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Liquid chromatography–electrospray ionization-mass spectrometric quantitation of juvenile hormone III in whole body extracts of the Formosan subterranean termite

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ABSTRACT

Juvenile hormone (JH) III is responsible for control of a variety of insect physiological and developmental states, including caste differentiation of the Formosan subterranean termite (*Coptotermes formosanus* Shiraki). We report here a simplified, efficient sample preparation and an optimized LC–ESI-MS method for quantifying JH III in whole body extracts. Sample preparation comprises hexane extraction (from termite whole bodies) and C18 cartridge purification. Previous LC–ESI-MS protocols exhibited the following two problems: (1) ion fragmentation differed when comparing spectra from insect samples and authentic JH III and (2) a JH III monitoring ion was not resolved from other unknown compounds in whole body samples from termites. To overcome these problems, we used a pentafluorophenyl LC column and water/acetonitrile containing ammonium acetate as solvent. In a mass chromatogram (*m*/*z* 235) of termite samples, a peak was detected at the retention time of authentic JH III, and MS^2 of this peak confirmed that the ion is a fragment of JH III, $[M–CH_3OH+H]^+$, being the base peak in both termites and authentic JH III samples. The protocol enables quantification of JH III in a single termite with signal/noise >10:1 and the limit of quantification is 21 pg.

1. Introduction

Juvenile hormones (JH) are a group of structurally related sesquiterpenes that regulate many aspects of physiology in insects, including development, reproduction, polymorphism, diapause, and pheromone production [1,2]. There are six known JHs in insects, however, many insect species contain only JH III, which has the structure: methyl 10*R*,11-epoxy-3,7,11-trimethyl-2*E*,6*E*-dodecadienoate (Fig. 1).

Quantification of JH III is important to ascertain its physiological roles in insects. Previous techniques included bioassays, immunological analysis, and radiochemical methods [3–5]. Gas chromatography–mass spectrometry (GC/MS) has been used as an accurate method for quantification of JH III. Bergot et al. used GC–electron impact (EI)-MS and selective ion monitoring (SIM) [6]. A required step for this method was to convert the monoepoxide JH III to its corresponding methoxy-hydrin derivative. In 2001, Teal et al. were able to avoid this step in hemolymph samples by using capillary GC–chemical ionization (CI)-MS [7]. They directly analyzed hexane extracts of Caribbean fruit fly hemolymph by GC–CI-MS, and could detect as little as 21.1 pg of JH III in the samples. However, their work comprised extraction of hemolymph, which is a much less complex sample than insect whole body extracts and does not sample glandular material or cuticle for JH III.

Recently, in addition to GC–MS methods, liquid chromatography (LC)–MS has also been applied to quantify JH III titer in insects, first by Westerlund and Hoffmann [8]. They chromatographed samples prepared from hemolymph on a reverse-phase C18 HPLC column using a water/methanol solvent system and the effluent from the HPLC was detected by electrospray ionization (ESI)-MS in the positive mode. This LC–ESI-MS protocol has been used in several other reports, e.g. Refs. [9–12]. However, in their report, ion fragmentation patterns of JH III were different when comparing samples of insect hemolymph and authentic JH III [8]. In mass spectra of hemolymph samples, the JH III sodium adduct ion (*m*/*z* 289) was the base peak [8,10,11]. In contrast, for their authentic JH III, a fragment ion of JH III (*m*/*z* 235), $[M–CH_3OH+H]^+$, was the base peak [9,10]. Westerlund and Hoffmann suggested that JH III sodium adduct ion

Abbreviations: JH, juvenile hormone; PFP, pentafluorophenyl.

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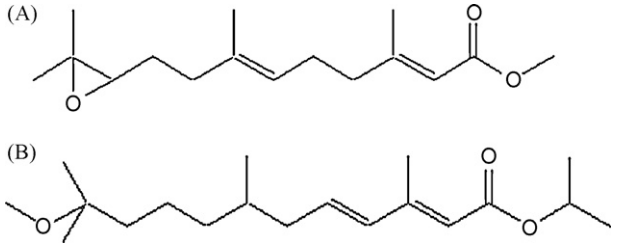

Fig. 1. Chemical structures of JH III ((A) $C_{16}H_{26}O_3$; MW: 266.38) and methoprene ((B) $C_{19}H_{34}O_3$; MW: 310.47).

