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Fast, ultra-trace detection of juvenile hormone III from mosquitoes using mass spectrometry

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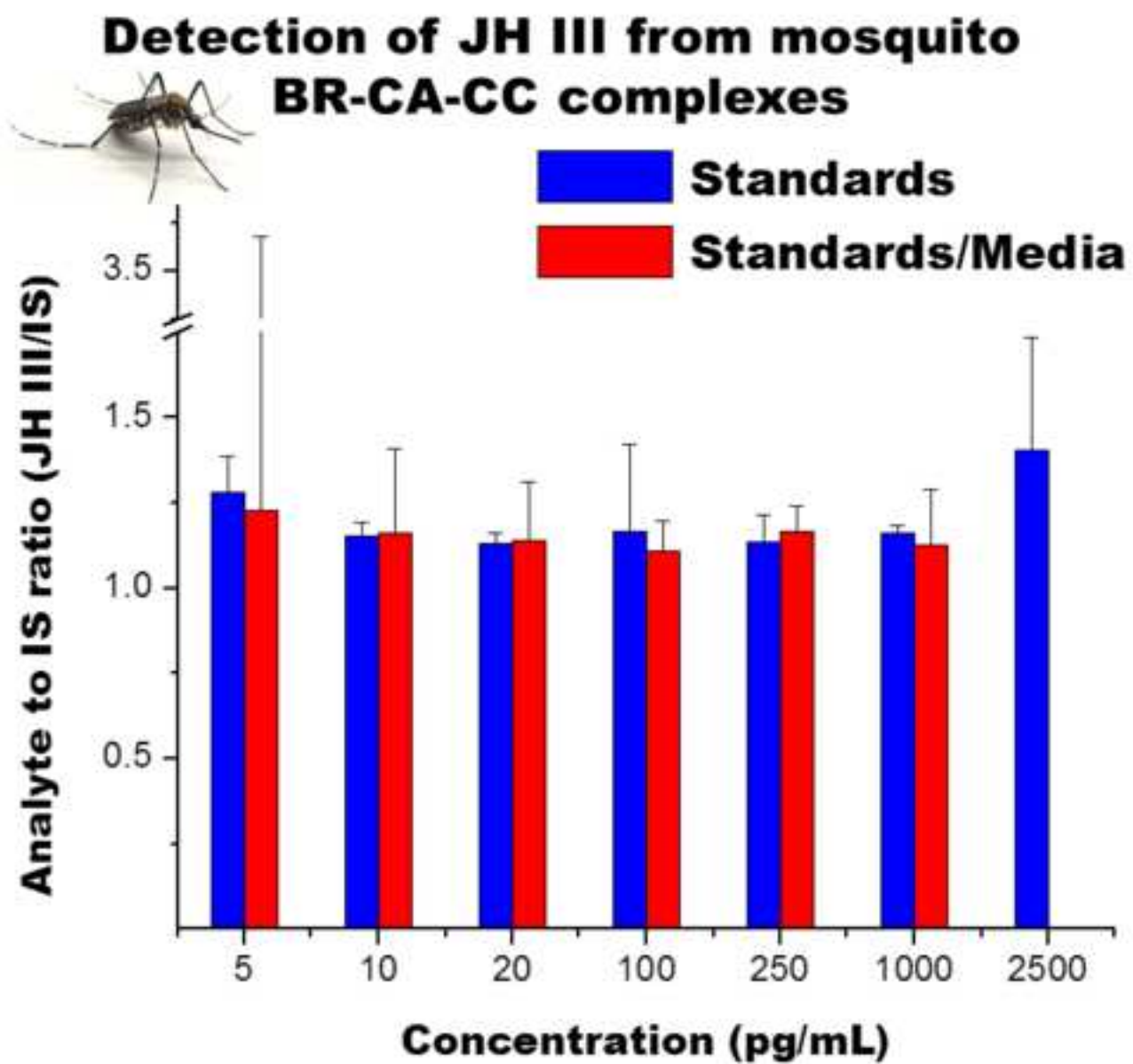
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Highlights

- A new protocol for fast separation and quantification of JH III from biological samples using liquid chromatography coupled to electrospray tandem mass spectrometry is described.
- the proposed protocol improves existing methodologies by combining a limited number of sample preparation steps with fast LC-MS/MS detection, providing a 8 pg/mL (0.32 pg on column) limit of detection (15-fold gain in sensitivity) with high inter and intraday reproducibility.
- A detailed description of the JH III fragmentation pathway is provided for the first time, based on isolation of the molecular ion and their intermediate fragments using in-source MS/MS, MS/MSⁿ and FT-ICR MS/MS measurements.
- The performance of the LC-MS/MS protocol is comparable to previously described JH III quantitation protocol based on fluorescence detection, with the added advantage that quantification is independent of the availability of fluorescent tags that are often unavailable or show quite diverse responses on a batch-to-batch basis.
- The JH III workflow was evaluated as a function of developmental changes, sugar feeding and farnesoic acid stimulation in mosquitoes.



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1 **Fast, ultra-trace detection of Juvenile Hormone III from mosquitoes using**
2 **mass spectrometry**

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10 **Notes**

11 The authors declare no competing financial interest.

12 **ABSTRACT**

13 In the present work, a new protocol for fast separation and quantification of JH III from
14 biological samples using liquid chromatography coupled to electrospray tandem mass
15 spectrometry is described. In particular, the proposed protocol improves existing methodologies
16 by combining a limited number of sample preparation steps with fast LC-MS/MS detection,
17 providing lower limits of detection and demonstrated matrix effect control, together with high
18 inter and intraday reproducibility. A limit of detection of 8 pg/mL (0.32 pg on column) was
19 achieved, representing a 15-fold gain in sensitivity with respect to previous LC-MS based
20 protocols. The performance of the LC-MS/MS protocol is comparable to previously described
21 JH III quantitation protocol based on fluorescence detection, with the added advantage that
22 quantification is independent of the availability of fluorescent tags that are often unavailable or
23 show quite diverse responses on a batch-to-batch basis. Additionally, a detailed description of
24 the JH III fragmentation pathway is provided for the first time, based on isolation of the

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25 molecular ion and their intermediate fragments using in-source MS/MS, MS/MSⁿ and FT-ICR
26 MS/MS measurements. The JH III workflow was evaluated as a function of developmental
27 changes, sugar feeding and farnesoic acid stimulation in mosquitoes and can be applied to the
28 detection of other juvenile hormones.

29 **Keywords: Juvenile hormone III, Liquid chromatography, MRM, quantification,**
30 **extraction.**

31 **INTRODUCTION**

32 Juvenile hormones (JHs) are synthesized by the *corpora allata* (CA) and play key roles in
33 many processes in insect development and reproduction, including inhibition of metamorphosis,
34 caste determination and differentiation, stimulation of flight and migration, stimulation of
35 reproduction, regulation of diapause, stress resistance, and aging[1-6]. Several JHs have been
36 identified and characterized in insects, with JH III being the most widespread[7-9, 6]. A common
37 structural feature for all JHs is the presence of an epoxide group near one end and a methyl ester
38 on the other. JH titers in small insects are often in the femtomole to picomole range, which
39 makes it challenging to detect by most typical analytical techniques[10-14].

