phase with acetonitrile instead of methanol as an organic modifier because it gave the stronger response and sharper peak for JH III and 20E. So, acetonitrile as the organic modifier was chosen to further optimize other components in the mobile phase. The influence of additives in mobile phase on the peak shape and sensitivity was investigated by injecting the mixed working standard solution containing variable concentrations of each modifier (0.01, 0.05, 0.1 and 0.5% for formic acid; 0.01, 0.05, 0.1 and 0.5% for acetic acid; 5, 10, 15 and 20 mM for ammonium formate; 5, 10, 15 and 20 mM for ammonium acetate, respectively) into the LC–MS/MS system and registering the responses in MRM mode. Eventually, it was found that the peak shapes and responses of JH III and 20E were satisfactory when the concentration of formic acid was in the range of 0.05–0.2% which can aid protonation and improve ionization efficiency of the JH III and 20E. However, poor ionization efficiency was achieved when the concentration of formic acid was below 0.05% (incomplete ionization) and above 0.2% (inhibited ionization). As a result, the combination of acetonitrile (0.1% formic acid) and pure water (0.1% formic acid) as the best compromise between sensitivity and resolution was used in the mobile phase.

Under optimized LC and mass spectrometry conditions, a gradient elution procedure with a total run time of 10 min including reconditioning phase for the next cycle was performed to quantify JH III and 20E. 20E and JH III were separated with retention times of 2.303 and 5.682, respectively.

The optimization of the MS/MS conditions was carried out by infusion of the individual standard solutions and spiked sample extracts at the concentration levels of 1 μg/mL for JH III and 2 μg/mL for 20E in electrospray ionization (ESI) positive mode. On the full scan mass spectra of JH III, the protonated molecular ion $[M+H]^+$ and sodium adduct ion $[M+Na]^+$ were observed at a mass to charge ratio (*m*/*z*) of 267.1 and 289.1, respectively. In the previous studies [16,21], $[M+Na]^+$ as the precursor ion and its two corresponding product ions were used for the identification and quantification of JH III. We tried to obtain the fragmentation ions of $[M+Na]^+$ by collision-induced dissociation (CID) for the individual standard solutions and spiked sample extracts. Either the precursor ion was not dissociated under low CE or large numbers of low abundance fragments were present with the small increase of CE, which made the identification more difficult and uncertain. So, two characteristic fragmentations of the protonated molecular ion $[M+H]^+$ were monitored for JH III and 20E, the most abundant one being used for quantification, while the second one was used as a qualifier to improve selectivity, which is of particular interest for confirmatory analysis of complicate matrix samples in comparison with standards. The protonated molecular ion of JH III in ESI^+ mode is *m*/*z* 267.1. The fragments of *m*/*z* 267.1 were *m*/*z* 235 ($[M-CH_3OH+H]^+$) and *m*/*z* 217.1 ($[M-CH_3OH-H_2O+H]^+$) where *m*/*z* 235 is the dominant ion. 20E gave a protonated molecular ion of *m*/*z* 481.1 where *m*/*z* 371 ($[M-2H_2O-C(CH_3)_3OH+H]^+$) and *m*/*z* 445 ($[M-2H_2O+H]^+$) were the predominant fragments by CID. The mass spectrometry parameters applied for the substances JH III and 20E with regard to the transitions from precursor to product ions are shown in Table 1. The main CID fragmentation pathways of the protonated JH III and 20E are shown in Fig. 1.

