Pesticide Biochemistry and Physiology 163 (2020) 164–174

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Capsaicinoid metabolism by the generalist *Helicoverpa armigera* and specialist *H. assulta*: Species and tissue differences

Jiang Zhu[a,b], Kai Tian[a,b], Christopher A. Reilly[c], Xinghui Qiu[a,*]

[a] State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] Department of Pharmacology and Toxicology, Center for Human Toxicology, College of Pharmacy, University of Utah, Salt Lake City, UT 84112, USA

ARTICLE INFO

Keywords:
Adaptation
Capsaicin
Dihydrocapsaicin
Helicoverpa armigera
Helicoverpa assulta
Metabolism

ABSTRACT

Helicoverpa armigera and *H. assulta* are two of the few insects that can feed on hot pepper fruits. Capsaicin and dihydrocapsaicin (*i.e.*, capsaicinoids) are the principal pungent compounds in hot peppers. To explore possible molecular mechanisms of adaptation that allow these two species to consume capsaicinoids, the capacity of the three detoxification tissues (fat body, midgut, and Malpighian tubule) of the two pests, to metabolically degrade capsaicin and dihydrocapsaicin, was compared. The results showed that capsaicin and dihydrocapsaicin were metabolized by crude enzyme preparations from all three tissues of the two pests. Five metabolites of capsaicin, and five metabolites of dihydrocapsaicin were identified. Tissue and species differences in the degree of capsaicin and dihydrocapsaicin metabolism were observed. The specialist *H. assulta* had an overall greater capacity to degrade the capsaicinoids compared to the generalist *H. armigera*. Further, the midgut was the most significant contributor to capsaicinoid metabolism. The notably high specific activity in Malpighian tubules of *H. armigera* also further highlights the significance of this organ in xenobiotic detoxification. Alkyl hydroxylation and dehydrogenation were the main pathways for the oxidative biotransformation of both capsaicin and dihydrocapsaicin by cytochrome P450s. This study provides evidence that enhanced metabolic decomposition of capsaicinoids may be an adaptation explaining dietary preferences for *Capsicum* fruits by these two pests.

1. Introduction

Chili pepper is an important and widely cultivated vegetable crop due to its unique spicy flavor and rich nutrition. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonanamide) are two of the main pungent ingredients of hot peppers (*Capsicum* spp.) (Cordell and Araujo, 1993; Al Othman et al., 2011), accounting for 80% to 90% of total capsaicinoid content of many hot peppers (Kosuge and Furuta, 1970; Perkins et al., 2002; Stipcovich et al., 2018). However, different varieties of peppers contain different aggregate and relative concentrations of the multiple known major and minor capsaicinoid analogues (Leete and Louden, 1968; Peña-Alvarez et al., 2009).

The cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) and the oriental tobacco budworm *Helicoverpa assulta* are significant agricultural pests. They are closely related, and it is very difficult to distinguish them by morphological characteristics, especially at the larval stage (Wang and Dong, 2001; Li et al., 2011). They have a similar appearance and feeding behavior, but quite different host ranges. *H. armigera* is a highly polyphagous herbivore, and it is known to infest > 200 different plants including those of the genus Solanaceae, Leguminosae and Malvaceae which includes cotton, corn, wheat, soybean, tobacco, peppers, and tomato (Fitt, 1989; Jallow et al., 2004; Srinivasan et al., 2006). Alternatively, *H. assulta* is an oligophagous species, mainly feeding on Solanaceae plants, such as chili pepper, tobacco and tomato (Mitter et al., 1993; Tang et al., 2006; Wu et al., 2006). While both species can successfully feed on hot pepper fruits, they differ in fitness (Cai et al., 2012; Jia et al., 2012). Specifically, *H. assulta* showed no significant difference in larvae, pupae and adult stages after feeding on pepper fruits with a content of capsaicin from 1.2 mg/L to 1097 mg/L (fresh weight), while the growth rate of *H. armigera* decreased with increasing amounts of capsaicin (Cai et al., 2012; Jia et al., 2012). Ahn et al (2011a) systematically compared the physiological tolerance of capsaicin among six species of the Noctuidae family, assessing larval development and survival, food consumption and utilization, and acute toxicity. It was shown that the host-plant specialist *H. assulta* was more tolerant to capsaicin than other noctuid species, including *H. armigera* (Ahn et al., 2011a). These results suggest

* Corresponding author.
E-mail address: qiuxh@ioz.ac.cn (X. Qiu).

https://doi.org/10.1016/j.pestbp.2019.11.013
Received 18 August 2019; Received in revised form 17 October 2019; Accepted 14 November 2019
Available online 19 November 2019
0048-3575/

that *H. assulta* has greater tolerance and perhaps unique physiological adaptations to allow it to consume capsaicinoids.

Due to its potential roles as an analgesic, anti-cancer, weight loss, hypoglycemic, lipid-lowering and anti-inflammatory molecule, capsaicin has attracted great attention (http://apps.webofknowledge.com/). However, there are only a few studies on interactions between capsaicinoids and insects. Capsaicin has been shown to affect the reproduction of the onion fly, *Delia antiqua* (Diptera: Anthomyiidae) by deterring oviposition (Cowles et al., 1989), and to induce a state of pharmacological blockade of heat sensitivity in the American cockroach (Maliszewska et al., 2018). Capsicum extracts also have been shown to be toxic to larvae of two species of mosquitoes, *Anopheles stephensi* and *Culex quinquefasciatus* (Madhumathy et al., 2007). Because of its toxicity against selected pests, capsaicin has been suggested as a potential botanical pesticide (Maliszewska and Tegowska, 2012). Previous biochemical studies have also demonstrated that capsaicin can be transformed into hydroxylated metabolites, capsaicin oxide, or capsaicin dimers by cytochrome P450s CYP6B6 and CYP9As (Tian et al., 2019), hydrolyzed into vanillylamine and 8-methyl-6-*trans*-nonenoic acid by carboxylesterase (Chanda et al., 2008), and conjugated with UDP-glucose to produce capsaicin β-glucoside, catalyzed by UDP glycosyltransferase (Ahn et al., 2011b). All of these reactions can be considered ways to inactivate capsaicinoids, through changing their key pharmaco/toxicophores, and enhancing elimination in many species, including mammals.

In insects, the fat body (FB), midgut (MG) and Malpighian tubule (MT) are recognized as the primary detoxification tissues. However, to our knowledge, few comparative studies have been conducted on the metabolism of capsaicinoids by these tissues in different insect species. The aim of this study was two-fold: First, we tested whether the specialist *H. assulta* was more capable of metabolizing capsaicinoids than the generalist *H. armigera*; second, we compared the relative metabolic capacity of the three detoxification tissues to assess which tissues were principally involved in capsaicinoid metabolism.

2. Materials and methods

2.1. Chemical reagents

Capsaicin (8-methyl-N-vanillyl-6-nonenamide, 99%), dihydrocapsaicin (8-methyl-N-vanillylnonanamide, 95%) and ethyl acetate (99.9%) were purchased from J&K chemical Co., Ltd. (Beijing, China). PTU (N-Phenylthiourea, 98%), β-NADPH (β-nicotinamide adenine dinucleotide phosphate, 95%), Ammonium formate (99%), DTT (4-Dithiothreitol, 99%), PMSF (Phenylmethylsulfonyl fluoride, 99%) and IPTG (Isopropyl β-D-thiogalactoside, 98%) were purchased from Aladdin Chemical Co., Ltd. (Shanghai, China). Formic acid (98%), HPLC grade acetonitrile and HPLC grade DMSO (Dimethyl sulfoxide, 99.9%) were purchased from Anpel Chemical Co., Ltd. (Shanghai, China). Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate, glycerol, EDTA·2Na and KCl were purchased from Xi Long Chemical Co., Ltd. (Beijing, China).