is detected as a base peak due to the high abundance of sodium ion in insect hemolymph, and Ichikawa et al. reported the addition of a trace amount of sodium salt to the mobile solvent could enhance intensity of the JH III sodium adduct ion in LC–ESI-MS [13]. It is our opinion that to optimally quantify JH III by LC–ESI-MS, it is necessary to find a method in which the ion fragmentation pattern of JH III is the same when comparing samples from insects and authentic JH III, and that samples and standards should be analyzed under similar ionic conditions so that an ion detected at either *m*/*z* 289 or 235 should be extant in both samples as a major peak exhibiting the same patterns of ion fragmentation. An obvious difference in the mass spectra between authentic JH III used for a standard curve and from complex insect samples was a nettlesome problem in displaying accurate quantification of JH III.

The aim of this study was to develop an optimized LC–ESI-MS protocol which would allow precise quantification of JH III in whole body extracts of Formosan subterranean termites (*Coptotermes formosanus* Shiraki). Previous methods for LC–ESI-MS were performed with much simpler insect samples, e.g. hemolymph [8–13]. In this study, we examined JH III from the entire insect which constitutes the most complex possible insect sample for any assay, the whole body extract. In termites, JH III is a candidate hormone for an important role in controlling caste differentiation [14,15]. Previously, GC–EI-MS methods were used in our laboratory for the quantification of JH III in Formosan termite samples which required >50 termite workers to obtain sufficient signal-to-noise to quantify JH III titer in whole body extracts [15,16]. A method for quantification of JH III in one or small numbers of individuals will allow us to examine individual variation in JH III titers and will help to understand control of caste differentiation and colony organization. Preparation of JH III samples using C18 cartridges for crude samples of whole body extracts of Formosan termite workers was followed by an LC–ESI-MS protocol using a pentafluorophenyl (PFP) LC column and a water/acetonitrile LC solvent system containing 1 mM ammonium acetate.

2. Experimental

2.1. Materials

Formosan subterranean termite workers were obtained from a colony collected in New Orleans on November 7, 2008. Synthetic JH III (purity 75% by HPLC), synthetic methoprene (for analytical standard), and acetonitrile (LC–MS grade), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (LC–MS grade) and chloroform (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). *n*-Hexane was purchased from Baker Chemical Co. (Phillipsburg, NJ, USA). Reverse-phase C18 cartridges (UNIBOND C18 SPICE Sample Preparation Cartridges) were purchased from ANALTECH, Inc. (Newark, DE, USA). HPTLC plates were purchased from E. Merck KgaA (Darmstadt, Germany). A reverse-phase PFP LC column (Pursuit® PFP, 2.0 mm i.d. × 250 mm) and a guard column (MetaGuard 2.0 mm Pursuit PFP) were purchased from Varian, Inc. (Palo Alto, CA, USA). All other chemicals were of analytical grade. Water of the required purity for the sample preparation and LC–MS was obtained using a Corning Mega Pure automatic water purification system.

2.2. Preparation of JH III from termite workers

2.2.1. Lipid extraction

According to the extraction methods from previous reports [16], Formosan subterranean termite workers were homogenized in a mixture of 1 ml of *n*-hexane, 0.5 ml of acetonitrile, and 0.5 ml of 2% NaCl with or without 40 ng of methoprene as an internal standard by using a W-385 sonicator (Heat Systems Ultrasonics Inc., Farmingdale, NY, USA) for 5 s three times on ice. The homogenates were centrifuged at 4900 × *g* for 2 min in a model IEC-MULTI RF centrifuge (Thermo Fisher Scientific Inc., MA, USA), and the hexane upper phase was collected. Two additional extractions with 1 ml of *n*-hexane were performed and the *n*-hexane upper phases were combined, after which the sample was dried under a stream of nitrogen.

2.2.2. C18 cartridge procedure

The dried samples were redissolved in 100 μl chloroform–methanol (1:1, v/v). This solution was applied to a C18 cartridge that had been prewashed with 4 ml of methanol. Then 2.5 ml of methanol was added to elute JH III and methoprene (Eluate 1). To confirm the recovery of the two compounds, 1.5 ml of methanol, followed by chloroform–methanol (1:1, v/v) were applied to the cartridge sequentially and the bound lipids were designated Eluates 2 and 3, respectively. After drying Eluate 1 under a stream of nitrogen, 16 μl of methanol was added to the tube and the methanol-soluble phase was used for LC–MS analysis.