It is essential to use stable isotope-labeled internal standard for the quantification of target analytes in LC–MS/MS analysis because it offers the similar performance with the target compounds in extraction efficiency, chromatographic behavior, ionization efficiency and fragmentation characteristics. However, there were no stable isotope-labeled JH III and 20E commercially available and the application of isotopic labeled analyte synthesized by in-house lab-

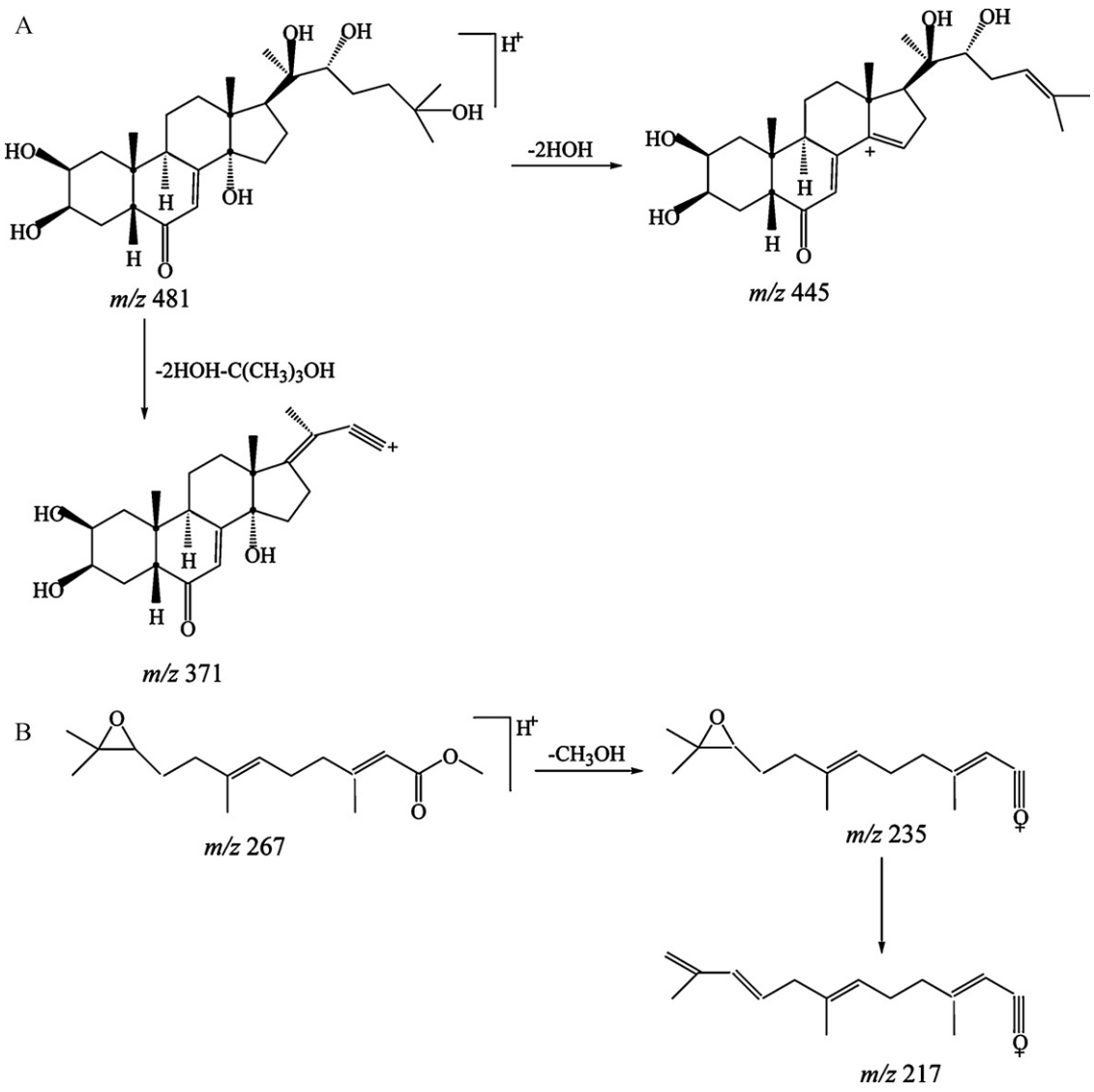

Fig. 1. Proposed fragmentation pathways of the protonated 20E (A) and JH III (B).

oratory was limited owing to the lack of certified values. Moreover, some alternatives such as similar compounds (ortho- or meta-substituted compound), structural analogues with similar structure and small differences in functional groups are used to compensate for some shortcomings of MS detector. However, ionization of these similar compounds or structural analogues as internal standard in real samples and the calibration are probably different especially under normal circumstances without the blank matrix in bio-analysis [29]. In light of the above, the internal standard was not used in this study, but an appropriate remedial measure was taken by programming different time segments to make impurities to waste which can avoid the ionization of interfering matrix in ion source part, eliminate the matrix effect.