2.2. Rearing of H. armigera and H. assulta

The cotton bollworms were procured from Jiyuan Baiyun Industrial Co., Ltd. (Henan, China), and maintained in the laboratory. The oriental tobacco budworms were generously provided by Dr. Junfeng Dong (Henan University of Science and Technology, China). All larvae were maintained at 25 ± 1 °C and 70% relative humidity with a 16:8 h (light: dark) photoperiod in individual tubes with wheat-germ-based artificial diets purchased from Jiyuan Baiyun Industrial Co., Ltd. (Henan, China).

Table 1
Rates of capsaicin depletion (nmol/mg·min) by crude enzymes from three detoxification tissues.

Samples	*H. armigera*	*H. assulta*
Fat body	0.20 ± 0.01b	0.29 ± 0.21a
Malpighian tubule	0.91 ± 0.17a**	0.54 ± 0.01 a
Midgut	0.39 ± 0.12b	0.47 ± 0.17a

The values in each column were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Significant differences between rates for each organ among a given species is marked by different letters ($p < .05$). The data in each line was compared by independent *t*-tests, and significant differences between metabolic rates for a given tissue between the two species are indicated with a star (**$p < .01$).

Table 2
Rates of dihydrocapsaicin depletion (nmol/mg·min) by crude enzymes from three detoxification tissues.

Samples	*H. armigera*	*H. assulta*
Fat body	0.15 ± 0.02c	0.50 ± 0.14 a**
Malpighian tubule	0.92 ± 0.12a*	0.65 ± 0.08 a
Midgut	0.50 ± 0.14b	0.55 ± 0.12a

The values in each column were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Significant differences between rates for each organ among a given species is marked by different letters ($p < .05$). The data in each line was compared by independent t-tests, and significant differences between metabolic rates for a given tissue between the two species are indicated with a star (*$p < .05$, **$p < .01$).

Table 3
Rates of capsaicin depletion (pmol/min·individual equvilent) by crude enzymes from three detoxification tissues.

Samples	*H. armigera*	*H. assulta*
Fat body	8.02 ± 0.43b	11.99 ± 2.52b
Malpighian tubule	4.97 ± 1.68c	2.31 ± 0.45c
Midgut	15.94 ± 1.19a	24.55 ± 0.89a**
Cumulative	28.93 ± 2.30	38.86 ± 3.45 **

The values in each column were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Significant differences between rates for each organ among a given species is marked by different letters ($p < .05$). The data in each line was compared by independent t-tests, and significant differences between metabolic rates for a given tissue between the two species are indicated with a star (**$p < .01$).

Table 4
Rates of dihydrocapsaicin depletion (pmol/ min·individual equvilent) by crude enzymes from three detoxification tissues.

Samples	*H. armigera*	*H. assulta*
Fat body	6.17 ± 0.33b	20.63 ± 4.34b*
Malpighian tubule	5.04 ± 1.70b	2.80 ± 0.54c
Midgut	20.65 ± 1.54a	28.38 ± 1.02a**
Cumulative	31.87 ± 2.52	51.82 ± 5.39 **

The values in each column were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Significant differences between rates for each organ among a given species is marked by different letters ($p < .05$). The data in each line was compared by independent t-tests, and significant differences between metabolic rates for a given tissue between the two species are indicated with a star (*$p < .05$, **$p < .01$).

2.3. Dissection of tissues from H. armigera and H. assulta

Each of three tissues (FB, MG and MT), was dissected from two-day-old sixth-instar nymphs of *H. armigera* and *H. assulta* on ice. Tissues from 30 or 60 sixth-instar larvae (30 for FB and MG, 60 for MT) were

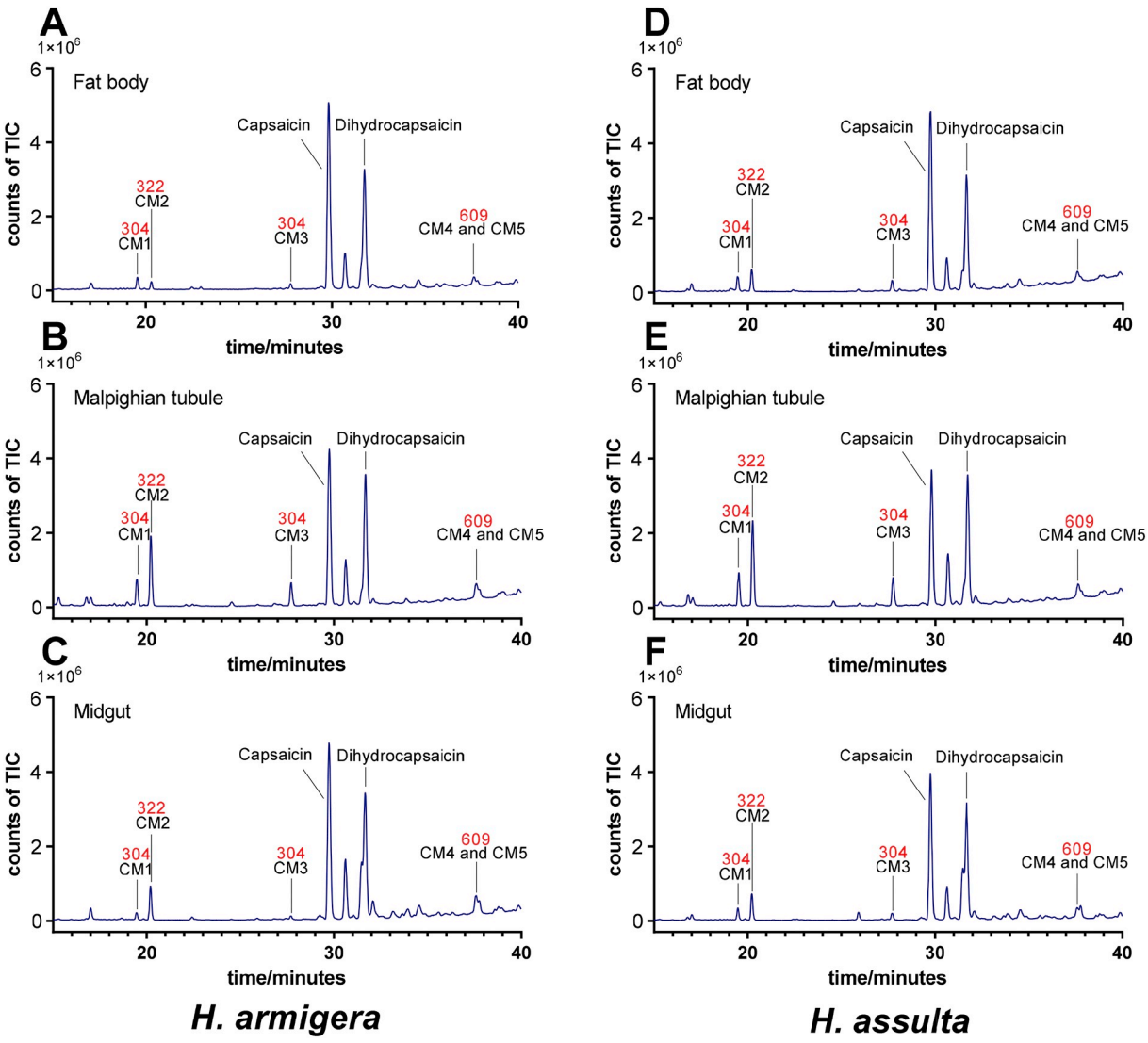


Fig. 1. Total ion chromatogram of capsaicin and metabolites detected by HPLC-Q-TOF. Representative TICs of capsaicin and its metabolites generated from incubations containing enzymes from the FB (A), MT (B) and MG (C) of *H. armigera*. Representative TICs of capsaicin and its metabolites generated from incubations containing enzymes from the FB (D), MT (E) and MG (F) of *H. assulta*. Five metabolites, CM1 at 19.6 min, CM2 at 20.3 min, CM3 at 27.8 min, CM4 at 37.8 min and CM5 at 38.5 min, are highlighted. Dihydrocapsaicin was used as the internal standard, eluting at 31.2 min.

cleaned in ice-cold 1.15% KCl solution and pooled as one replicate. The clear dissected tissues were immediately transferred to ice-cold homogenization buffer (0.1 M phosphate buffered saline, pH 7.6, containing 1 mM EDTA-2Na, 1 mM PMSF, 1 mM PTU, 0.1 mM DTT and 20% glycerol) for preparing crude enzymes. A minimum of three independent batches of tissues were prepared.