2.3. High performance thin-layer chromatography (HPTLC)

Eluates 1, 2, and 3 were prepared from hexane extraction of JH III (50 μg) spiked methoprene (50 μg) and 200 Formosan termite workers. After drying the eluates under a stream of nitrogen, they were completely redissolved in 100 μl of chloroform–methanol (2:1, v/v). Then, 10 μl of standards and a 5 μl sample of termite whole body extracts were spotted on a silica gel HPTLC plate, which was developed in a glass chamber with a solvent system of *n*-hexane:ethyl ether:acetic acid (70:30:1, v/v/v). Spots were detected by spraying 3% copper II acetate containing 8% phosphoric acid and heating at 120 °C on a hot plate.

2.4. Liquid chromatography–mass spectrometry (LC–MS)

Samples were chromatographed on a reverse-phase PFP LC column (2.0 mm i.d. × 250 mm) with a guard column using VARIAN 212-LC pumps (Varian, Inc.). Each sample (aliquot 10 μl) was injected by an autosampler (Model 430, VARIAN Inc.) using μl pickup mode. The separations were performed using solvent A (0.2% acetonitrile in water containing 1 mM ammonium acetate) and solvent B (95% acetonitrile in water containing 1 mM ammonium acetate) on a programmed gradient (50% B for 8 min, 50–100% B for 15 min, 100% B for 5 min, and 50% B for 7 min) at a flow rate of 200 μl/min and a LC temperature of 40 °C.

The effluent from the HPLC was introduced on-line into an ion trap mass spectrometer (Model 500-MS LC Ion Trap, VARIAN Inc.). MS and MS^2 analyses were accomplished by using electrospray ionization (ESI) in the positive mode under the following conditions: needle voltage, 5000 V; spray shield voltage, 600 V, and capillary voltage, 60.0 V in the standard mode. The spray was stabilized with nitrogen gas at 50.0 psi, and the drying gas pressure was 30 psi heated to 320 °C. Mass ranges of *m*/*z* 150–350 and 230–290 were

scanned for determination of optimal LC conditions and quantification of JH III, respectively. MS^2 was performed for the ion at m/z 235.0 ion by setting the fragmentation amplitude at 0.8 V, using a mass range of m/z between 84 and 245.

JH III was quantified using a mass chromatogram of m/z 235 in each sample. JH III in samples from 5 and 30 Formosan termite workers was quantified using a high range standard curve generated with quantities of 59, 118, 236, 472, 944, and 1888 pg of authentic JH III containing 40 ng of methoprene. The range of signal/noise (S/N) was between 14:1 (59 pg) and 180:1 (1888 pg), and the linearity had an R^2 of 0.9975. To quantify JH III in single termite workers, samples of 8, 16, 32, and 64 pg of authentic JH III containing 40 ng of methoprene were used for generating a lower range standard curve. The range of signal/noise (S/N) was between 10:1 (8 pg) and 22:1 (64 pg), and the linearity was an R^2 of 0.9973. All presented data from termite samples represented mean ± standard error of three independent experiments.

3. Results and discussion

3.1. Sample preparation

As shown in Fig. 2A, lane 5, hexane extracts of Formosan termite workers contain large quantities of lipids whose R/f values correspond to cholesterol esters (*a*), triglycerides (*b*), free fatty acids (*c*), cholesterol (*d*), and phospholipids (*e*) on HPTLC. There was no visible band co-migrating with JH III and methoprene. Since non-polar lipids such as cholesterol esters, triglycerides, and cholesterol are not eluted from a reverse-phase LC column in a water/acetonitrile solvent system, cleanup of the extracts was required before LC. In this study, we utilized reversed-phase C18 cartridges for cleanup of termite samples. The mixture of authentic JH III (50 μg) and methoprene (50 μg) dissolved in methanol was applied to a C18 cartridge and fractionated as described in Section 2. HPTLC indicated that JH III and methoprene quantitatively passed through the C18 cartridge (Fig. 2A, lanes 2–4) and LC–ESI-MS confirmed that each recovery into Eluate 1 was 100%. After preparation of termite samples by C18 cartridges using the same protocol, HPTLC shows that Eluate 1 (Fig. 2A, lane 6) did not contain the cholesterol esters, triglycerides, and cholesterol detected in Eluates 2 and 3 (Fig. 2A, lanes 7 and 8). This C18 cartridge protocol was very effective in the preparation of termite samples for LC–ESI-MS analysis.

