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Epicuticular hydrocarbons of the sugarcane borer *Diatraea saccharalis* (Lepidoptera: Crambidae)

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Abstract. The epicuticular hydrocarbons of the larval, pupal and adult stages of the sugarcane borer *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) are analysed. Dramatic changes are observed between the stages studied. Adult hydrocarbons are mostly saturated, with a predominance of 1–4 methyl-branched straight carbon skeletons of 37–47 atoms; the major components are isomeric mixtures of internally branched trimethylderivatives of C39, C37 and C41 carbon backbones. By contrast, very small amounts of methyl-branched components are detected in the pupae, although straight chain hydrocarbons of 23–35 carbons are the prevailing structures (70.7 ± 3.4%) with *n*-C29 and *n*-C27 as the major components. Unsaturated hydrocarbons (29.0 ± 3.5%) of similar chain lengths elute by gas chromatography of epicuticular extracts as complex mixtures of mono-, di- and trienes; with the degree of unsaturation increasing with chain length. This is the first report of very long chain unsaturated hydrocarbons in cuticular extracts of a larval lepidopteran (93.3 ± 0.6% of the lipid components), with chain lengths in the range 37–53 carbons and up to four double bonds; the major component being C49:3, which co-elutes with C49:4 and C49:2.

Key words. Developmental stages, epicuticle, gas chromatography, hydrocarbons, Lepidoptera, mass spectrometry.

Introduction

Hydrocarbons are the most extensively studied insect cuticle lipid components, with reported chain lengths that typically vary from approximately 20 to more than 50 carbons. The most well-known function of the cuticular lipid layer is as barrier against water loss, and hence the avoidance of lethal desiccation (Hadley, 1984; Gibbs, 1998). Cuticle lipids also serve as protection against environmental chemicals and microorganisms (Juárez, 1994; Juárez & Calderón-Fernández, 2007; Pedrini *et al*., 2009), as well as in chemical communication, usually as contact pheromones (Ginzel & Hanks, 2003; Ferveur, 2005; Cocchiararo-Bastias *et al*., 2011). Although there are thousands of scientific reports on insect hydrocarbons (Blomquist & Bagnères, 2010), relatively few of these studies are in the order Lepidoptera, which comprises one of the most species-rich and diverse groups (Howard & Baker, 2004). The sugarcane borer *Diatraea saccharalis* Fabricius (Pyralidae, Crambidae) is native to the Americas, spreading from southeast U.S.A., throughout the Caribbean, Central America, and South America, to northern Argentina. Larvae of *D. saccharalis* bore into the sugarcane stalks producing mechanical and physiological damage. The extent of the damage depends on seasonality; they attack the leaf whorl early in the season; at later stages, the larvae tunnel through the stalk, causing the plant to be prone to breakage (Rodriguez-del-Bosque *et al*., 1990). In Argentina, economic losses close to 20% of the corn production are reported, although the pest's population has diminished significantly after the introduction of genetically-modified (Bt) corn in the last decade (Serra & Trumper, 2006).

Dramatic transformations occurring along the life cycle of lepidopteran insects might be correlated with major changes in cuticle components relevant to insect fitness. The present study aims to identify the major epicuticular hydrocarbon

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components of *D. saccharalis* through its developmental stages, from larva to pupa, and adult, to understand the changes in cuticle composition and their relation to the major physiological transitions through its life cycle.

Materials and methods

Insects

Larvae and pupae of *D. saccharalis* were obtained from a colony reared at CIRPON (Centro de Investigaciones sobre el Control de Poblaciones de Organismos Nocivos, Fundación Miguel Lillo, Argentina). Larvae were maintained in the laboratory under a LD 12 : 12 h photocycle at 25 ± 2 °C and $70 \pm 5\%$ relative humidity and on an artificial diet (Hensley & Hammond, 1968 comprising soybean pellet (150 g), wheat germ (100 g), sugar (140 g), vitamin solution (15 mL), ascorbic acid (5 g), Formol (2.5 mL) and water (1.0 L), fortified with vitamin B_{12}, choline chloride (1.0 g), agar (17.5 g) and streptomycin (1.0 g). Fifth larval stage (5 days old), 7-day-old pupae and adults were used for lipid analyses. Pupae were maintained in separate containers and sexed upon emergence, and adults (males/females separate) were analysed on day 2.

Chemicals

Solvents (*n*-hexane, diethyl ether) and iodine were obtained from Carlo Erba (Italy). Dimethyl disulphide (DMDS) and *n*-alkane standards of 10–44 carbons were purchased from Sigma Aldrich (St Louis, Missouri). Sodium thiosulphate pentahydrate ($Na_2S_2O_3$) was obtained from Merck (Germany).