2.4. Crude enzyme preparation

Each tissue sample was transferred to a 1-mL glass tissue grinder containing 600 µL of ice-cold homogenization buffer and gently homogenized using a glass pestle (30 up-and-down strokes) on ice. The homogenous liquid was centrifuged at 12,000 x*g* for 25 min at 4 °C, and 100 µL of the supernatant was used as the crude enzyme preparation for capsaicinoid metabolism assays. The protein content of the crude enzyme preparations was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a protein standard.

2.5. Metabolism of capsaicin and dihydrocapsaicin

Incubations (200 µL) consisted of PBS buffer (0.1 M) at pH 7.4, 1 mM NADPH, 50 µM substrate (capsaicin or dihydrocapsaicin), and 200 µg crude enzyme protein. Stock solutions of substrates were prepared in HPLC grade DMSO and were stored at −20 °C. Reactions were initiated by the addition of substrate and were incubated at 30 °C in a shaking water bath at 250 rpm for 30 min. The reactions were terminated by the addition of triple volume (600 µL) ice-cold ethyl acetate and vortexed at room temperature for 20 min, then centrifuged at 12,000 x*g* for 5 min and the upper organic layer (500 µL) was collected. This extraction process was repeated three times, and the organic extracts were pooled and evaporated under nitrogen. Samples were reconstituted to a volume of 200 µL using acetonitrile, filtrated through 0.22 µm PTFE filters into auto-sampler vials, and analyzed by HPLC and HPLC-Q-TOF mass spectrometry. Two control incubations were performed in parallel: One without the addition of the crude enzyme preparation (0 enzyme), and the second with heat inactivated crude enzymes, prepared by boiling the enzyme for 10 min. The amount of capsaicinoid depletion was calculated using the amount of capsaicinoids remaining in the heat inactivated enzyme control as the initial amount. The enzyme activity was calculated as both the amount of capsaicin or dihydrocapsaicin (nmol) depleted per unit time (min) per milligram (mg) of total protein (Tables 1 and 2), and the amount of

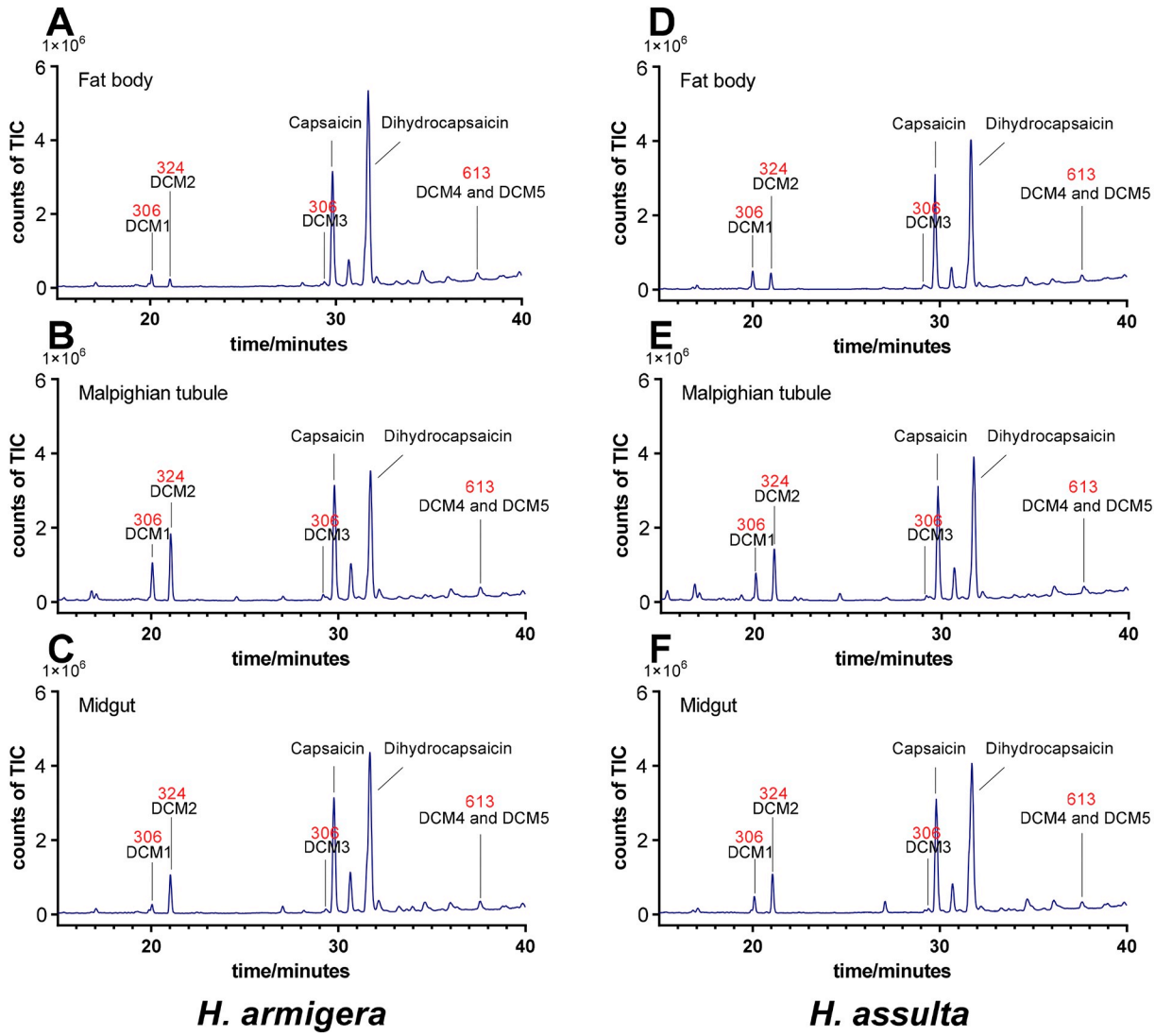


Fig. 2. Total ion chromatograms of dihydrocapsaicin and metabolites detected by HPLC-Q-TOF. Representative TICs of dihydrocapsaicin and its metabolites generated from incubations containing enzymes from the FB (A), MT (B) and MG (C) of *H. armigera*. Representative TICs of dihydrocapsaicin and its metabolites generated from incubations containing enzymes from the FB (D), MT (E) and MG (F) of *H. assulta*. Five metabolites, DCM1 at 20.0 min, DCM2 at 21.0 min, DCM3 at 29.2 min, DCM4 at 40.7 min and DCM5 at 41.5 min, are highlighted. Capsaicin was used as the internal standard, eluting at 29.7 min.

capsaicin or dihydrocapsaicin (nmol) depleted per unit time (min) per tissue equivalent (Tables 3 and 4).