40 The most widely used analytical methods for identification and detection of JHs include
41 nuclear magnetic resonance (NMR), infrared spectroscopy (IR), and gas chromatography (GC)–
42 mass spectrometry (MS) [14-19]. During GC-MS, fragment ions from the electron or chemical
43 ionization process are typically used to identify the JH molecules; however, this analysis
44 typically requires lengthy preparation steps[20, 19, 21-23]. More recently, several studies have
45 shown the advantages of a number of additional techniques for the identification and
46 quantification of JHs, such as direct analysis in real time-MS (DART-MS), high performance
47 liquid chromatography-MS/MS (HPLC- MS/MS), HPLC with fluorescence detection (HPLC-
48 FD) and ultra-performance liquid chromatography-MS (UPLC-MS)[18, 24-32]. A variety of
49 ionization sources have been utilized prior to the MS analysis, such as electrospray ionization
50 (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization
51 (APPI) and atmospheric pressure thermospray ionization (APTSI); allowing the detection of the
52 JH III molecular ion in the protonated and sodiated forms[33]. While some studies showed the

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4 53 advantages of using the sodiated species for quantification in single ion monitoring mode (SIM),
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6 54 only the protonated species allowed for multiple reaction monitoring (MRM)[25].
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8 55 In the present work, we describe an extraction protocol followed by an HPLC-ESI-
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10 56 MS/MS analysis that increased sensitivity and reproducibility, while reducing the analysis time
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12 57 for JH III detection in mosquito samples. The features of the method proposed are compared
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14 58 with previously established MS- and FD-based methods. Additionally, a detailed description of
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16 59 the fragmentation behavior of JH III $[M+H]^+$ molecular ion is described for the first time.
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18 60 MATERIALS AND METHODS

21 61 Materials and reagents

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24 62 Certified standard solutions for JH III and its deuterated analog (JH III-D3) were
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26 63 obtained from Toronto Research Chemicals (Toronto, Canada). Sodium chloride, potassium
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28 64 chloride, hydrochloric acid, sodium hydroxide, ammonium acetate, ammonium formate and
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30 65 ammonium hydroxide salts were analytical grade or better (Fisher Scientific, Pittsburgh, PA).
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32 66 Water, methanol, hexane and acetonitrile were all Optima grade or better (Fisher Scientific).
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34 67 Chromatographic mobile phases (0.1% formic acid in water, and 0.1% formic acid in
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36 68 acetonitrile) of Optima LC-MS grade were also purchased from Fisher Scientific, and used as
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38 69 received. Tissue culture media Gibco M-199, silanized LC vials and silanized LC vials with
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40 70 fused 250 μ L inserts were also purchased from Fisher Scientific. The tuning mix calibration
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42 71 standard (G24221A) was obtained from Agilent Technologies (Santa Clara, CA).
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44 72 Sample preparation and storage

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46 73 Biological samples were prepared following the protocol described in Figure 1. Briefly,
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48 74 preparations were of intact *corpora allata-corpora cardiaca* (CA-CC) complexes connected to
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50 75 the brain and head capsule, and are denoted as BR-CA-CC complexes. BR-CA-CC were
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52 76 dissected in a drop of mosquito saline-buffer containing 138 mM NaCl, 8.4 mM KCl, 4 mM
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54 77 $CaCl_2$, 12 mM NaH_2PO_4 and 42.5 mM sucrose[34]. After dissection, the BR-CA-CC complexes
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56 78 were incubated in 150 μ L of tissue culture media M-199, containing 2% Ficoll 400 and 50 μ M
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58 79 methionine. Incubations of BR-CA-CC complexes were carried out in a humid chamber in
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60 80 silanized 2 mL vials for 4 h in the dark at 32°C, and under continuous gentle agitation. After
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4 81 incubation, 10 μ L of 6.25 ppb JH III-D3 in acetonitrile were added to each sample, followed by
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6 82 600 μ L of Hexane. Samples were vortexed for 1 minute, and spun for 5 minutes at 4°C and 2000
7
8 83 g. The organic phase was transferred to a new silanized vial and dried under nitrogen flow. Dried
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10 84 extracts were re-suspended in 100 μ L of acetonitrile, vortexed 1 minute, transferred to a new
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12 85 silanized vial with a fused 250 μ L insert and stored at -20°C.
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14 86 **Mass Spectrometry Analysis**

17 87 The mass spectrometry analyses were carried out in-house using HPLC-MS/MS. Briefly,
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19 88 sample injections (40 μ L) and LC separations were performed by a Prominence LC-20AD Ultra-
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21 89 Fast Liquid Chromatograph (Shimadzu, Kyoto, Japan), equipped with a Dionex Acclaim 120
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23 90 C18 Column (250x2.1 mm, 5 μ m) obtained from Thermo Scientific (Sunnyvale, CA). Column
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25 91 temperature was kept at 40°C. A 15 minutes binary gradient program between 0.1% formic acid
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27 92 dissolved in water (mobile phase A) and 0.1% formic acid dissolved in acetonitrile (mobile
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29 93 phase B) was run according to the following timetable: hold 5% B for 0.5 min; ramp to 98% B in
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31 94 7.5 min; hold 98% B for 3 min; return to 5% B in 0.5 min; hold 5% B for 3.5 min. Flow rate was
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33 95 constant at 0.8 mL/min. Detection was performed by a QTRAP® 5500 triple quadrupole mass
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35 96 spectrometer (AB Sciex, Ontario, Canada) equipped with a Turbo V™ ion source. The mass
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37 97 spectrometer was operated under ESI positive mode ionization with multiple reaction monitoring
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39 98 (MRM) of two transitions per compound. MRM detection and electrospray source parameters
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41 100 were optimized by infusing 1 mg/L solutions of each compound in 0.1% formic acid in
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43 101 acetonitrile. Source parameters were: curtain gas = 10 psi; spray Voltage = 5000 V; temperature
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45 102 400°C; ion source gas 1= 40 psi; ion source gas 2= 50 psi; entrance potential = 7.0 V. MRM
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47 103 transitions parameters are listed in Table 1.

48 104 JH III and JH III-D3 chemical structures and purities were confirmed by MS/MS and
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50 105 accurate mass measurements using Fourier transform ion cyclotron resonance-MS (nanoESI-FT-
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52 106 ICR MS). In-source fragmentation and MS/MSⁿ capabilities in the QTRAP® 5500 triple
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54 107 quadrupole mass spectrometer (AB Sciex, Ontario, Canada) were used to determine the
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56 108 fragmentation pathways of JH III and JH III-D3 (see Table 1 and 2). nanoESI FT-ICR MS/MS
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58 109 experiments were performed in positive ion mode in a 7T Solarix FT-ICR MS spectrometer
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60 110 (Bruker Daltonics, Inc., Billerica, MA). Ion transmission was optimized for high sensitivity for
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62 JH III and its fragments in the m/z 100–300 range. Ions were accumulated in the collision cell (2
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4 111 MHz, 1000 Vpp) for 4s. FT-ICR MS spectra were acquired over 200-time domain acquisitions at
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6 112 2 Mword (1 s transient). FT-ICR signals were processed using a half-sine apodization followed
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8 113 by Fast-Fourier transform and broadband phase correction (absorption spectra using absorption
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10 114 mode processing, AMP), resulting in a 2-fold increase in mass resolution.
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12 115 **RESULTS AND DISCUSSION**

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14
15 116 The main aim of this study was to develop a protocol for fast and accurate measurement
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17 117 of JH III from biological samples using mass spectrometry. In particular, our efforts were
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19 118 focused on: 1) minimizing the number of steps during sample preparation prior to the MS
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21 119 analysis, 2) developing a fast LC-MS/MS protocol, 3) improving sample storage protocols, 4)
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23 120 evaluate inter and intraday analysis variation, 5) refining the interpretation of the JH III MS/MS
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25 121 fragmentation pathway, 6) enhancing limits of detection while reducing matrix effects, and 7)
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27 122 comparing the performance with a previously utilized JH III quantitation protocol based on
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29 123 fluorescence detection[30].
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31 124 **Mass Spectrometry Analysis**