3.2. Optimization of extraction conditions

Selection of the extraction solvent could be a critical point due to the presence of many proteins and lipids in queen larvae and drone pupae which could interfere with the extraction of JH III and 20E and lead to low recovery and inaccurate quantification. In order to obtain optimal extraction efficiency, different extraction solvents or their combinations were investigated. Various solvents including methanol, acetonitrile, ethyl acetate, hexane, diethyl ether, isooctane and acetone were tested for the extraction of JH III and 20E from queen larvae and drone pupae samples. The extract chromatograms obtained by using acetonitrile, acetone or ethyl acetate as the extraction solvent presented many interfering peaks but only small amounts of JH III and 20E were obtained from the spiked queen larvae and drone pupae samples. After preliminary experimentation, it was found that methanol and diethyl ether can more effectively extract the 20E from queen larvae and drone pupae samples. In the final, methanol was used for the extraction of 20E due to the anesthetic toxicity of diethyl ether to human health even at low concentration. JH III and specific glycoprotein are fairly easy to combine into the juvenile hormone binding protein (JHBP), which acts as a carrier to deploy the hormone to target tissues [30]. Hexane and isooctane, as the appropriate extraction solvent, can also sequester JH III and obstruct its coagulation with proteins [23]. However, the poor recoveries for JH III were achieved by using hexane as the extracting solvent maybe due to the low ability to release JH III from the JHBP. A suitable combination of two solvents, *viz.* methanol and isooctane, was confirmed to extract specifically target compounds from queen larvae and drone pupae samples and to allow the best recovery.

As a traditional extraction technique, shake extraction is once widely used although it presented some disadvantages such as

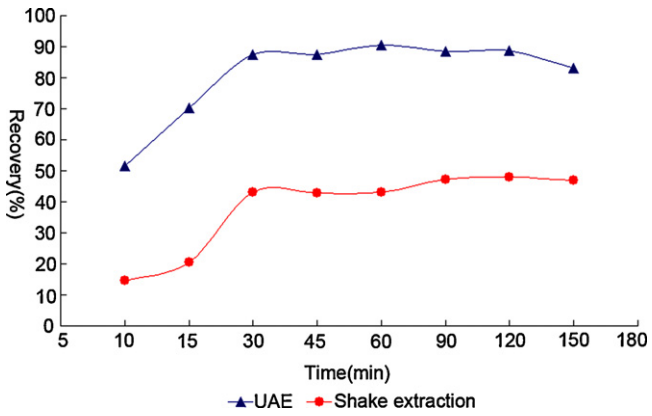

Fig. 2. Time of sonication and shake extraction on the recovery of JH III and 20E in queen larvae and drone pupae samples spiked at level of 0.5 ng/mL for JH III and 20 ng/mL for 20E.