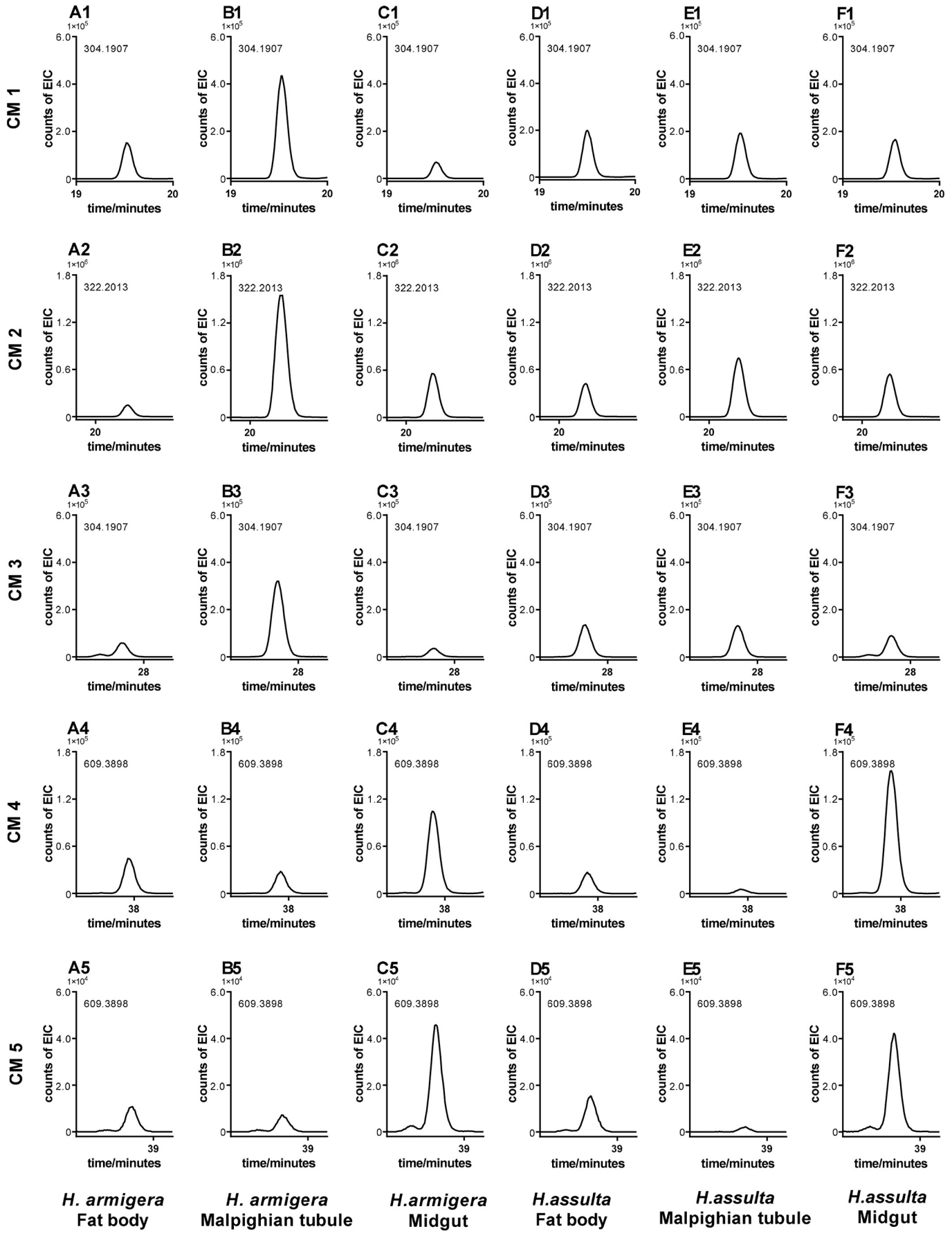
2.6. HPLC and HPLC-Q-TOF conditions

Chromatographic separation of analytes was performed using an Agilent 1260 series instrument (equipped with the Acclaim™ 120 C18 reverse column, 150 mm*4.6 mm, 3 μm, Agilent, USA) with a diode array detector (DAD). Gradient elution (0–1 min, 5% B; 1–41 min, 100% B; 41–42 min, 100% B; 42–45 min, 5% B; 55–50 min, 5% B) using water (A) and acetonitrile (B) was used. The injection volume of samples was 30 μL, the HPLC flow rate was 0.5 mL/min, and the column temperature was 28 °C. Capsaicin and dihydrocapsaicin were detected at 210 nm.

Metabolites were identified using LC/MS/MS using a 6520 quadrupole/time-of-flight (Q-TOF) instrument interfaced with an Agilent 1200 HPLC system. The mass spectrometer was equipped with an electrospray ionization source (Agilent) and operated in positive ionization mode, scanning the range of *m/z* 50–1000. Other instrument settings were: Capillary voltage (3500 V), fragmenter voltage (120 V), drying gas temperature (300 °C), gas flow (nitrogen; 10 L/min), and nebulizer pressure (40 psi). For MS/MS analysis, the collision voltage was 15 V. Samples (5 μL/injection) were chromatographed over a ZORBAX Eclipse Plus C18 (4.6*150 mm 5 μm, Agilent, USA) reversed-phase column, and eluted at a flow rate of 0.5 mL/min using a gradient of water (A) and acetonitrile (B) (0–1 min, 5% B; 1–40 min, 95% B; 40–51 min, 95% B; 51–53 min, 5% B; 53–60 min, 5% B). The column temperature was maintained at 28 °C and the mass spectrometer parameters were optimized for the detection of capsaicin and its characteristic fragment ions.

2.7. Quantitative analysis of analytes

Degradation of capsaicin or dihydrocapsaicin was quantified using the normalized peak (analyte/internal standard) area from HPLC profiles. The relative abundance of the metabolites by mass spectrometry was assessed using relative peak area due to the lack of reference standards for quantifying the various metabolites. Specifically, peak area values were normalized to that of the spiked internal standard (capsaicin was used as internal standard for dihydrocapsaicin, and *vice versa*). The values of peak area are reported as mean ± SD (n = 3).



(caption on next page)

Fig. 3. Extracted ion chromatograms (EIC) for capsaicin metabolites present in incubations containing enzymes from the FB, MT, and MG of *H.armigera and H.assulta*. Representative EICs for the five capsaicin metabolites generated from FB (column A, rows 1–5) MT (column B, rows 1–5) and MG (column C, rows 1–5) of *H.armigera*. Representative EICs for the five capsaicin metabolites generated from FB (column D, rows 1–5), MT (column E, rows 1–5) and MG (column F, rows 1–5) of *H.assulta*. The *m/z* used for the EIC of CM1 was 304.1907; CM2 (322.2013), CM3 (304.1907), CM4 (609.3898) and CM5 (609.3898).

2.8. Data analysis

Statistical comparison of differences between capsaicinoid metabolism by the three tissues was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Data were further compared with an independent *t*-test to identify significant differences occurring between species. In addition, normalized peak area data of each of the observed metabolites were statistically analyzed using ANOVA followed by Tukey's HSD test among different preparations. All statistical analyses were performed using SPSS.21 and a significant difference is defined as having a $p < .05$.

3. Results

3.1. Metabolism of capsaicin and dihydrocapsaicin by crude enzymes

Rates for the metabolic depletion of capsaicin and dihydrocapsaicin by crude enzyme preparations of the three metabolic organs of *H. assulta* and *H. armigera* are shown in Tables 1 and 2. No significant substrate depletion or metabolite formation was observed in the controls lacking enzymes or containing heat inactivated enzymes (Supplemental Figs. 1–3). The rates (nmol/mg·min) of capsaicin and dihydrocapsaicin metabolism by the FB, MG and MT enzyme preparations from *H. armigera* ranged from 0.20 to 0.91, and 0.15 to 0.92, respectively. The rates of capsaicin and dihydrocapsaicin metabolism by the FB, MG and MT enzyme preparations from *H. assulta* were 0.29 to 0.54 and 0.50 to 0.65 nmol/mg·min, respectively. In general, the MT showed the highest specific activity for capsaicinoid metabolism for both species, followed by the MG and then FB. However, a significant difference in the metabolic rates for the two capsaicinoids among the three tissues was only observed for *H. armigera*; comparable intermediate rates of capsaicinoid metabolism were observed for all three tissues from *H. assulta*. Comparisons between the corresponding tissues from different species showed that the *H. armigera* MT had significantly higher activity towards the two substrates than *H. assulta*, while *H. assulta* FB transformed dihydrocapsaicin more efficiently than FB *of H. armigera.*

In terms of total metabolic capacity per individual, rates for both capsaicin and dihydrocapsaicin depletion (pmol /min/individual equivalent) were highest in MG, followed by FB then MT (Tables 3 and 4). Compared with *H. armigera,* significantly higher activities were observed in *H. assulta* MG for capsaicin and in FB and MG for dihydrocapsaicin. As a consequence, *H. assulta* displayed significantly greater overall capacity to metabolize capsaicinoids than *H. armigera*; *H. assulta* showed 1.34- and 1.63- fold higher cumulative depletion rates than *H. armigera* for capasaicin and dihydrocapsaicin, respectively.