Sample extraction

For hydrocarbon extraction and analysis, epicuticular lipids were extracted by immersing three specimens of each insect stage (larvae, pupae and adults) in three successive portions of redistilled *n*-hexane (6 mL g^{-1}) for 5 min each ($n = 5$). The lipid extracts were concentrated under a stream of nitrogen gas. Epicuticular hydrocarbons were separated from other components by adsorption chromatography on a mini-column (2.5×0.5 cm) of activated Supelcosil A (Supelco, Bellefonte, Pennsylvania), eluted with redistilled *n*-hexane (6 mL mg^{-1} lipid), then concentrated under a nitrogen atmosphere (Juárez *et al*., 2001). Hydrocarbons of the artificial diet were extracted similarly. The epicuticular lipids of each developmental stage were analysed by thin-layer chromatography on silica gel plates (Polygram Sil G/UV254, 4×8 cm; Macherey-Nagel, Germany), using two solvent development mixtures: *n*-hexane (100%) followed by *n*-hexane/ethyl ether/acetic acid (80 : 20 : 1, v/v/v). Plates were sprayed with 5% sulphuric acid in 95% ethanol and lipid bands were visualized after charring at 180–200 °C for 20 min. Unsaturation of the epicuticular hydrocarbons of each stage was assessed using silica gel plates that were impregnated with silver nitrate in acetonitrile (20% w/v), and with *n*-hexane/ethyl ether (80 : 20, v/v) as the developing solvent. Bands were visualized after charring as before.

Unsaturated hydrocarbon derivatization

The locations of double-bonds were determined after derivatization of the hydrocarbons according to Carlson *et al*. (1989); hydrocarbon extracts in *n*-hexane (100 μL) were treated with 100 μL of DMDS and 50 μL of iodine solution in diethyl ether (60 mg mL^{-1}). The reaction mixture was held for 4 h at 40 °C, diluted with 0.5 mL of *n*-hexane and treated with $Na_2S_2O_3$ (10% w/v) until the iodine colour disappeared. The organic phase was then separated and evaporated under nitrogen.

Capillary gas chromatography-mass spectrometry

Analysis of epicuticular hydrocarbons by capillary gas chromatography-mass spectrometry was performed using a Hewlett Packard 6890 gas chromatograph equipped with a HP-5MS capillary column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 μm) (Hewlett-Packard, Palo Alto, California) and coupled with a mass selective detector (5975C VL; Agilent, Santa Clara, California). The injector port was operated in splitless mode at 280 °C, and the oven temperature was programmed from 50 °C (hold time 1 min) to 200 °C (50 °C min^{-1}) and then to 300 °C at 3 °C min^{-1} (hold time 10 min). The carrier gas was helium, at constant flow (1.5 mL min^{-1}). The mass selective detector was operated in the SCAN mode with a mass range of m/z 35–650, electron impact mode at 70 eV and transfer line at 320 °C; the ionization chamber and quadrupole were operated at 230 and 150 °C, respectively. A high temperature Zebron ZB-5HT Inferno column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 μm; Phenomenex, Torrance, California) was used for very high molecular weight components. For the high-temperature analyses, some operating conditions were modified; the injector port temperature was set at 360 °C and the oven was programmed from 50 °C (hold time 1 min) to 200 °C (50 °C min^{-1}), then to 360 °C (7 °C min^{-1}), with a holding time of 20 min. The mass range of the mass selective detector was modified to m/z 35–850, the transfer line was set at 360 °C, and the temperature of the ionization chamber was set at 300 °C. Analysis of commercially available *n*-alkane standards of 22–44 carbons was performed similarly for estimation of the Kovats index (KI) (Kovats, 1965). The methyl branching assignments of epicuticular hydrocarbons were based on their KI values and mass fragmentation patterns (Juárez *et al*., 2001). The relative amounts of each component (mean ± SE) were calculated by dividing the corresponding peak area by the total hydrocarbon peak area. Shorthand nomenclature is used in the text and tables to identify the hydrocarbons. CXX indicates the total number of carbons in the straight chain; linear alkanes are denoted by *n*-CXX, whereas the location of methyl branches is described as x-Me for monomethyl alkanes, x,x-DiMe for dimethyl alkanes, x,x,x-TriMe for trimethyl alkanes, and x,x,x,x-TetraMe for tetramethyl alkanes. Unsaturated hydrocarbons are shown as x-CXX:1, x,x-CXX:2, x,x,x-CXX:3 and x,x,x,x-CXX:4 to indicate alkenes, alkadienes, alkatrienes and alkatetraenes, respectively.

Results

Epicuticular lipids of larvae, pupae and adults of *D. saccharalis* differed qualitatively after thin-layer chromatography analyses. Hydrocarbons were the major components in pupae and larvae. By contrast, free fatty acids and triacylglycerols were predominant in adults, together with minor amounts of hydrocarbons, waxes and cholesterol esters. The corresponding hydrocarbon fractions were checked for unsaturation on $AgNO_3$-impregnated plates, showing the presence of saturated, mono- and multiple unsaturated bands in pupae and larvae; adult hydrocarbons were mostly saturated.