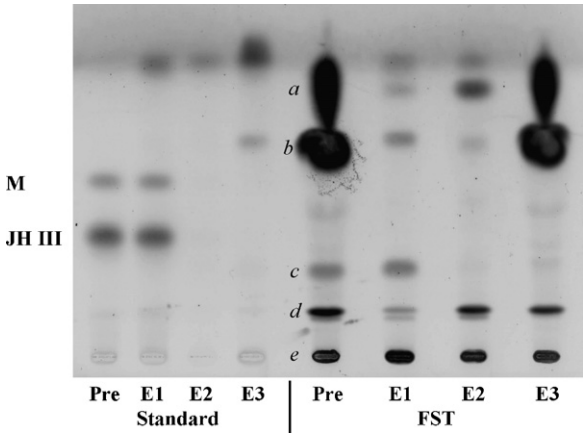

Fig. 2. The HPTLC plate shows lipids obtained from hexane extracts before (pre) and after the C18 cartridge protocol (E1, E2, and E3) of a mixture of authentic JH III and methoprene (M) and from a Formosan termite worker. Effluents 1, 2, and 3 were obtained from the cartridges by addition of 2.5 ml of methanol (E1), 1.5 ml of methanol (E2), and 4 ml of chloroform/methanol (1:1, v/v) (E3), respectively. The locations of cholesterol ester (*a*), triglyceride (*b*), free fatty acids (*c*), cholesterol (*d*), and phospholipids (*e*) are indicated.

After drying Eluate 1 from termites under a stream of nitrogen, the dried lipids were not completely solubilized using only methanol, and a white precipitate was observed. Since synthetic JH III and methoprene were easily redissolved in methanol after drying, only the methanol-soluble phase was transferred to autosampler tubes and analyzed by LC–ESI-MS for quantification of JH III.

3.2. LC–ESI-MS optimization

Previous studies with insect samples were performed by an LC–ESI-MS using a C18 LC column and a water/methanol solvent system without supplemental ions [8–12]. In the previous protocol for hemolymph, the JH III sodium adduct ion (m/z 289) was monitored and used for quantification. Based on these reports, we first tested the same protocol for whole body extracts of Formosan termite workers and found that the protocol did not work effectively in the whole body termite samples because of the following two problems. The first problem was that ion fragmentation differed when comparing spectra from termite samples (base peak, m/z 289) and authentic JH III (base peak, m/z 235) (Supplemental Fig. 1D and F). By usual accepted protocols, the same base peak (m/z 289 or 235) should be observed in mass spectra of both unknowns and standards. A second problem was that JH III does not separate as a single peak from another peak containing unknown compounds in whole body samples from termites in a mass chromatogram of m/z 289 (Supplemental Fig. 1B).

To overcome these problems, we firstly examined the effect of ion supplements on ion fragmentation of JH III. The previous study reported that intensity of the JH III sodium adduct ion (m/z 289) increased by addition of sodium ions into the solvent system [13]. However, it is widely known that the non-volatile sodium acetate precipitates at the spray needle in the ESI source and causes blockage of the flow system. We also found a confounding problem with inlet fouling because the non-volatile sodium acetate precipitates at the spray needle in the ESI source after sequential analysis of a few samples. Therefore, we abandoned sodium acetate as a supplement in LC–ESI-MS, and tested the effect of volatile chemicals, including formic acid, acetic acid, and ammonium acetate, by LC–ESI-MS using a PFP LC column and isocratic elution of water/acetonitrile (40/60). $[M+H]^+$ was not increased upon addition of 0.05% acetic acid or 0.05% formic acid (data not shown). Optimally, 1 mM ammonium acetate was employed as a supplement that enhances ionization of JH III, resulting in m/z 235 (loss of one CH_3OH from the methyl ester) becoming the base peak in both termite and authentic JH III samples with very good reproducibility (Figs. 3 and 5).

Upon chromatography of termite whole body samples using a C18 column and the solvent system including 1 mM ammonium acetate, a single peak of JH III was detected in a mass chromatogram of m/z 235, but its peak shape was broad and unsuitable for integration quantification (Fig. 4, C18). Thus, we changed the solid phase material from C18 to pentafluorophenyl (PFP) to improve the monitoring ion peak separation of JH III from other compounds. PFP columns are known to enhance interaction with aromatics, halogens, conjugated systems, and epimers and are able to often separate impurities that co-elute on a C18 LC column. In addition, we also changed the solvent system from water/methanol to water/acetonitrile, due to high backpressure on the PFP LC column. A mass chromatogram of m/z 235 showed the peak of JH III to be sharper on the PFP LC column than on the C18 LC column (Fig. 4), indicating more efficient chromatography. Based on these results, we adapted the PFP LC column as a solid improvement for quantification of JH III in termite whole body samples.