3.2. Detection of metabolites by HPLC-Q-TOF

The total ion chromatograms obtained from the analysis of capsaicin and dihydrocapsaicin and their metabolites are shown in Fig. 1 and Fig. 2, respectively. Under the conditions described in this study, five significant metabolite peaks of capsaicin (CM1-CM5), and five significant metabolite peaks of dihydrocapsaicin (DCM1-DCM5) were detected. None of these metabolites were observed in the negative control samples (Fig. S1-S3).

Absolute quantitation of the metabolites was not possible due to the lack of availability of authentic standards. However, to assess the amount of each metabolite, the area value of each metabolite peak (Figs. 5 and 6) was integrated based on the extracted ion chromatogram (Figs. 3 and 4) and normalized to the internal standard. From the extracted ion chromatogram (EIC) of capsaicin, the most abundant metabolite of capsaicin was CM2 particularly in MT from *H. armigera* (Figures 3B2 and 5), and the least was CM5 in MT from *H. assulta* (Figures 4E5 and 5). Similarly, the most abundant dihydrocapsaicin metabolite was DCM2 in MT from *H. armigera* (Figures 4B2 and 6), while the least was DCM3 in MG from *H. armigera* (Figures 4C3 and 6).

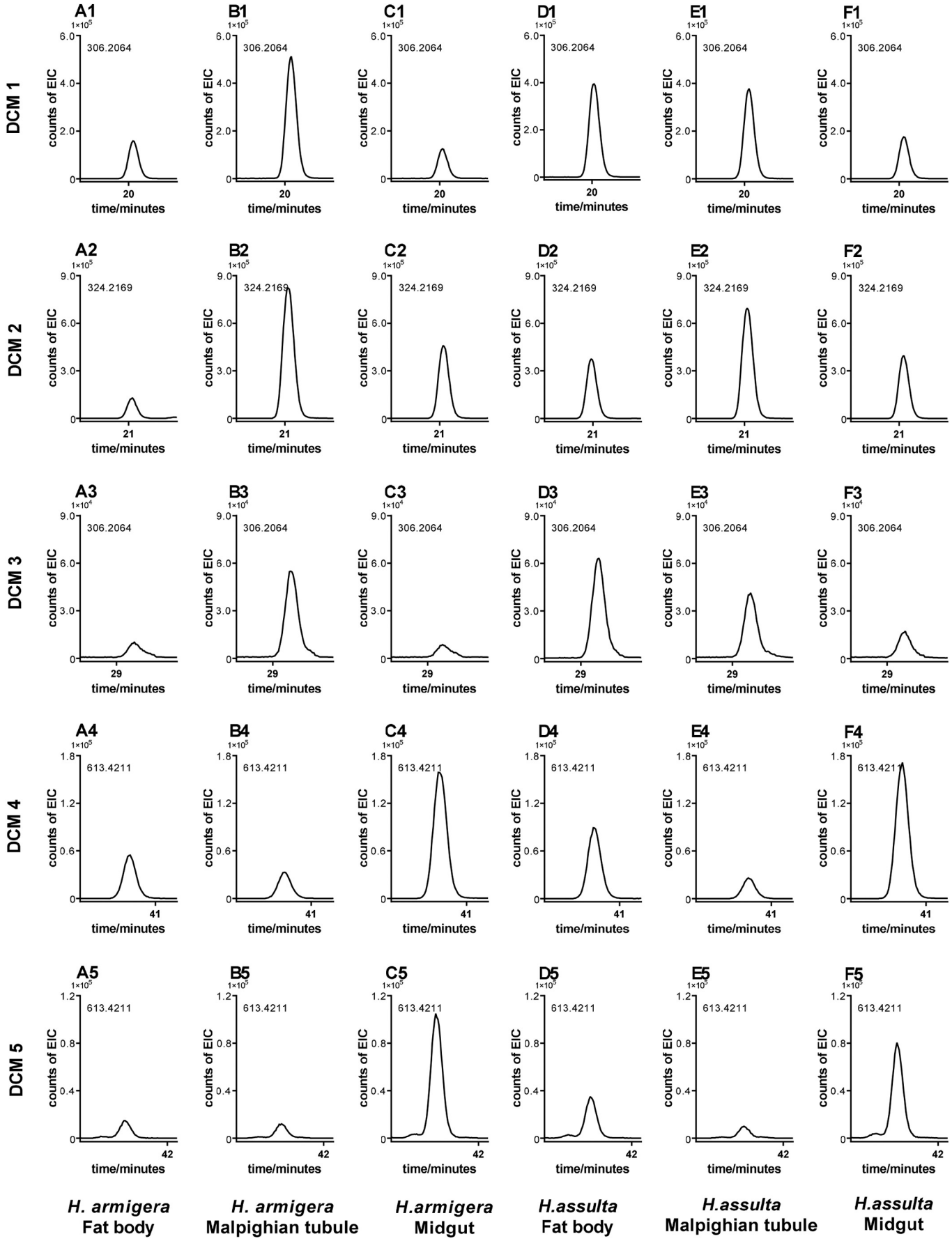
3.3. Putative identification of metabolites by HPLC-Q-TOF

Structures of metabolites detected in this study are proposed (Fig. 7), based on results from previous studies(Reilly et al., 2003; Reilly and Yost, 2006; Reilly, 2013). The metabolites exhibited base peak *m/z* values of 304, 322, 304, and 609, and MS/MS spectra that were wholly consistent with the formation of an N-macrocycle (*m/z* 304), ω-hydroxylation (m/z 322), terminal alkyl dehydrogenation (*m/z* 304), and dimerization (m/z 609). The mass of CM1 (m/z 304.1907) and CM3 (*m/z* 304.1907) were 2 amu less than capsaicin (m/z 306.2064), indicating that they were dehydrogenated metabolites; of note, these metabolites eluted at times different from hydroxylated metabolites indicating they were not dehydration products formed in the mass spectrometer. In support of these structural assignments, the characteristic fragment ion m/z 137 in the MS/MS spectra of CM1, CM2 and CM3 indicated that metabolism occurred on the alkyl portion of capsaicin. Additionally, the fragment ions of m/z 440 and 273 in the spectra of CM4 and CM5 indicated a dimer of the vanilloid rings with the loss of one and two unmodified alkyl chains.

Previous research using human cytochrome P450 enzymes found that dehydrogenation of capsaicin generated an N-macrocycle and an ω-dehydrogenated metabolite (Reilly et al., 2003). Considering that CM1 eluted earlier than CM3, and based on nearly identical MS/MS spectra being observed for these metabolites and those reported by Reilly (2013), it was concluded that CM1 was the macrocycle product while CM3 was the terminal alkyl dehydrogenated product. CM2 (*m/z* 322.2013) was identified as an alkyl hydroxylated metabolite of capsaicin based on the presence of a fragment ion of m/z 137. Additionally, the characteristic products ion m/z 292 and 168 corresponding to the loss of terminal CH_2O (m/z 30) from m/z 322 and the corresponding alkyl chain fragment ion, was observed. These observations implied that CM2 was the ω-hydroxylated metabolite of capsaicin.

The m/z values of CM4 and CM5 (609.3898) were 303 amu greater than capsaicin (m/z 306.2064) (Figs. 1, 3 and S4). Based on this mass shift and the MS/MS spectra, CM4 and CM5 were concluded to be dimers of capsaicin. However, it was not feasible to determine the specific structures of CM4 and CM5 based on the MS/MS results alone. Therefore, we speculate that CM4 and CM5 may be isomers or alternative products of radical dimerization of the vanilloid rings (5,5′-dicapsaicin or 4'-O-5-dicapsaicin). The structure of the previously identified P450- and peroxidase-generated capsaicin 5,-5′ dimer (Goodwin and Hertwig, 2003; Reilly et al., 2013) is shown in Figs. 7E and J.