time- and solvent-consuming nature. In this study, the poor recoveries (less than 47.4%) were obtained by using shake extraction. It is difficult to release JH III and 20E from queen larvae and drone pupae samples with methanol and isooctane though these solutions were effective solvents of target analytes, so it was necessary to select a new way to extract them. Ultrasound-assisted extraction (UAE) was applied as a simple, inexpensive method applicable to a wide range of biological matrix, as an another alternative for sample pretreatment because the energy imparted facilitates and accelerates some steps, such as dissolution, fusion and leaching, and so on. In queen larvae and drone pupae samples analysis, sonication was used in sample pretreatment for better dissolution of JHBP. Some parameters such as time (min), temperature (°C) and amount of extraction solution (mL) were optimized to increase the extraction efficiency, with minimum solvent consumption and minimum duration of the extraction time in this study. Fig. 2 shows the effect of experimental duration on the extraction efficiency of JH III and 20E in queen larvae and drone pupae samples. It can be seen that the recoveries of JH III and 20E increases with the extraction time from 10 to 30 min in both methods and then level off over 30 min. The recoveries of JH III and 20E in UAE were higher than that in shake extraction at all the time. *F*-Test showed that the extraction time within 30 min had a significant effect on the recoveries of JH III and 20E ($p < 0.05$), and the effect of ultrasound and shake extraction on the recovery also was significant ($p < 0.01$). This process indicates that UAE is more effective than shake extraction to extract the JH III and 20E in queen larvae and drone pupae samples. The most likely reason is that power of ultrasound wave possesses the stronger penetration ability compared with shake extraction to enhance the internal diffusivity of queen larvae and drone pupae samples and release JH III and 20E.

The mixed solution (5 mL of methanol and 9 mL of isooctane) at 30 °C was found to be optimum as the appropriate extraction solution under the requirement to guarantee recovery and decrease solvent consumption (Fig. 3). The amount of extraction solvents (methanol/isooctane) and temperature had similar effect on the recoveries of JH III and 20E in queen larvae and drone pupae samples. Both the recoveries of JH III and 20E extracted by UAE and shake extraction were found to increase with the increase of the amount of extraction solvents and temperature. But the recoveries of them do not significant change with increasing temperature and the amount of extraction solvents when the temperature changed from 30 °C to 40 °C and the amount of extraction solvents changed from 14 mL (methanol/isooctane; 5 + 9, v/v) to 24 mL (methanol/isooctane; 9 + 13, v/v). So, the amount of extraction solvents (methanol/isooctane) of 14 mL and 30 °C seem to be appropriate for UAE. In the final, the recovery of JH III and 20E obtained by UAE was better than that obtained by shake extraction at the same extraction conditions.