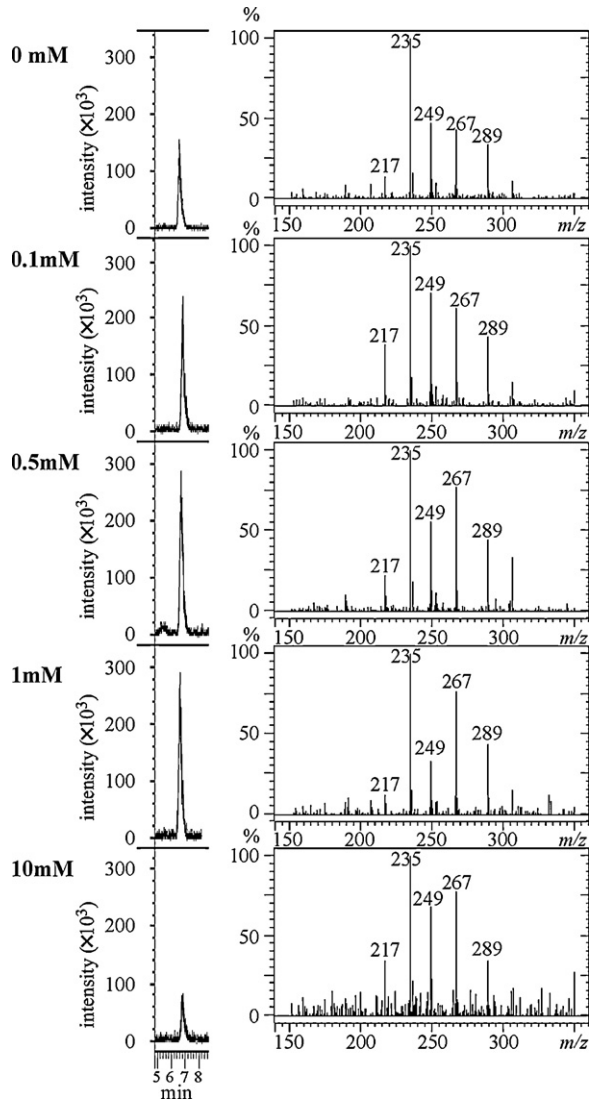

Fig. 3. The effect of ammonium acetate on ion fragmentation of JH III. JH III (1 ng) was analyzed by LC–ESI-MS using a PFP LC column and 60% acetonitrile containing 0–10 mM ammonium acetate. Left panels show the mass chromatograms of *m/z* 235. JH III is detected at 6.9 min. Right-hand panels show mass spectra. Maximum intensity of a fragment ion of JH III, $[M–CH_3OH+H]^+$ (*m/z* 235), is observed in 1 mM ammonium acetate.

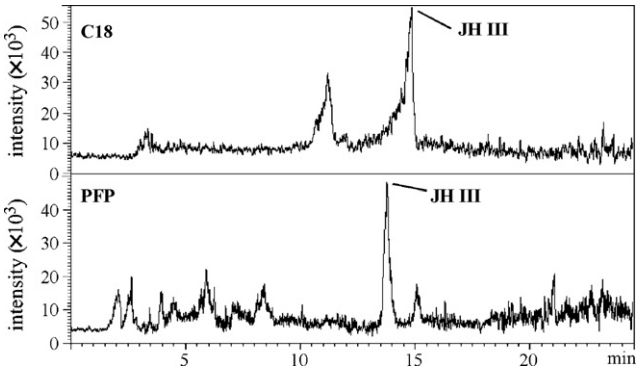

Fig. 4. Mass chromatogram of *m/z* 235 of whole body extracts of Formosan termite workers analyzed by a C18 LC column (upper panel) and a pentafluorophenyl (PFP) LC column (lower panel). The termite sample was prepared from 200 termite workers as described in Section 2 and the sample was dissolved in 100 μl of methanol and 10 of the 100 μl were analyzed by LC–ESI-MS protocols for C18 and PFP LC columns that are described in Supplemental Fig. 1 and Section 2, respectively.