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34 125 JH III can be typically detected in the protonated and sodiated forms (e.g., $m/z = 267$
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36 126 $[M+H]^+$ and $m/z = 289 [M+Na]^+$), depending on the spraying and solvent conditions[24]. Since
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38 127 the fragmentation of the sodiated molecular ion $[M+Na]^+$ does not produce diagnostic fragment
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40 128 ions, previous studies have preferred LC-MS for JH identification and quantitation, with the
41
42 129 caveat that quantification accuracy may be compromised by potential interferences due to the
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44 130 complex nature of the biological sample[28, 29]. Alternatively, the fragmentation of the
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46 131 protonated form $m/z = 267 [M+H]^+$ provides a variety of signature fragmentation pathways that
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48 132 can be used to identify the JH III structure (Figure 2 top). The $[M+H]^+$ molecular ion can
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50 133 undergo fragmentation via collision induced activation using in-source MS/MS and the MS/MSⁿ
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52 134 linear trap region of the LC-QQQ (see Table 2). Inspection of the in-source and MS/MSⁿ data
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54 135 permitted the construction of the JH III $[M+H]^+$ fragmentation pathway (Figure 3). Upon
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56 136 collision induced activation, the JH III $[M+H]^+$ main fragmentation pathways lead to the
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58 137 observation of $m/z = 235 [M+H-CH_3OH]^+$ and $m/z = 249 [M+H-H_2O]^+$ ions, corresponding to the
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60 138 loss of one methanol group and one water molecule, respectively. Further activation of $m/z = 235$
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62 139 and 249 generates $m/z = 217 [M+H-CH_3OH-H_2O]^+$ and $m/z = 207 [M+H-CH_3OH-CO]^+$,
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4 140 corresponding to the combined loss of one methanol group and water, and to the loss of a CO
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6 141 group, respectively. In contrast to previous reports[25], our data support the generation of $m/z =$
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8 142 217 as a product of 267->235->217 and 267->249->217. The activation of $m/z = 217$ produces
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10 143 $m/z = 189$ $[M+H-CH_3OH-H_2O-CO]^+$, which further fragments into $m/z = 147$ $[M+H-CH_3OH-$
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12 144 $H_2O-CO-CH_3CH=CH_2]^+$. Complementary analysis using nESI-FT-ICR MS/MS supports the
13
14 145 above proposed mechanism. The nESI-FT-ICR MS/MS showed the fragmentation of $[M+H]^+$
15 146 ($m/z = 267.195461$, $C_{16}H_{27}O_3$) into $[M+H-H_2O]^+$ ($m/z = 249.18492$, $C_{16}H_{25}O_2$), $[M+H-CH_3OH]^+$
16
17 147 ($m/z = 235.16929$, $C_{16}H_{23}O_2$), $[M+H-CH_3OH-H_2O]^+$ ($m/z = 217.158786$, $C_{15}H_{21}O$), $[M+H-$
18
19 148 $CH_3OH-CO]^+$ ($m/z = 207.174417$, $C_{14}H_{23}O$), $[M+H-CH_3OH-H_2O-CO]^+$ ($m/z = 189.163858$,
20
21 149 $C_{14}H_{21}$) and $[M+H-CH_3OH-H_2O-CO-CH_3CH=CH_2]^+$ ($m/z = 147.116827$, $C_{11}H_{15}$) with sub-ppm
22
23 150 mass accuracy (see Table 2). Previous studies utilizing chemical ionization ion trap mass
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25 151 spectrometry, performed the quantification based on the summed intensities of six diagnostic
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27 152 ions ($m/z = 235, 217, 189, 147, 125, \text{ and } 111$)[19]. Although this approach is also feasible in the
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29 153 case of LC-QQQ instruments, using a lower number of MRM transitions allows the monitoring
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31 154 of a higher number of points across the LC peak relative to the aforementioned MRM strategies
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33 155 and therefore provides higher sensitivity. The selection of the transitions to perform the JH III
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35 156 quantification was based on their relative abundance. That is, the most abundant fragmentation
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37 157 transitions utilized were 267->235 (primary) and 267-> 147 (secondary). Previous reports have
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39 158 also utilized 267-> 235 as a primary and 267-> 217 as secondary MRM transitions for LC-
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41 159 MS/MS analysis[25, 27].

42 160 The heavy isotopomer JH III-D3 was utilized as an internal standard to normalize all
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44 161 sample preparation, extraction and analysis steps in order to accurately quantify the amount of
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46 162 JH III hormone produced by the BR-CA-CC complexes. The JH III-D3 molecular ion was
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48 163 observed in the protonated form $[D+H]^+$, and the stoichiometry was confirmed utilizing nESI-
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50 164 FT-ICR MS ($m/z = 270.21420$) with sub-ppm accuracy. The fragmentation of the JH III-D3
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52 165 $[D+H]^+$ molecular ion showed multiple similarities to JH III $[M+H]^+$ (Figure 2). That is, the loss
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54 166 of the deuterated methanol group led to the observation of $m/z = 235$ $[M+H-CD_3OH]^+$; further
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56 167 activation showed the same fragmentation pattern as the JH III $[M+H]^+$ molecular ion.

57 168 A plausible mechanistic sequence for the generation of JH III fragment ions is shown in
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59 169 Figure 3 and in the supplementary materials. Briefly, it is proposed that opening of the oxirane to
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61 170 form a tertiary alcohol and an alkene would facilitate the elimination of water (perhaps, via a 6-

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4 171 electron cyclic transition state) leading to the formation of $m/z = 249$ $[M+H-H_2O]^+$ from $m/z =$
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6 172 267 $[M+H]^+$ and $m/z = 217$ $[M+H-CH_3OH-CO]^+$ from $m/z = 235$ $[M+H-CH_3OH]^+$. Similarly,
7
8 173 elimination of methanol accounts for the formation of both $m/z = 235$ $[M+H-CH_3OH]^+$ from m/z
9
10 174 $= 267$ $[M+H]^+$ and $m/z = 217$ $[M+H-CH_3OH-CO]^+$ from $m/z = 249$ $[M+H-H_2O]^+$. The loss of
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12 175 methanol is confirmed by the aforementioned isotopic labeling study with JH III-D3. In each
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14 176 case, the exclusion of the methanol generates a molecular ion in which the positive charge will
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16 177 reside on the $C\equiv O$ oxygen. The subsequent loss of CO from $m/z = 217$ $[M+H-CH_3OH-CO]^+$ to
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18 178 form $m/z = 189$ observed by the nESI-FT-ICR MS/MS analysis is unlikely to occur on the basis
19
20 179 of direct cleavage from an sp^2 carbon. Thus, it is proposed that a rearrangement of the C-2 double
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22 180 bond occurs, allowing the CO to leave with the generation of a resonance-stabilized allylic
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24 181 carbocation that has the potential to undergo a Wagner-Meerwein hydride shift to form an even
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26 182 more stable allylic carbocation in which the positive charge is delocalized over an entire 7-
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28 183 carbon conjugated triene array. Whether the rearrangement and elimination are separate steps or
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30 184 a concerted process is not clear. Further rearrangement of this system allows the elimination of
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32 185 propene as a neutral fragment generating $m/z = 147$ $[M+H-CH_3OH-H_2O-CO-CH_3CH=CH_2]^+$ as
33
34 186 an even more delocalized allylic carbocation in which the positive charge can be delocalized
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36 187 over the entire 9-carbon tetraene unit. The loss of CO from $m/z = 235$ $[M+H-CH_3OH]^+$ to
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38 188 generate $m/z = 207$ $[M+H-CH_3OH-CO]^+$ is proposed to occur via a similar initial rearrangement
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40 189 of the C-2 double bond. The resultant allylic carbocation will also be resonance stabilized. It is
41
42 190 unclear whether the loss of CO occurs on the oxirane or the rearranged oxirane or both.