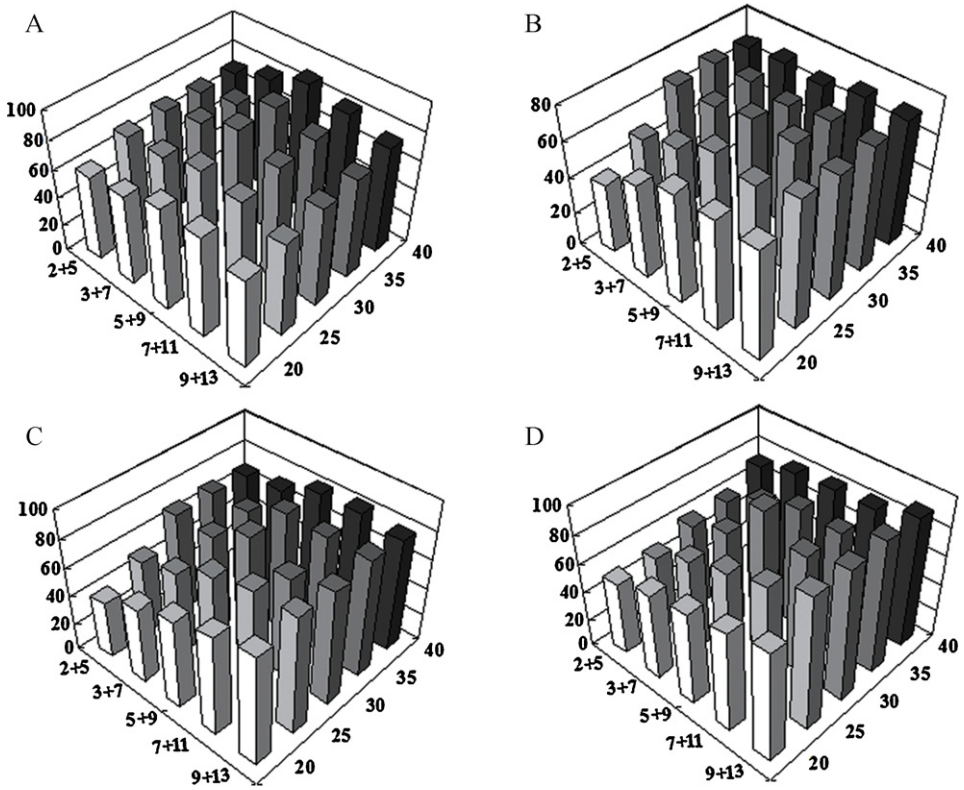

Fig. 3. Effect of extraction solution volume and temperature on the average recovery of JH III and 20E in queen larvae and drone pupae samples spiked at level of 0.5 ng/mL for JH III and 20 ng/mL for 20E ((A) JH III in queen larvae; (B) 20E in queen larvae; (C) JH III in drone pupae; (D) 20E in drone pupae).

Table 2
Regression and data and LOQs for JH III and 20E ($n = 15$).

	Compound	
	JH III	20E
Slope ($a \pm S_a$)	4802.6 ± 241.7	110.0 ± 8.6
y-Intercept ($b \pm S_b$)	483.7 ± 45.7	641.6 ± 51.3
Coefficient of correlation	0.9996	0.9995
LOQ (μg/kg)		
Queen larvae	0.10	5
Drone pupae	0.15	5

a, slope; b: intercept; S_a, standard deviation of slope; S_b, standard deviation of intercept.

Solid phase extraction is routinely used in many different biological matrixes to isolate analytes of interest from a wide variety of interference impurities before instrumental analysis. In this study, only the simple extraction procedure without solid phase extraction step was carried out and satisfactory recoveries were achieved by decreasing the loss of target compounds in the process of sample pretreatment.

3.3. Method validation

3.3.1. Linearity

The use of matrix-matched calibration curve implies that there is similar matrix effect among calibration solutions and samples. In fact, it is rather difficult to obtain such ideal condition for the active compounds analysis of samples due to species diversity of samples and the presence of unknown concentration of the target analytes in the sample. Thus, non-matrix-matched external standard method in this study was used.

Calibration standards with seven concentration levels of JH III and 20E were prepared and subsequently analyzed in quintuplicate in three separate analytical runs by plotting the peak area each compound against the concentration of analyte and performing a linear regression for the construction of calibration curves. Linear range was tested following the developed procedure in the MRM mode and the ranges studied were 0.05–20 ng/mL for JH III and 2–200 ng/mL for 20E. Good linearity was obtained for JH III and 20E with correlation coefficients (r) greater than 0.999 with weighting factor ($1/y$), respectively (Table 2). The analysis of variance (ANOVA) with lack-of-fit test was run to validate the regression data of calibration curves, tested to allow the determination of whether the selected model is adequate to describe the obtained data. The linear model appeared to be adequate for both JH III and 20E since the p-values for lack-of-fit were greater or equal to $\alpha = 0.05$ at a confidence level of 95%.

3.3.2. Recovery and matrix effects

The recoveries and matrix effects through the method were carried out by spiking queen larvae and drone pupae samples with known amounts of standard solutions at three concentration levels (0.05, 0.10, 1 ng/g for JH III and 2, 10, 50 ng/g for 20E, respectively) in quintuplicate. In parallel, corresponding control samples (no spiked queen larvae and drone pupae samples) were performed to distinguish the native amount of JH III and 20E in samples from the one spiked. The amount of endogenous analytes in control samples was expressed as "D" for the sake of the convenient calculation.