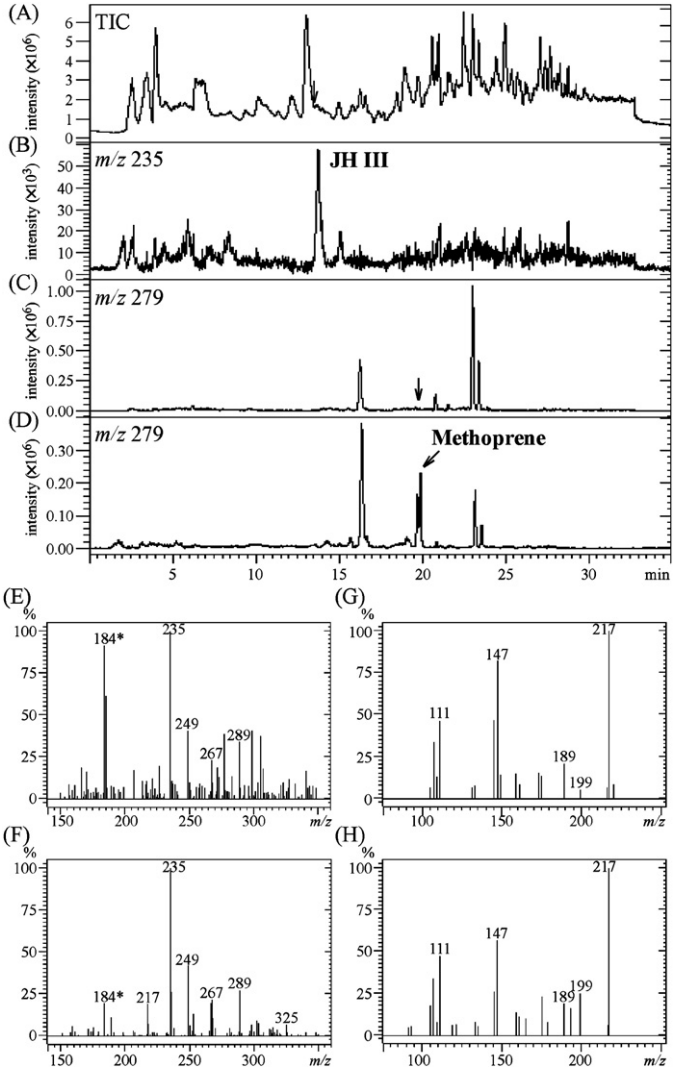

Fig. 5. LC–ESI-MS/MS^2 of whole body extracts of Formosan termite worker. (Panel A) A total ion chromatogram, (Panel B) a mass chromatogram of *m/z* 235, (Panel C) a mass chromatogram of *m/z* 279, obtained from termite samples without methoprene, and (Panel D) a mass chromatogram of *m/z* 279, obtained from termite samples with methoprene. The termite sample was prepared from 200 termite workers with or without methoprene as described in Section 2. The sample was dissolved in 100 μl of methanol and 10 of the 100 μl was analyzed by the optimized LC–ESI-MS protocol. In panel A, an arrow shows the retention time of authentic JH III. In panel B, a fragment ion of JH III, $[M–CH_3OH+H]^+$, is detected as a single peak at 14.9 min. In panel C, an arrow indicates the retention time of methoprene not detected in termite samples. In panel D, a fragment ion of methoprene, $[M–CH_3OH+H]^+$, is detected as a peak at 19.8 min. Panels E and F show mass spectra of termite and authentic JH III samples detected at 14.9 min, respectively. Panels G and H show the MS^2 spectra generated from the ion at *m/z* 235, from the termite sample detected at 14.9 min and from $[M–CH_3OH+H]^+$ of authentic JH III, respectively.

3.3. LC–ESI-MS/MS^2 analysis of authentic JH III

Fig. 5F shows a mass spectrum of authentic JH III (236 pg), analyzed by the optimized LC–ESI-MS protocol as described above. A peak containing ions from JH III, $[M–CH_3OH+H]^+$ (*m/z* 235, base peak), $[M+Na]^+$ (*m/z* 289), $[M+H]^+$ (*m/z* 267), $[M–H_2O+H]^+$ (*m/z* 249), and $[M–CH_3OH–H_2O+H]^+$ (*m/z* 217) was detected at 14.9 min. In this system, the limit of detection (LOD) of JH III is 4 pg with a minimum S/N value of 7.