42 191 **HPLC-MS/MS method validation**

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44 192 The HPLC-MS/MS workflow was developed using standards and biological samples,
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46 193 with the extraction protocol described in Figure 1. The HPLC consisted of a short 15 minutes run
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48 194 with JH III eluting at around 8.2 minutes (Figure 4). As expected, JH III and its heavy analog JH
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50 195 III-D3 co-eluted, and their detection was based on monitoring the $267 \rightarrow 235$ and $270 \rightarrow 235$ as
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52 196 primary and $267 \rightarrow 147$ and $270 \rightarrow 147$ as secondary transitions. Notice that the primary MS/MS
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54 197 channel is 4-5 fold more abundant than the secondary channel. Comparison between the standard
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56 198 and the biological samples showed no changes on retention time, as well as on the ratio between
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58 199 transitions, allowing the use of these parameters for analyte confirmation purposes.

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4 200 The effect of analyte extraction was evaluated by comparing the instrument response of
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6 201 analyte to internal standard ratio (JH III/ JH III-D3), for pure- and media-extracted standards
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8 202 over a wide concentration range (Figure 5a). An extraction recovery of near 55% was routinely
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10 203 observed regardless of the analyte concentration. No improvements were observed by
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12 204 performing a double extraction with hexane (Figure 5b). The reduced sensitivity caused by the
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14 205 extraction efficiency is evidenced in the reduced slope on the linear response of the instrument as
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16 206 a function of analyte concentration (Figure 5c), justifying the use of JH III-D3 to normalize this
17 207 step.

18
19 208 The HPLC-MS/MS method limit of detection (LOD), defined as 3 times the background
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21 209 analytical response, was determined experimentally to be 8 pg/mL (0.32 pg on column) by
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23 210 spiking increasing quantities of JH III in ACN (at 5, 10, 20, 100, 250 and 1000 pg/mL in
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25 211 triplicates, n=18), and calculating the uncertainty on the intercept of the obtained curve. A LOD
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27 212 of 19 pg/mL (0.76 pg on column) was obtained by spiking the analyte into M-199 medium and
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29 213 submitting the prepared solutions to the complete extraction protocol; this higher LOD is a
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31 214 consequence of a reduced signal after extraction. However, the obtained protocol LOD is
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33 215 approximately 10 times lower than previous LC-MS based methodologies for the detection of JH
34 216 III in hemolymph (LOD = of 4 -5 pg on column[29, 27, 31]).

35 217 Inter and intra-day reproducibility was evaluated by extracting and analyzing spiked M-
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37 218 199 media with 500 pg/mL of JH III. The protocol was halted at the drying step (Figure 1) and
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39 219 the extracted standards were stored dry for 0 (reference, n=32), 1, 4, 5 and 7 days (n=5 each).
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41 220 There were no significant differences between the measurements in terms of peak areas or
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43 221 recovery (Figure 6a), which suggested that the analyte extracted from spiked media could be
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45 222 stored dry for at least 7 days. Most intra-day variability occurred between one standard deviation
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47 223 from the pooled mean of all measurements. A relative standard deviation (RSD) of 10% or lower
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49 224 was obtained for each analysis day, while an RSD of 16% was obtained in the complete dataset.

50 51 225 **Analysis of JH III from biological samples**

52
53 226 In order to evaluate the applicability of this analysis protocol, JH III synthesized *in vitro*
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55 227 by mosquito BR-CA-CC complexes under different developmental and physiological conditions
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57 228 were analyzed. Samples were prepared following the protocol previously described (Figure 1).
58
59 229 Calibration curves were obtained by plotting the peak area ratio of JH III to JH III-D3 as a

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function of the analyte concentration. Linearity was observed over a wide concentration range ($R^2 > 0.999$; 5 to 2500 pg/mL, 0.2 pg to 100 pg on column). The concentration of JH III-D3 was constant in biological samples and calibration solutions (625 pg/mL). Calibration standards were analyzed in duplicate at the beginning and at the end of each analytical sequence, with calibration verification performed every 12 runs by injecting 250 pg/mL of JH III in acetonitrile buffer, allowing a 15% deviation. A control blank (non-extracted) was analyzed with every batch, consistently displaying the absence of JH III signal. The analysis sequence also included carryover tests by verifying the absence of a JH III signal upon injecting the control blank after the highest calibration standard, as well as after every calibration verification standard.

In each LC-QQQ analysis batch, duplicates of a negative control and two concentrations of positive controls (50 pg/mL and 500 pg/mL) were extracted according to Figure 1 and measured, with deviations of 15% or lower consistently observed. JH III compound identifications were considered positive when a signal-to-noise ratio larger than 3 were present in both the quantification and confirmation MRM transitions, with a difference of less than 0.05 min in retention time relative to that of the JH III-D3. The results of the analysis of over 1500 samples revealed that the confirmation ratios for internal standards and biological samples were constantly within two standard deviations (Figure 5d).

In order to investigate the effect of sample storage on analyte signal, 10 biological samples were split after hexane extraction and the resulting extract aliquots were dried under nitrogen. One of the dried aliquots was stored at 4°C and the second was reconstituted in acetonitrile for immediate analysis. Five of the stored aliquots were reconstituted and analyzed after 5 days, while the remaining samples were analyzed after 30 days of storage. There were no significant differences in terms of recovery relative to the initial measured concentration (Figure 6b) as a function of storage time, suggesting that dried biological samples can be correctly quantified after a month of dry storage. Previous work has shown that JH III is stable for up to one month and up to six 6 months in methanol if stored at 4°C and -18°C, respectively[25]. Similar stabilities were detected when JH III was stored in hexane and acetonitrile. However, the possibility to store dry samples largely facilitates the handling and shipping for inter-laboratory comparisons and complementary measurements.

Further validation of the JH III quantitation protocol was attained by analyzing JH III synthesis by BR-CA-CC adult female mosquito preparations *in vitro* as a function of

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4 261 developmental changes, sugar feeding and farnesoic acid stimulation. The results of these
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6 262 experiments were compared with previous studies using HPLC-FD[35-37]. The comparison of
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8 263 the MS and the FD results showed good agreement for all the cases, considered reinforcing the
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10 264 value of the new developed protocol to assess JH III levels in mosquitoes (Figure 7). The new
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12 265 LC-MS/MS protocol showed similar high sensitivity, accuracy and reproducibility compared to
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14 266 the previously reported HPLC-FD[30], with the advantage of having selective MS detection that
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16 267 enables absolute quantification by using a heavy analog as an internal standard. The HPLC-FD
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18 268 protocol, while also sensitive, depends on commercially made tags that are often unavailable,
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20 269 require derivatization steps and show quite diverse responses on a batch-to-batch basis. On the
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22 270 contrary, this LC-MS/MS protocol includes a straightforward extraction protocol followed by a
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24 271 sensitive and reproducible HPLC-ESI-MS/MS analysis for absolute JH III detection in mosquito
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26 272 samples.

27 **273 CONCLUSIONS**

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30 274 An analytical workflow for fast, ultra-trace quantitation of JH III from biological samples was
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32 275 developed. The fragmentation pathway of JH III [M+H]⁺ molecular ion was studied, and a
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34 276 mechanistic model is proposed. A HPLC-MS/MS workflow based on MRM using 267-> 235
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36 277 and 267 -> 147 was optimized for quantitative analysis, with higher sensitivity and a reduced
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38 278 number of sample preparation steps. A better analytical performance of the proposed protocol
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40 279 was demonstrated in terms of reproducibility, sensitivity and routine applicability, with higher
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42 280 sensitivity than previous LC-MS applications for the detection of JH III and with similar
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44 281 sensitivity to spectrofluorometric methods without the need of lengthy derivatization steps.
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46 282 Results showed that the storage protocol allows for quantification of samples with at least one
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48 283 month of dry storage. Further developments of the proposed workflow can be used to the
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50 284 analysis of other juvenile hormones.