The recoveries are determined by comparing the MRM response of queen larvae and drone pupae that was spiked with a fixed concentration of JH III and 20E standard solution before extraction (B) relative to the response of the queen larvae and drone pupae samples first subjected to the extraction procedure and then spiked with the same amount of JH III and 20E (A); thus, the recovery is equal to $[((B - D)/(A - D)) \times 100]$. The main purpose of recovery calculation method was to minimize or eliminate the contribution of the matrix effect and the endogenous JH III and 20E to some extent. The recovery ranges of JH III and 20E in queen larvae and drone pupae samples were indicated in Table 3. Typical MRM chromatograms of JH III and 20E in queen larvae and drone pupae samples assay were shown in Fig. 4.

Matrix effects are assessed by comparing the MS/MS responses of known concentrations of JH III and 20E standard solution in neat solvent (C) and response of the analytes spiked into a matrix sample that has been carried through the sample preparation process in advance with the same concentration (A). For matrix effect factor (MEF, $[((A - D)/C) \times 100]$), MEF = 100 indicates no matrix effects, MEF < 100 indicates ion suppression and MEF > 100 indicates ion enhancement. Finally, about 10 different portions of samples for each matrix were analyzed to show that the matrix effects ranged from 93.1 to 103.4 for JH III and from 94.8 to 101.9 for 20E. Slight ion suppression was observed for JH III in queen larvae and for 20E in drone pupae at low spiked concentrations due to the presence of specifically endogenous substances in different matrix. However, there were no significant matrix effects observed for JH III in queen larvae and for 20E in drone pupae at high spiked concentrations, JH III in drone pupae and for 20E in queen larvae at any concentration. The most likely reason is due to the fact that competitive ability of droplet surface charges of the trace content of endogenous target analytes is weaker than that of high spiked concentrations in the ion source part of mass spectrometry.

3.3.3. Precision

The precision expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of the assay was determined by analysis of spiked queen larvae and drone pupae samples containing the JH III at 0.05, 0.1, 1.0 ng/g and 20E at 2, 10, 50 ng/g and the corresponding control samples (no spiked queen larvae and drone pupae samples). The RSD values of differences among measured concentrations were used to show the precision of the method. Intra-day precision was evaluated for the determination of six aliquots of each sample fortified at three concentration levels in quintuplicate on the same day. Inter-day precision was analyzed for the determination of three aliquots each sample on five successive days in quintuplicate. Limits of acceptable intra- and inter-day precision were set at RSD % <15%. Data for intra- and inter-day precision of the method were presented in Table 3. Intra-day precision ranged between 2.57% and 10.8%, Inter-day precision ranged between 4.39% and 13.1%.

3.3.4. Limit of quantification

The limits of quantification (LOQs) were calculated with the MRM chromatograms of JH III and 20E from queen larvae and drone pupae extraction solutions using the quantification transitions. The LOQ ($LOQ = 10 \cdot S_{xy}/a$, where S_{xy} is the standard deviation of intercepts and a is the mean slope of the calibration curve) is the lowest concentration of analyte that can be determined [31]. The results are shown in Table 2.

3.3.5. Stability

It is well known that instability of the target analytes during storage or analysis may significantly affect the precision and recovery of the final results. So, stability must be taken into account during the method validation. To check for the stability of target analytes in pure solvent and mobile phase, JH III and 20E were dissolved in methanol or mixed solution of water (0.1% formic acid): acetonitrile (0.1% formic acid) (10:90, v/v), and then stored at room temperature, 4 °C and −18 °C, respectively.