At 19.8 min, a peak producing the ions from methoprene, $[M–CH_3OH+H]^+$ (*m/z* 279, base peak), $[M–CH_3OH–CH_3CCH_3+H]^+$

(m/z 237), $[M–CH_3OH–CH_3CCH_3–H_2O+H]^+$ (m/z 219), and $[M–CH_3OH–CH_3CCH_3–H_2O–CO+H]^+$ (m/z 191), was detected (data not shown).

MS^2 spectra from the $[M–CH_3OH+H]^+$ (m/z 235), fragment ion of authentic JH III showed product ions at m/z 217, 199, 189, 147, and 111 (Fig. 5H), which were assigned to be $[M–CH_3OH–H_2O+H]^+$, $[M–CH_3OH–2H_2O+H]^+$, $[M–CH_3OH–H_2O–CO+H]^+$, $[M–CH_3OH–C_2H_4O_2–C_3H_8O+H]^+$, $[C_7H_{10}O$ (scission between C6 and C7 after loss of $CH_3OH)+H]^+$, respectively, according to a previous report [7].

3.4. LC–ESI-MS/MS^2 analysis of termite samples

Fig. 5A shows a typical total ion chromatogram (TIC) of a termite sample (10/100 μl injection) that was prepared from 200 Formosan termite workers without methoprene. MS of the peak at 14.9 min shows ion m/z 235 as the base peak (Fig. 5E). The MS also shows lower abundance ions at m/z 289, 267, and 249. Although m/z 184 is detected at high intensity, it was due to a background peak also detected in standard samples.

MS^2 confirmed that the single peak detected at 14.9 min in a mass chromatogram (m/z 235) of termite samples originated from JH III. MS^2 spectra generated from m/z 235 of the termite sample provided identical fragment ions with authentic JH III (Fig. 5G), confirming that m/z 235 detected in the termite sample at 14.9 min is a non-contaminated fragment ion generated from JH III. Based on the results that m/z 235 detected in termite samples at 14.9 min is the base peak of authentic JH III and was separated as a single peak in a mass chromatogram of m/z 235 (Fig. 5B), we quantified its peak area for JH III titer. The limit of quantification (LOQ) of JH III was 22 pg with S/N values of 11 in whole body termite samples.

In MS of the termite samples, m/z 313 was detected as a major peak at 19.8 min, but no ion identical to methoprene, $[M–CH_3OH+H]^+$ (m/z 279), was detected (Fig. 5C). Therefore, we added methoprene as an internal standard (Fig. 5D).

3.5. The useful detection limit of JH III in termite samples

By using the above methods for quantification of JH III in whole body extracts of Formosan termite workers, we analyzed JH III titers of 5, 10, 20, and 30 individuals in triplicate to show linearity with actual insect samples. Each termite sample was cleaned up by a C18 cartridge adsorption after hexane extraction, dissolved in 16 μl of methanol, and 10 μl of the sample was injected for LC–ESI-MS. A mass chromatogram of m/z 235 produced a single peak of JH III at 14.9 min with S/N values of 23 ± 7:1, 51 ± 6:1, 58 ± 3:1, and 76 ± 9:1 in 5, 10, 20, and 30 termite workers, respectively (Supplemental Fig. 2). The JH III amounts found in 5, 10, 20, and 30 termite workers were 190.2 ± 44.1, 346.6 ± 33.1, 561.9 ± 11.2, and 789.0 ± 30.6 pg, respectively. The amount of JH III was only 4-fold higher in 30 termites than 5 termites, although their numbers were 6-fold different. The values of JH III for 5 termites were 117.2, 196.0, and 274.6, and these values give a wide range of S.E. as compared to that of 20 and 30 termites. We suggest the reason for this, as mentioned by previous reports, that there is a significant individual variation in JH III titer in each worker termite and such variation may be involved in caste differentiation in the species. On the other hand, such a gap disappears when sample numbers increased: JH III was 1.4-fold higher in 30 termites than 20 termites when the numbers were increased to 1.5-fold higher.

Next, we attempted detection of JH III in single termites. Fig. 6B shows an example of a mass chromatogram at m/z 235 in a sample prepared from a single termite worker (wet body weight; 3.4 mg). A peak corresponding to the principal fragment ion of JH III, $[M–CH_3OH+H]^+$ (m/z 235), was detected at the retention time of authentic JH III with a S/N value of 12. The JH III titer of this termite worker was 34.7 pg (10.2 pg/mg in wet body weight) is in the order of magnitude with a previous report that the average titer of JH III in Formosan termite workers (by examining an aggregate from a number of termites) was 13 pg/mg in wet body weight [16]. This opens the door for study of individual differences according to maturity, caste development, and colony condition.