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56 287 FGN and R00GM106414 to FFL) and the Advanced Mass Spectrometry Facility of Florida
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58 288 International University.

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4 289 **SUPPORTING INFORMATION**

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7 290 The proposed mechanisms of fragmentations are outlined in the supplementary material. Figure
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9 291 S1. Tentative fragmentation pathway of m/z 267 to m/z 235 and m/z 217. Figure S2. Tentative
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11 292 fragmentation pathway of m/z 235 to m/z 207. Figure S3. Tentative fragmentation pathway of
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13 293 m/z 217 to m/z 189.

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15 294 **FIGURE CAPTIONS**

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18 295 **Figure 1.** Protocol for extraction and analysis of JH III synthesized *in vitro* by mosquito BR-CA-
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20 296 CC preparations. BR-CA-CC: brain *corpora allata-corpora cardiaca*. ACN: acetonitrile.

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23 297 **Figure 2.** MS/MS fragmentation spectra for JH III [M+H]⁺ and JH III-D3 [D+H]⁺.

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26 298 **Figure 3.** Fragmentation scheme for JH III [M+H]⁺ and JH III-D3 [D+H]⁺ proposed based on
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28 299 isolation of the molecular ion and fragment ions using in- source MS/MS, MS/MSⁿ and FT-ICR
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30 300 MS/MS.

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33 301 **Figure 4.** Typical ion extracted chromatogram for JH III from a biological sample (top) and from
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35 302 a standard solution (bottom) using the 267-> 235 (primary) and the 267->147 (confirmation)
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37 303 MRM transitions. In the inset, the corresponding chromatograms for JH III-D3 are shown.

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39 304 **Figure 5.** Response curves as a function of the JH III concentration in the sample. A) Analyte to
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41 305 internal standard ratio (JH III/ JH III-D3) for non-extracted and extracted samples; b) extraction
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43 306 recovery for single and double extraction; c) method response for non-extracted and extracted
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45 307 samples; and d) confirmation ratio for internal standards and biological samples.

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47 308 **Figure 6.** Intra- and inter-day effects of sample storage on analyte signal responses from
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49 309 standards and biological extracts as a function of storage time. a) Comparisons of peak areas of
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51 310 JH III and JH III-D3 after samples were dried and stored at 4°C. b and c) Comparison of analyte
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53 311 recovery (expressed as %) as a function of storage time when standards (JH III) (b) or biological
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55 312 samples (c) were analyzed.

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58 313 **Figure 7.** Comparison of FD and MS methods to quantify JH III synthesis as a function of: A)
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60 314 Developmental changes. Synthesis was evaluated from BR-CA-CC preparations dissected from

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315 pupae and adult female mosquitoes at different hours before and after adult eclosion. FD values
316 are from reference[35]. B) Effect of sugar feeding. MS: Newly eclosed adult females were fed
317 for 4 days on 3% or 20% sucrose. FD values are from reference[37]. C) MS: BR-CA-CC
318 preparations were dissected from 4 day-old adult female mosquitoes and stimulated with 40µM
319 farnesoic acid (FA). FD values are from reference[36].

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Table 1. Common MRM parameters utilized for JH III and JH III-D3 detection on the QTRAP® 5500 triple quadrupole mass spectrometer.

Compound	DP ¹ (V)	Q1 (Da)	CE ² (V)	Q3 (Da)	CXP3 (V)
JH III	46	267	11	235	30
			19	147	16
JH III-D3	46	270	11	235	28
			17	147	6

¹ Declustering Potential; ² Collision Energy; ³ Collision cell exit potential.

Table 2. Typical fragment ion and dissociation pathways observed using in-source MS/MS, MS/MSⁿ and FT-ICR MS/MS for JH III [M+H]⁺ molecular ion.

	Parent m/z	Fragment m/z
In-source MS/MS	267	249, 235, 217, 189, 147
	249	217, 189, 147
	235	217, 189, 147
	217	189, 147
	189	147
MS/MSⁿ	267 -> 235	217, 207, 189
	267 -> 249	217, 189, 147
	267 -> 217	189, 147
	267 -> 189	147
FT-ICR MS/MS	267.195461 C ₁₆ H ₂₇ O ₃	249.18492 C ₁₆ H ₂₅ O ₂
		235.169298 C ₁₅ H ₂₃ O ₂
		217.158786 C ₁₅ H ₂₁ O
		207.174417 C ₁₄ H ₂₃ O
		189.163858 C ₁₄ H ₂₁
		147.116827 C ₁₁ H ₁₅

Figure 1

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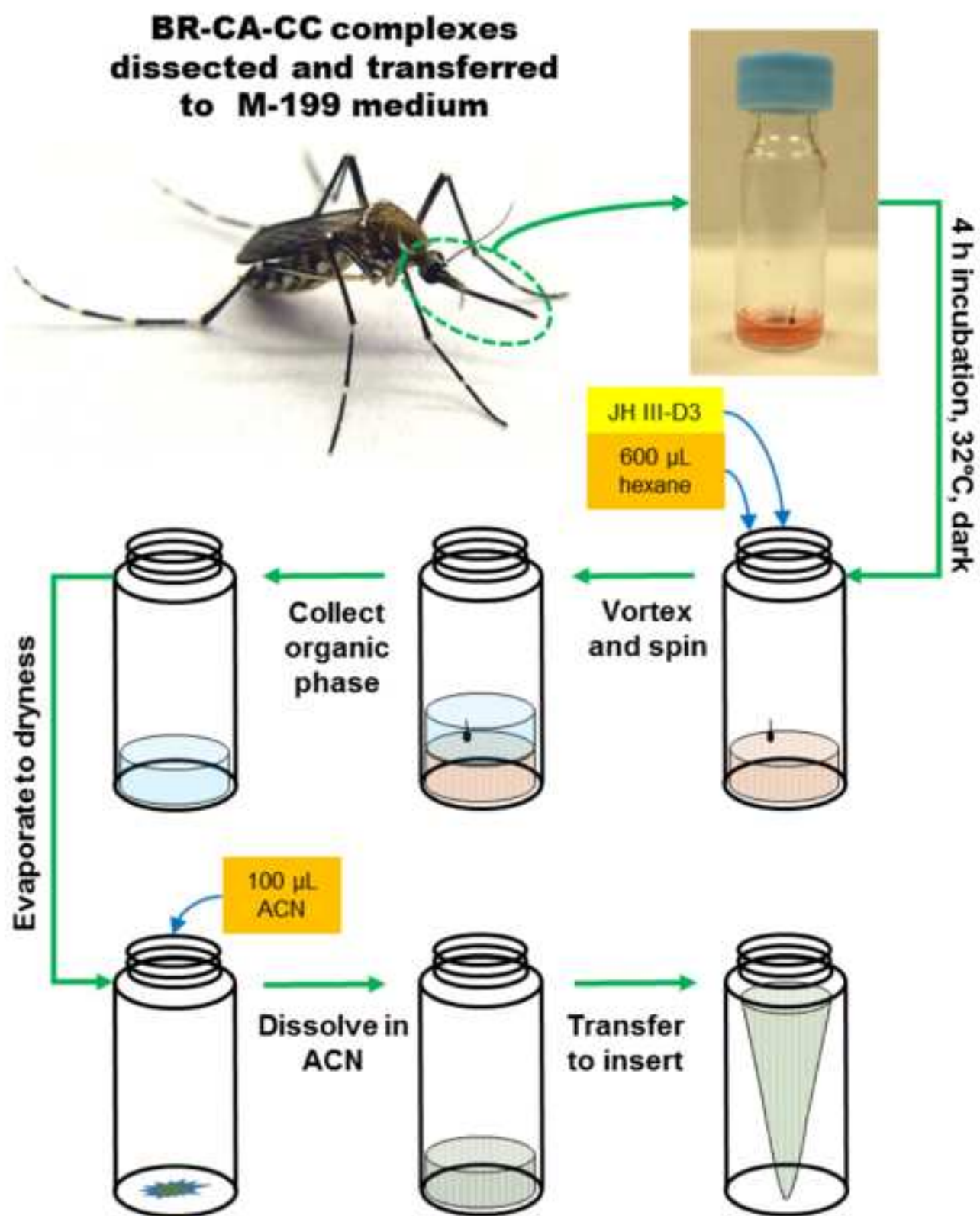