Similar to capsaicin, metabolites of dihydrocapsaicin had base peak m/z values of 306, 324, 306, 613 and 613 (Figs. 2, 4 and S5), also consistent with the formation of corresponding N-macrocycle (m/z 306), ω-hydroxylated (m/z 324), alkly dehydrogenated (m/z 306), and dimerized products. Due to the structural similarities between capsaicin and dihydrocapsaicin, and the MS/MS data, DCM1 and DCM3 were identified as the macrocycle and terminal alkyl dehydrogenated metabolites, DCM2 as the ω-hydroxylated metabolite, and DCM4 and DCM5 as isomers of the dihydrocapsaicin dimer. Accurate mass fragment ion data for all metabolites are shown in Table S1.



(caption on next page)

Fig. 4. Extracted ion chromatograms (EIC) for dihydrocapsaicin metabolites present in incubations containing enzymes from the FB, MT, and MG of *H.armigera and H.assulta*. Representative EICs for the five dihydrocapsaicin metabolites generated from FB (column A, rows 1–5) MT (column B, rows 1–5) and MG (column C, rows 1–5) of *H.armigera*. Representative EICs for the five dihydrocapsaicin metabolites generated from FB (column D, rows 1–5), MT (column E, rows 1–5) and MG (column F, rows 1–5) of *H.assulta*. The m/z used for the EIC of DCM1 was 306.2064; DCM2 (324.2169), DCM3 (306.2064), DCM4 (613.4211) and DCM5 (613.2169).

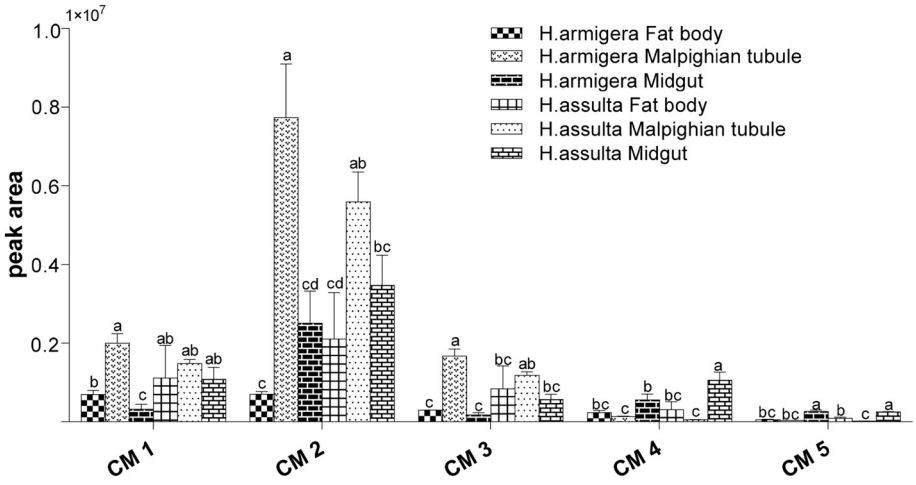


Fig. 5. Normalized peak area values (mean ± SD) for capsaicin metabolites. One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to identify statistical differences for each metabolite between the six enzyme preparations. Significant difference is indicated by different letters ($p < .05$).

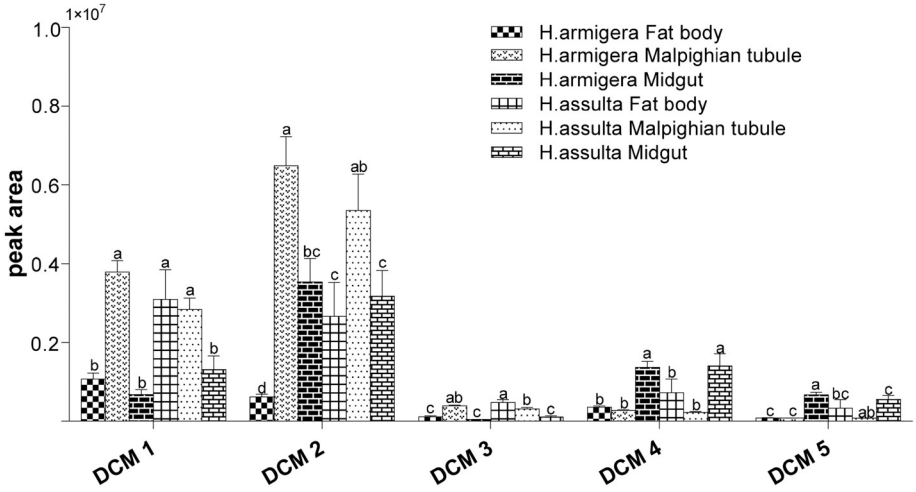


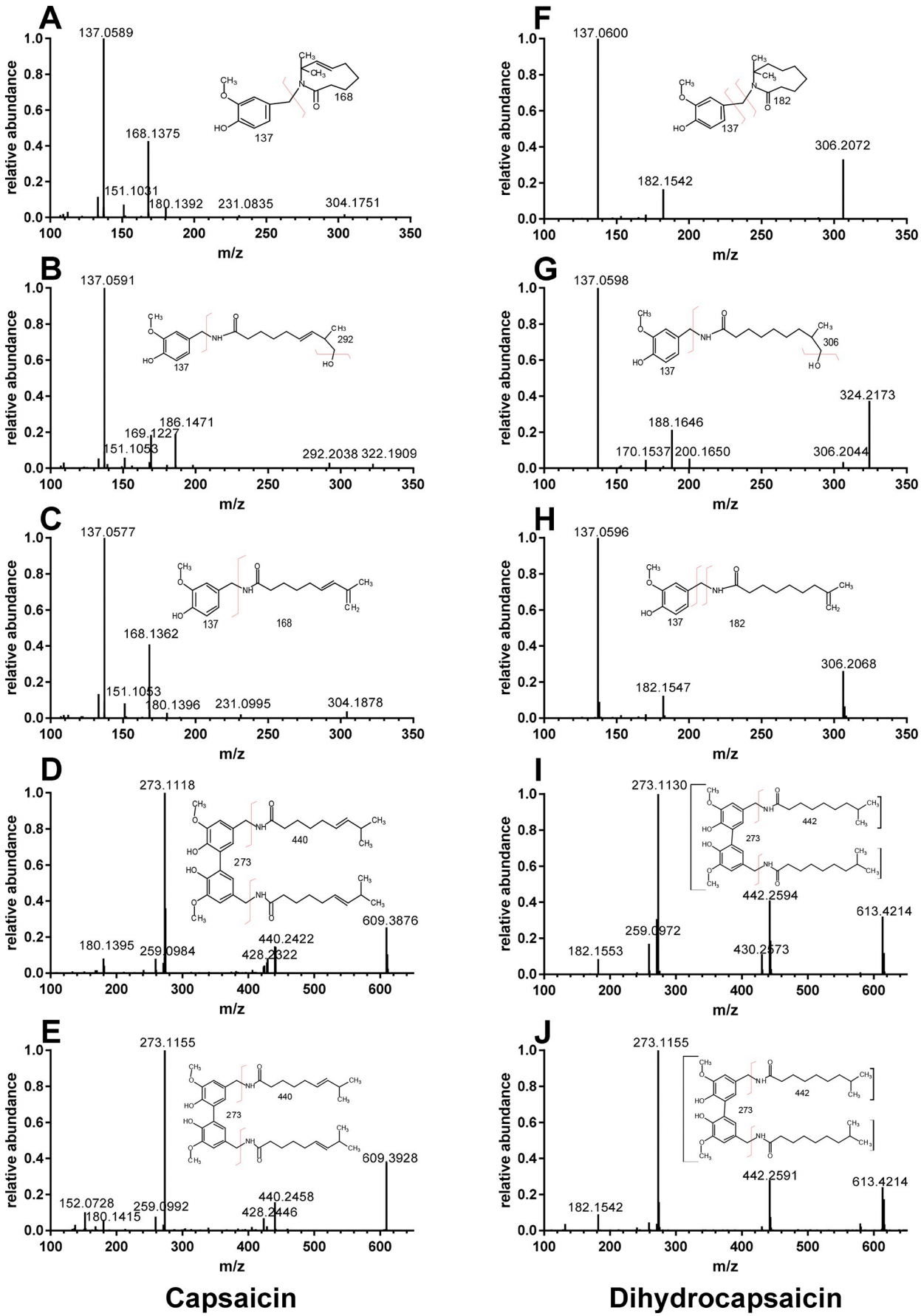
Fig. 6. Normalized peak area values (mean ± SD) of dihydrocapsaicin metabolites. One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to identify statistical differences for each metabolite between the six enzyme preparations. Significant difference is indicated by different letters (p < .05).