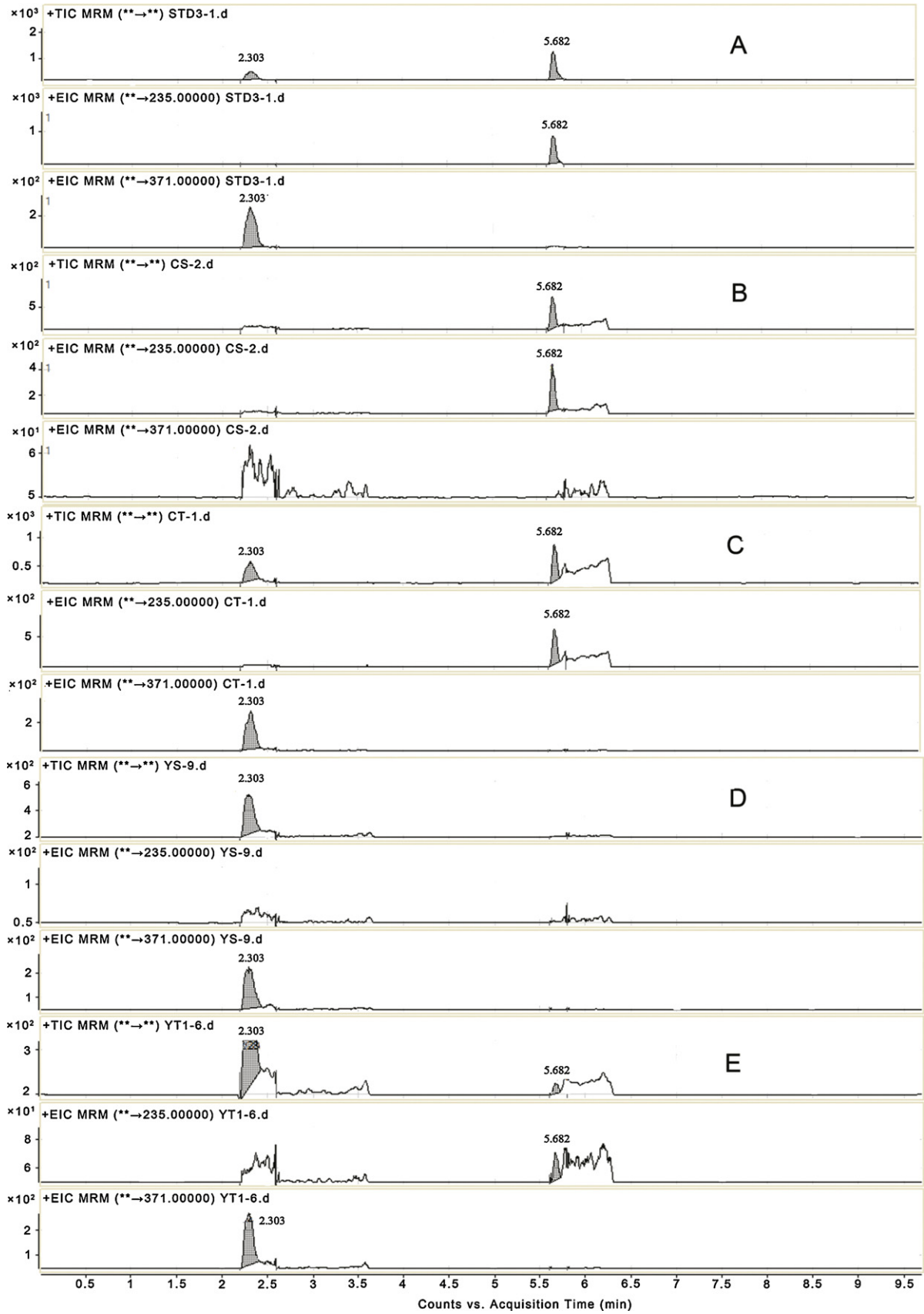

Fig. 4. Representative MRM chromatograms (A) at 0.5 ng/mL for JH III and 20 ng/mL for 20E; (B) queen larvae samples; (C) spiked queen larvae samples at 0.1 ng/mL and 10 ng/mL for JH III and 20E, respectively; (D) drone pupae samples; (E) spiked drone pupae samples at 0.1 ng/mL and 10 ng/mL for JH III and 20E, respectively.

Table 3
Recovery and precision results of JH III and 20E in queen larvae and drone pupae samples based on the developed method.