Figure 2
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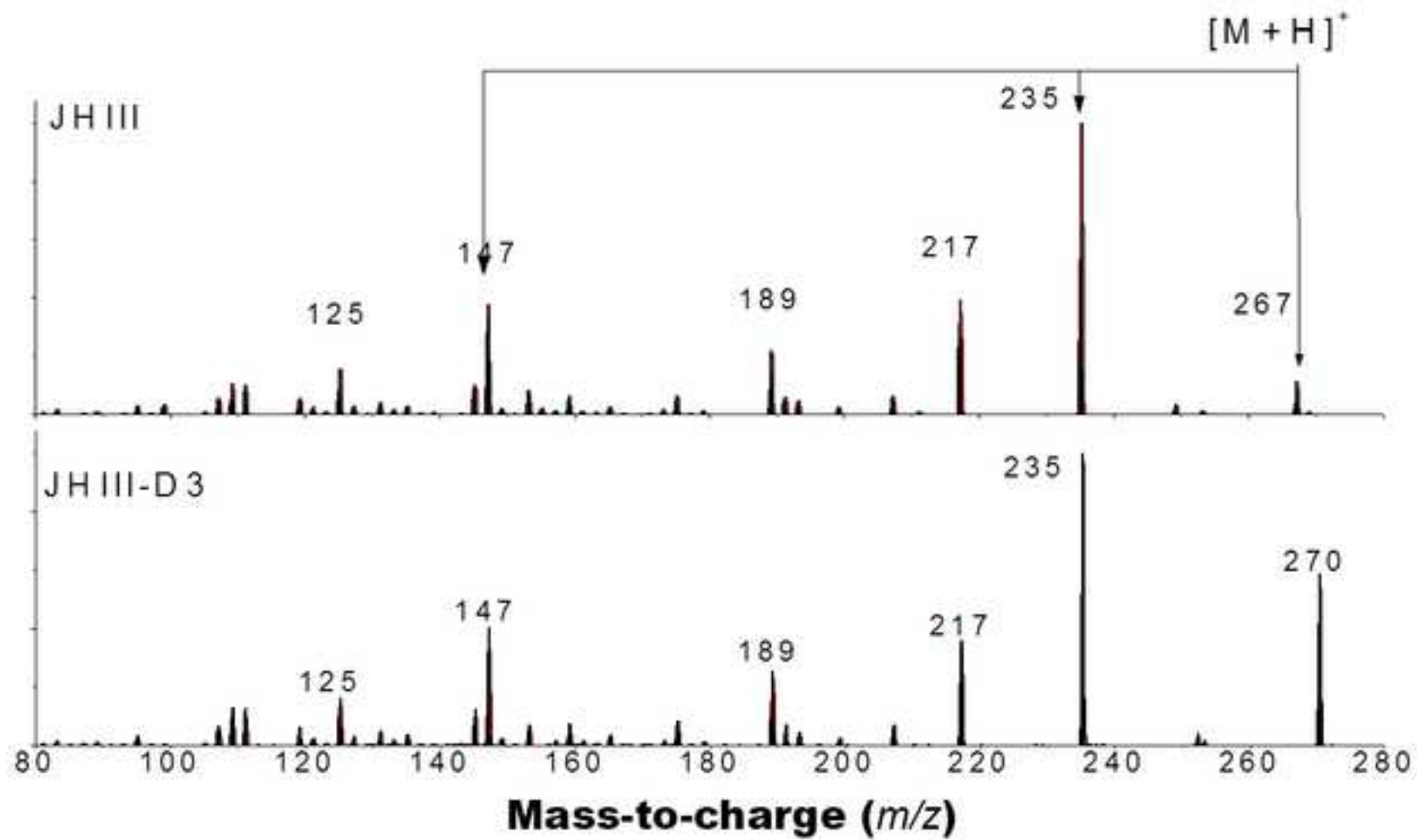


Figure 3

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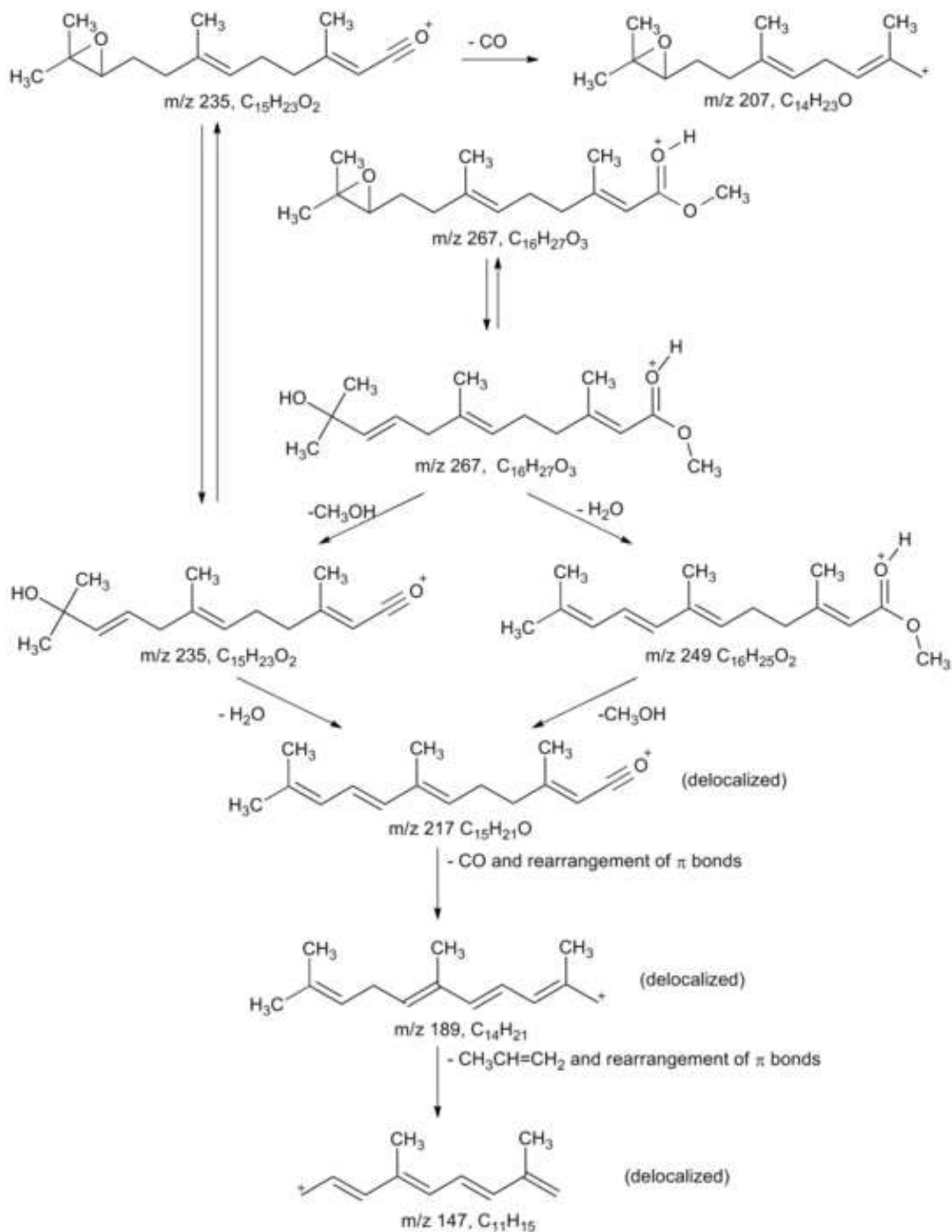