4. Discussion

The generalist *H. armigera* and the specialist *H. assulta* are two of the few insects that can feed on hot pepper fruits, but mechanisms that allow these pests to tolerate capsaicinoids are largely unknown. In this work, the capacity of the three main detoxification organs (FB, MG and MT) from the two closely related species *H. armigera* and *H. assulta,* to metabolically decompose two capsaicinoids was compared.

The data in Tables 1 and 2 show that crude enzyme preparations from the FB, MG and MT tissues from *H. armigera* had different specific activity for capsaicinoid metabolism, with the three tissues of *H. assulta* being more comparable. Notably, MT possessed the highest specific activity for metabolism of both capsaicin and dihydrocapsaicin in *H. armigera* (Tables 1 and 2). Comparing the two species, *H. assulta* FB transformed dihydrocapsaicin more efficiently than *H. armigera*, while the depletion rates of both substrates in MT were significantly higher in *H. armigera* than *H. assulta*. This species- and tissue-selective activity was also observed for capsaicin glucosylation by Ahn et al. (2011b). Although MT have been well recognized as key excretory and osmoregulatory organs of insects (Dow et al., 2018), its role in xenobiotic detoxification has become increasingly more apparent (Esquivel et al., 2014;Wang et al., 2004; Overend et al., 2015; Esquivel et al., 2016; Li et al., 2017;Yuan et al., 2018). Our data further support a key role of the MT as a detoxification organ involved in capsaicinoid transformation.

Considering that biomass and protein content of different tissues of the larvae are different, the capsaicinoid metabolism activity was alternatively calculated on the basis of individual equivalent (Tables 3 and 4). Expressing the data in this way illustrated that the MG was the most significant contributor to capsaicinoid depletion in both species. In total, *H. assulta* had a significantly higher capacity to metabolize the capsaicinoids than *H. armigera.* This result is consistent with that of an *in vivo* study (Ahn et al., 2011b), which demonstrate that *H. assulta* has a higher overall detoxification ability than other two generalist species (*H. armigera* and *H. zea*), and provides evidence that may explain the observed differences in tolerance to capsaicinoids between the two pests.



(caption on next page)

Fig. 7. Representative MS/MS spectra of the capsaicinoid metabolites. The MS/MS spectra of CM1, CM2, CM3, CM4, CM5, DCM1, DCM2, DCM3, DCM4 and DCM5 were shown in panels A, B, C, D, E, F, G, H, I and J, respectively. Proposed metabolite structures are shown within each panel where the red line illustrates a common fragmentation pathway to yield diagnostic vanilloid ring-derived and alkyl-derived fragment ions used in the identification of each metabolite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The structures of capsaicin and dihydrocapsaicin are very similar (Usman et al., 2014). Both contain vanillamide groups, in which reactions such as oxidation, reduction, demethylation and glucuronic acid conjugation may occur. Additionally, the alkyl side chains of both capsaicinoids are susceptible to enzymatic oxidation. Here we show that the alkyl terminus was a preferred site of metabolism by the insect enzymes, with the formation of multiple terminal hydroxylated and dehydrogenated metabolites. In this study, a total of five metabolites of capsaicin and five metabolites of dihydrocapsaicin were produced by the crude enzymes from the three tissues of both *Helicoverpa* pests (Fig. 1 and Fig. 2). The identification of the metabolites reveals that the metabolic pathways of capsaicin and dihydrocapsaicin by enzymes in the three detoxification tissues are extremely similar, largely due to their similarity in structure (Fig. 7, Table 3), and comparable to human CYP450 enzymes, as previously reported by multiple prior studies.

The species- and tissue-specific difference in capsaicinoid biotransformation was also illustrated by variations in the relative abundance of the metabolites (Figs. 3 to 6). Differential metabolite abundance is likely a consequence of differences in the expression of capasaicinoid-metabolizing enzymes in each tissue. Our results (Figs. 3 to 7) indicate that hydroxylation and dehydrogenation are the two main pathways in the NADPH-driven biotransformation of the two capsaicinoids. These five metabolites of capsaicin were characterized in our previous study using recombinant individual cytochrome P450, showing that CM1, CM4 and CM5 can be generated by CYP6B6 and CYP9As, CM2 by CYP6B6, and CM3 by CYP9As (Tian et al., 2019). Considering that the five metabolites of capsaicin are products of cytochrome P450 catalyzed reactions, we hypothesize that the observed metabolism is mediated by cytochrome P450s in the preparations from different tissues, and that tissue- and species-specific differences in the expression of these and possibly other CYPs explain the observed differences in metabolism. While speculative, it is possible that induction of CYPs, particularly CYP6B6 which produces CM2, may be higher in the FB and MG of *H. assulta*.

In conclusion, our data demonstrate that the cumulative capsaicinoid metabolic capacity of the FB, MG and MT of *H. assulta* was greater than that of *H. armigera*. Capsaicinoids were metabolized by these three detoxification tissues into multiple alkyl hydroxylated and dehydrogenated metabolites, with ω-hydroxylation and ω-dehydrogenation being most significant pathways in the biotransformation of both capsaicin and dihydrocapsaicin. Tissue differences in the substrate specificity and relative contribution in capsaicin and dihydrocapsaicin metabolism were observed and the notably high specific activity was found for the Malpighian tubules of *H. armigera.* This result highlights the significance of this organ in xenobiotic detoxification. Additionally, the midgut is the first tissue exposed to xenobiotics during feeding, and it is seemingly logical that the most significant contributor to capsaicinoid detoxification for both pests was the midgut tissue. Overall, this study suggests that the fitness advantages observed for the specialist *H. assulta* over the generalist *H. armigera* when capsaicinoids are present in the food source may be related to an enhanced metabolic capacity for capsaicinoid degradation by *H. assulta,* potentially explaining their relative preference for capsicum fruits.

Acknowledgments

The authors are grateful to Dr. Junfeng Dong (Henan University of Science and Technology, China) for providing the oriental tobacco budworms used in this study. This work is supported by the National Natural Science Foundation of China (No. 31871999) to X. Qiu.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2019.11.013.