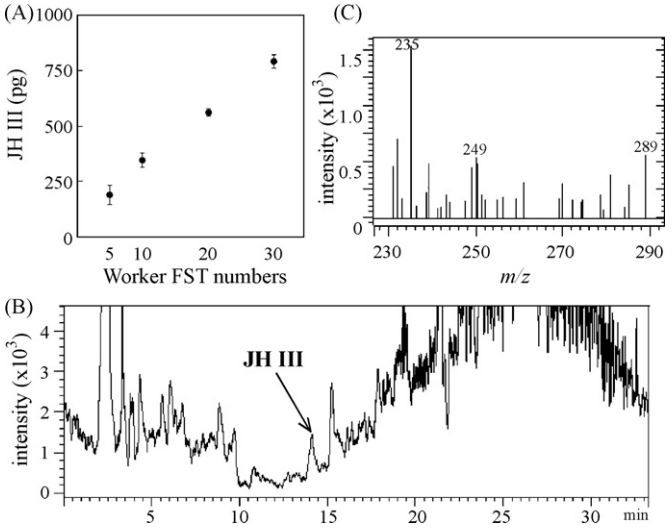

Fig. 6. The detection limit of JH III in termite samples. (Panel A) JH III amounts determined from whole body extracts of 5, 10, 20, and 30 Formosan termite workers by LC–ESI-MS. Bars represent mean ± S.E. (n = 3). (Panel B) A mass chromatogram of m/z 235 of a sample from a termite worker. JH III is detected at the retention time of authentic JH III. (Panel C) Mass spectra detected at 14.2 min in the single termite sample.

In this study, we performed ESI-MS using the range between m/z 230 and 290 for quantification of JH III. The MS range was chosen to include the major ions of JH III, m/z 235, 249 and 267, in addition to the fragment ion of methoprene, m/z 279. We also tested the multiple reaction monitoring (MRM) method for the quantification and found that that detection limits for JH III were higher in the MRM method (156 pg) than in MS^1 (8 pg) in standard JH III samples. Comparing signal/noise in 20 termite samples, the MRM method gave a slightly better result (S/N = 56, by using the sum of all product ions, m/z 111 + 147 + 189 + 199 + 217, from precursor ion, m/z 235) than the MRM method (S/N = 25, by using only a fragment ion, m/z 217) and MS, m/z 235 (S/N = 52) (Supplemental Fig. 3). We suggest that the reason is that an ion for MS^2, m/z 235, is a fragment ion from a precursor ion $[M+H]^+$. Therefore, we concluded that MS^1 is better for high sensitivity detection of JH III than the MRM method in termite samples.

Previous studies have used GC–EI-MS to quantify JH III in whole body extracts of Formosan termites [15,16]. This study shows the C18 cartridge preparation and LC–ESI-MS using a PFP LC column and a water/acetonitrile solvent containing 1 mM ammonium acetate can be used to quantify JH III at higher sensitivity and more rapidly as compared to GC–EI-MS protocol. Sample preparation for termites for GC–EI-MS comprised four preparation steps, including hexane extraction, aluminium oxide gel chromatography, d3 methoxy-hydrin derivatization, followed by a second aluminium oxide gel chromatography, and requiring more than 50 termite workers to quantify JH III. In contrast, the herein described LC–ESI-MS protocol comprises two steps of sample preparation, hexane extraction and reverse-phase C18 cartridge extracts, resulting in a simple protocol that enables us to quantify JH III in a single For-

mosan termite worker, with excellent linearity as tested up to 30 termites.

4. Conclusion

A simplified and optimized method for sample preparation and LC–ESI-MS quantitative analysis of JH III in insect whole body extractions is presented. A pentafluorophenyl LC column was used with a water/acetonitrile solvent containing ammonium acetate. The mass chromatogram at m/z 235 was monitored to detect JH III, $[M–CH_3OH+H]^+$, for quantification of JH III. A single termite worker contains enough JH III for quantitation by this method with a S/N >10:1. While signal-to-noise levels for ultimate sensitivity of 3:1 are often cited, this is not an useful level. Individual variations in all castes and inter-developmental changes in JH III levels in castes can now be examined under a number of environmental conditions and caste ratios. We believe that this optimized LC–ESI-MS method for quantification of JH III will be also useful for investigators working on other insects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.08.008.

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