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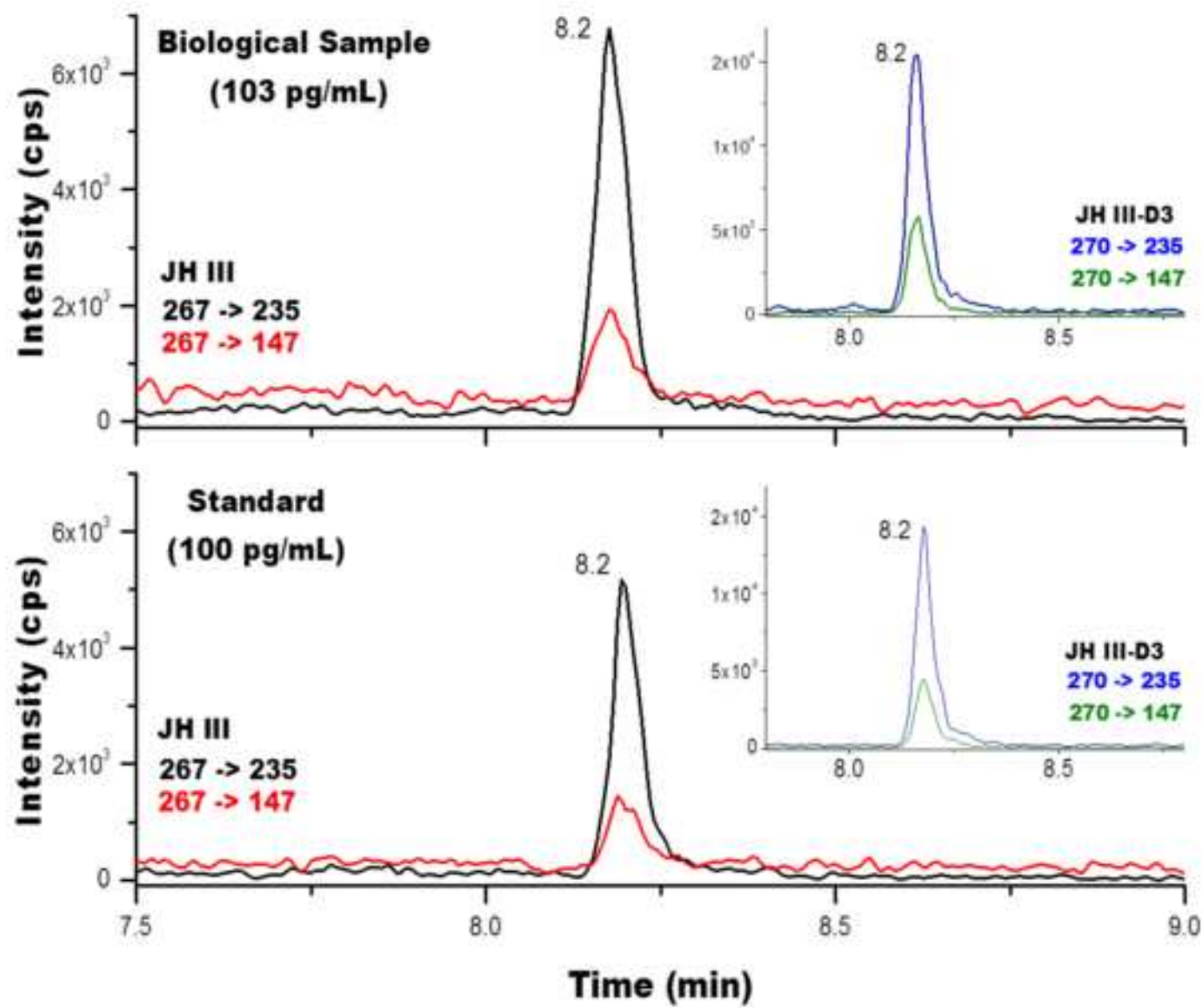


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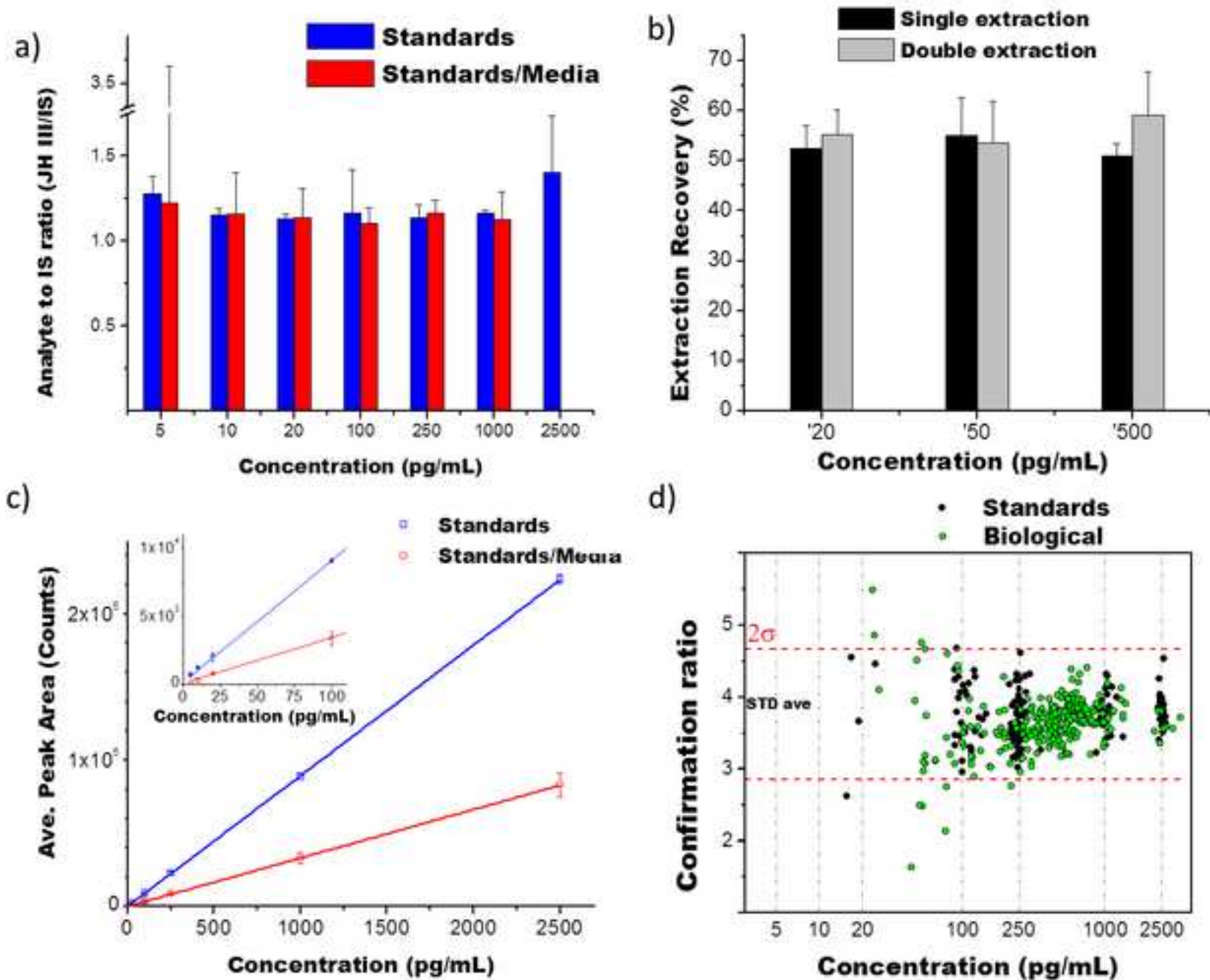