	JH III			20E		
	0.05 ng/mL	0.5 ng/mL	1 ng/mL	2 ng/mL	10 ng/mL	50 ng/mL
Queen larvae						
Recovery (%)	76.2	89.1	93.7	63.9	74.7	87.4
Matrix effect (%)	93.1	93.9	99.4	101.9	99.3	99.2
Intra-day precision (%) $n = 30$	10.8	7.91	3.68	10.6	5.99	2.57
Inter-day precision (%) $n = 75$	13.1	8.62	6.61	10.1	6.51	4.79
Drone pupae						
Recovery (%)	73.7	84.9	98.2	81.7	99.3	97.2
Matrix effect (%)	103.4	99.4	99.7	94.8	98.9	99.8
Intra-day precision (%) $n = 30$	7.94	8.11	2.83	12.4	9.47	5.68
Inter-day precision (%) $n = 75$	10.9	8.69	4.39	13.9	9.10	6.74

The concentrations of JH III and 20E in the two solvents were quantified in triplicate at regular intervals and were compared with freshly prepared standard solutions in the darkness. The standard solutions in methanol (JH III: 1 ng/mL, 20E: 20 ng/mL) were found to be stable for one month at 4 °C, but about 15% of them was degraded after two months. The content of JH III and 20E decreased by about one-third when kept at room temperature for one week and seventy percent after one month. However, JH III and 20E standard solutions were stable at −18 °C for six months, but just decreased by 10% for ten months in darkness.

The purified extracts in the mixed solution of water (0.1% formic acid) and acetonitrile (0.1% formic acid) was stored at −18 °C, 4 °C and room temperature, respectively. It was observed that the JH III and 20E contents of samples were unstable over one week at 4 °C. The storage stability showed that no amount of JH III and 20E was lost when extractants was stored at −18 °C for two months. However, the degradation rate of JH III and 20E in real samples reached about 10% of them each day at room temperature.

Stability differences of target analytes in two different solvents were observed with higher degradation speed in solution containing formic acid than those in pure organic solvent. The reason for that is likely due to the fact that JH III and 20E were unstable in the acidic conditions relative to the pure organic solvent system.

3.3.6. Application of the validated method for real-world samples

The above-said validated LC–MS/MS method was used to determine the levels of JH III and 20E in queen larvae and drone pupae samples collected from the apiary of bee research institute of Chinese academy of agricultural sciences. All samples were processed according to the method developed. The concentrations of JH III and 20E were calculated as the arithmetic mean of six experiments under the optimum working conditions. Finally, JH III was detected in queen larvae samples at a mean level (0.20 ± 0.06 ng/g; mean ± SD) and the mean content of 20E with 18.0 ± 0.1 ng/g (mean ± SD) levels was achieved in drone pupae samples, while non detectable levels of JH III and 20E were obtained for drone pupae samples and queen larvae samples, respectively. The most likely cause is that drone pupae samples do not contain JH III and queen larvae samples do not also contain 20E.

Previous studies have successfully developed the analysis method of JH III in *Coptotermes formosanus* and whole body extracts of the *Formosan subterranean termite* [25,26]. This newly established method of simultaneous analysis of JH III and 20E in drone pupae and queen larvae samples showed a more comprehensive evaluation methodology to validate the reliability of developed method. Moreover, in this study the solid phase extraction was not employed for the clean-up of the samples, which not only simplify the whole sample preparation procedure without losing sensitivity but also minimize the loss of analytes.

4. Conclusion

A simple and sensitive LC–MS/MS method was developed that allows for the first time the simultaneous quantitative analysis of JH III and 20E in queen larvae and drone pupae samples. The actual contents of JH III and 20E in queen larvae and drone pupae samples was first reported on the basis of validated analytical method. This method possessed acceptable parameters for method validation such as linearity, limit of quantification, recovery, matrix effects, intra- and inter-day precision and stability and was thus adequate for the rapid analysis of real samples without cumbersome pretreatment procedures. This method can be used to analyze JH III and 20E in both queen larvae and drone pupae samples.

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