References

Ahn, S.J., Badenes-Pérez, F.R., Heckel, D.G., 2011a. A host-plant specialist, *Helicoverpa assulta*, is more tolerant to capsaicin from *Capsicum annuum* than other noctuid species. J. Insect Physiol. 57 (9), 1212–1219.
Ahn, S.J., Badenes-Pérez, F.R., Reichelt, M., Svatoš, A., Schneider, B., Gershenzon, J., Heckel, D.G., 2011b. Metabolic detoxification of capsaicin by UDP-glycosyltransferase in three *Helicoverpa* species. Arch Insect Biochem 78 (2), 104–118.
Al Othman, Z.A.A., Ahmed, Y.B.H., Habila, M.A., Ghafar, A.A., 2011. Determination of capsaicin and dihydrocapsaicin in *Capsicum* fruit samples using high performance liquid chromatography. Molecules 16 (10), 8919–8929.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1–2), 248–254.
Cai, Y., Jia, Y., Wang, T., Luo, M., Guo, X., Yuan, G., 2012. Effects of four pepper varieties on development and fecundity of *Helicoverpa assulta* (Guenée). Acta Phytophy Sin 39 (3), 211–216.
Chanda, S., Bashir, M., Babbar, S., Koganti, A., Bley, K., 2008. *In vitro* hepatic and skin metabolism of capsaicin. Drug Metab. Dispos. 36 (4), 670–675.
Cordell, G.A., Araujo, O.E., 1993. Capsaicin: identification, nomenclature, and pharmacotherapy. Ann. Pharmacother. 27 (3), 330–336.
Cowles, R.S., Keller, J.E., Miller, J.R., 1989. Pungent spices, ground red pepper, and synthetic capsaicin as onion fly ovipositional deterrents. J. Chem. Ecol. 15 (2), 719–730.
Dow, J.A.T., Pandit, A., Davies, S.A., 2018. New views on the Malpighian tubule from post-genomic technologies. Curr. Opin. Insect Sci. 29, 7–11.
Esquivel, C.J., Cassone, B.J., Piermarini, P.M., 2014. Transcriptomic evidence for a dramatic functional transition of the Malpighian tubules after a blood meal in the Asian tiger mosquito *Aedes albopictus*. Plos Neglect Trop D 8 (6), e2929.
Esquivel, C.J., Cassone, B.J., Piermarini, P.M., 2016. A de novo transcriptome of the Malpighian tubules in non-blood-fed and blood-fed Asian tiger mosquitoes *Aedes albopictus*: insights into diuresis, detoxification. and blood meal processing. Peerj 4 (2), e1784.
Fitt, G.P., 1989. The ecology of *Heliothis* species in relation to agroecosystems. Annu. Rev. Entomol. 34 (1), 17–53.
Goodwin, D.C., Hertwig, K.M., 2003. Peroxidasecatalyzed oxidation of capsaicinoids: steady-state and transient-state kinetic studies. Arch. Biochem. Biophys. 417, 18–26.
Jallow, M.F.A., Cunningham, J.P., Zalucki, M.P., 2004. Intra-specific variation for host plant use in Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae): implications for management. Crop Prot. 23 (10), 955–964.
Jia, Y., Cheng, X., Cai, Y., Luo, M., Guo, X., Yuan, G., 2012. Difference of the fitness of *Helicoverpa armigera* (Hübner) fed with different pepper varieties. Acta Phytophy Sin 32 (1), 159–167.
Kosuge, S., Furuta, M., 1970. Studies on the pungent principle of Capsicum. Agric. Biol. Chem. 34 (2), 248–256.
Leete, E., Louden, M.C.L., 1968. Biosynthesis of capsaicin and dihydrocapsaicin in *Capsicum frutescens*. J. Am. Chem. Soc. 90 (24), 6837–6841.
Li, Q.Q., Li, D.Y., Ye, H., Liu, X.F., Shi, W., Cao, N., Duan, Y.Q., 2011. Using COI gene sequence to barcode two morphologically alike species: the cotton bollworm and the oriental tobacco budworm (Lepidoptera: Noctuidae). Mol. Biol. Rep. 38 (8), 5107–5113.
Li, Y., Piermarini, P.M., Esquivel, C.J., Drumm, H.E., Schilkey, F.D., Hansen, I.A., 2017. RNA-Seq comparison of larval and adult Malpighian tubules of the yellow fever mosquito *Aedes aegypti* reveals life stage-specific changes in renal function. Front. Physiol. 8, 283.
Madhumathy, A.P., Aivazi, A.A., Vijayan, V.A., 2007. Larvicidal efficacy of *Capsicum annum* against *Anopheles stephensi* and *Culex quinquefasciatus*. J Vector Dis. 44 (3), 223–226.
Maliszewska, J., Marcinkowska, S., Nowakowska, A., Kletkiewicz, H., Rogalska, J., 2018. Altered heat nociception in cockroach *Periplaneta americana* L. exposed to capsaicin. J. Comp. Physiol A 204 (6), 605–611.
Maliszewska, J., Tegowska, E., 2012. Capsaicin as an organophosphate synergist against Colorado potato beetle (Leptinotarsa decemlineata Say). J. Plant Res. 52 (1), 28–34.
Mitter, C., Poole, R.W., Matthews, M., 1993. Biosystematics of the Heliothinae (Lepidoptera: Noctuidae). Annu. Rev. Entomol. 38 (1), 207–226.
Overend, G., Cabrero, P., Halberg, K.A., Ranford-Cartwright, L.C., Woods, D.J., Davies, S.A., Dow, J.A., 2015. A comprehensive transcriptomic view of renal function in the malaria vector, *Anopheles gambiae*. Insect Biochem Molec 67, 47–58.
Peña-Alvarez, A., Ramírez-Maya, E., Alvarado-Suárez, L., 2009. Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase microextraction–gas chromatography–mass spectrometry. J. Chromatogr. A 1216 (14), 2843–2847.
Perkins, B., Bushway, R., Guthrie, K., Fan, T., Stewart, B., Prince, A., Williams, M., 2002.

Determination of capsaicinoids in salsa by liquid chromatography and enzyme immunoassay. J. AOAC Int. 85 (1), 82–85.
Reilly, C.A., Yost, G.S., 2006. Metabolism of capsaicinoids by P450 enzymes: a review of recent findings on reaction mechanisms, bio-activation, and detoxification processes. Drug Metab. Rev. 38 (4), 685–706.
Reilly A., C., 2013. Cytochrome P450-dependent modification of capsaicinoids: pharmacological inactivation and bioactivation mechanisms. Role of capsaicin in oxidative stress and cancer. Springer, pp. 107–129.
Reilly, C.A., Ehlhardt, W.J., Jackson, D.A., Kulanthaivel, P., Mutlib, A.E., Espina, R.J., Moody, D.E., Crouch, D.J., Yost, G.S., 2003. Metabolism of capsaicin by cytochrome P450 produces novel dehydrogenated metabolites and decreases cytotoxicity to lung and liver cells. Chem. Res. Toxicol. 16 (3), 336–349.
Reilly, C.A., Henion, F., Bugni, T.S., Ethirajan, M., Stockmann, C., Pramanik, K.C., Srivastava, S.K., Yost, G.S., 2013. Reactive intermediates produced from metabolism of the vanilloid ring of capsaicinoids by P450 enzymes. Chem. Res. Toxicol. 26 (1), 55–66.
Srinivasan, R., Uthamasamy, S., Talekar, N.S., 2006. Characterization of oviposition attractants of *Helicoverpa armigera* in two Solanaceous plants. *Solanum viarum* and *Lycopersicon esculentum*. Curr. Sci. 90 (6), 846–850.
Stipcovich, T., Barbero, G.F., Ferreiro-González, M., Palma, M., Barroso, C.G., 2018. Fast analysis of capsaicinoids in Naga Jolokia extracts (*Capsicum chinense*) by high-performance liquid chromatography using fused core columns. Food Chem. 239, 217–224.
Tang, Q.B., Jiang, J.W., Yan, Y.H., van Loon, J.J., Wang, C.Z., 2006. Genetic analysis of larval host-plant preference in two sibling species of *Helicoverpa*. Entomol. Exp. Appl. 118 (3), 221–228.
Tian, K., Zhu, J., Li, M., Qiu, X., 2019. Capsaicin is efficiently transformed by multiple cytochrome P450s from Capsicum fruit-feeding *Helicoverpa armigera*. Pestic Biochem Phys 156, 145–151.
Usman, M., Rafii, M., Ismail, M., Malek, M., Latif, M., 2014. Capsaicin and dihydrocapsaicin determination in chili pepper genotypes using ultra-fast liquid chromatography. Molecules 19 (5), 6474–6488.
Wang, C., Dong, J., 2001. Interspecific hybridization of *Helicoverpa armigera* and *H. assulta* (Lepidoptera: Noctuidae). Chin. Sci. Bull. 46 (6), 489–491.
Wang, J., Kean, L., Yang, J., Allan, A.K., Davies, S.A., Herzyk, P., Dow, J.A., 2004. Function-informed transcriptome analysis of *Drosophila* renal tubule. Genome Biol. 5 (9), R69.
Wu, K., Gong, P., Ruan, Y., 2006. Is tomato plant the host of the oriental tobacco budworm, *Helicoverpa assulta* (Guenée)? Acta Entomologica Sin 49 (3), 421–427.
Yuan, Y.Y., Li, M., Fan, F., Qiu, X.H., 2018. Comparative transcriptomic analysis of larval and adult Malpighian tubules from the cotton bollworm *Helicoverpa armigera*. Insect Sci. 25 (6), 991–1005.