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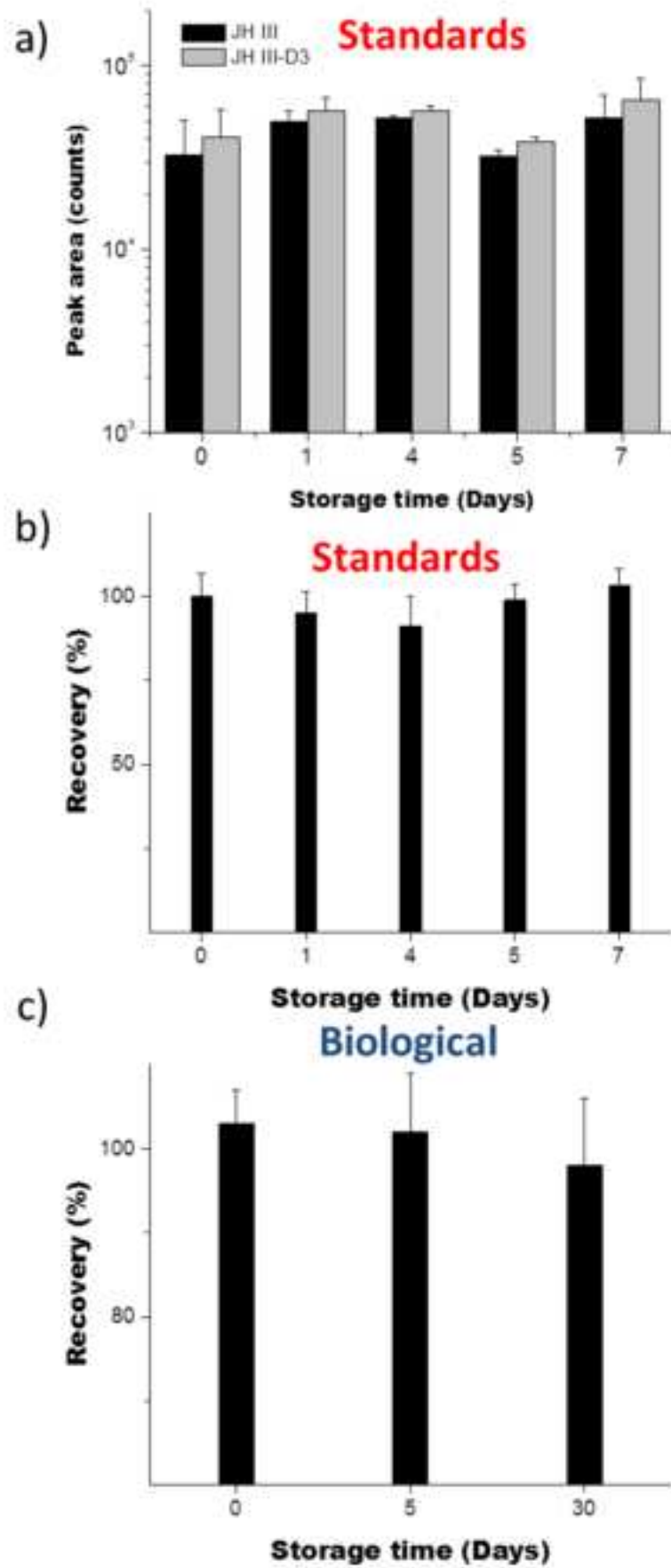
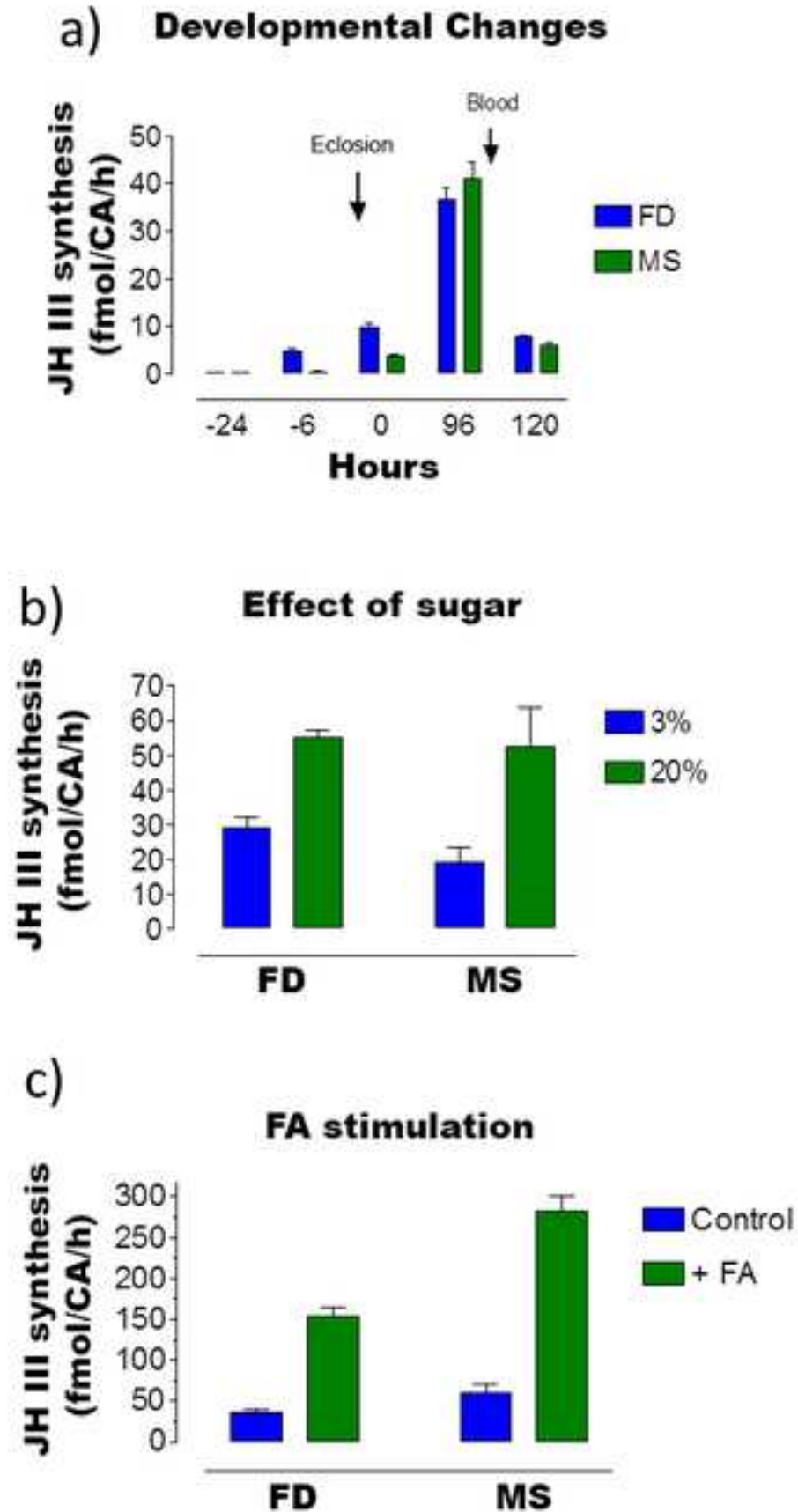


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