The three insect stages differed significantly in the structure of their hydrocarbon components. Odd-numbered chains prevailed, with KI from 2300 to 5300. Straight chain saturated components ranged from 23 to 35 carbons; methyl-branched chains varied from 37 to 47 carbons, with 1–4 methyl branches. Up to four double bonds were detected in the unsaturated fraction, varying from 23 to 53 carbons (Tables 1 and 2). Trace amounts of saturated hydrocarbons (*n*-C23 to *n*-C33) were detected in the lipid extracts of the artificial diet.

Adult stage

Saturated very long chain methyl-branched components were the predominant structures of the epicuticular hydrocarbons of adult *D. saccharalis* (71.0 ± 0.7%), together with 26.7 ± 0.1% of straight chain components and 2.3 ± 0.5% of unsaturated straight chain hydrocarbons (Tables 1 and 2). Isomeric mixtures of mono-, di-, tri- and tetramethyl derivatives of very long chain methyl-branched components, with 37–47 carbons, mostly in odd-numbered straight chain backbones, were detected. Small amounts of monomethyl branched components with odd-numbered chains (39–45 carbons) with the CH_3 group inserted internally in odd carbons (mostly 11- to 21-) were detected together with trace amounts of MeC44 isomers with the CH_3 inserted in odd and even positions. Isomeric mixtures of internally branched dimethyl derivatives were also identified with carbon skeletons of 37–47 carbons. The major components were 13,23-DiMeC43 and smaller amounts of the 13,25- isomer (peak 35; Fig. 1), together with 13,17-DiMeC45 and minor amounts of the 13,23- and 13,25- isomers (peak 41; Fig. 1). Isomeric mixtures were more complex at increasing chain lengths, as in DiMeC41, DiMeC42 and DiMeC44 with up to eight to nine components, with the CH_3 group inserted both in odd- and even-numbered carbons (Table 1). The higher molecular weight component of this fraction was 13,17-DiMeC47 (peak 42; Fig. 1 and and Table 1). Trimethylalkanes were the major components, with odd-numbered chains usually predominating over even-numbered chains. A series of internally branched isomeric mixtures with the 13,17,21- or 11,15,19-trimethylderivative in carbon backbones of 39, 37 and 41 atoms were the dominant structures, with the 11,15,19-TriMeC39 being the major isomer (peak 22; Fig. 1 and Table 1) followed by a mixture of 11,15,19- and other isomers of DiMeC41 (peak 30; Fig. 1 and Table 1). Considerable amounts of trimethylderivatives with the CH_3 group inserted in even carbons (12,16,20- and 12,16,22-) of even-numbered chain components between 38 and 44 carbons were also detected. Small amounts of tetramethyl derivatives of odd and even chains between 38 and 41 carbons in the straight chain skeleton were detected as well, usually eluting in the tail of the corresponding trimethyl derivative, as shown for the predominant isomer 9,13,17,21-TetraMeC39 (peak 23; Fig. 1 and Table 1); the higher KI (4171) detected corresponded to 11,15,19,23-TetraMeC41 (peak 31; Fig. 1 and Table 1). Small amounts of odd-numbered monounsaturated hydrocarbon chains (27–33 carbons) were identified. After DMDS derivatization, the double bond location was detected in 5-, 7- and 9-positions, both in the male and female mono-unsaturated fraction. Trace amounts of mono-unsaturated components with double bond location in 10-, 11-, 12-, 13-, 14-, 15- and 16-position were only detected in females; isomers with central double bond location were predominant eluting ahead of terminal double bond isomers. Di-unsaturated components were also detected in trace amounts, although the high molecular weight of the corresponding DMDS derivative prevented identification. In the straight chain fraction, relatively shorter odd-numbered chains of 23–33 carbons were detected, the major component was C29 (peak 7; Fig. 1), followed by C27 and C31 (peaks 5 and 9, respectively; Fig. 1).

Pupae

Saturated, mostly odd-numbered straight chains ranging from 23 to 35 carbons represented 70.7 ± 3.4% of the hydrocarbon fraction in pupae, with *n*-C27 and *n*-C29 as the major components. Very small amounts of methyl-branched hydrocarbons (0.3 ± 0.2%), with a structure similar to that of the major methyl-branched components of adults, were also detected. In the unsaturated fraction (29.0 ± 3.5%), one to three double bonds were detected in hydrocarbons of chain lengths similar to that of the major saturated components (Fig. 1 and Table 2). Usually, multicomponent peaks contained different mixtures of a large variety of positional isomers, sometimes co-eluting with mono- and di-unsaturated components. Trienes were also detected in the extracted ion chromatogram eluting together with dienes of the same chain length. Components with three double bonds were undetectable or present in small amounts of relatively shorter chains up to 35 carbons. However, the relative abundance of trienes increased steadily with chain length, reaching to more than 30% of the C35 peak; at the same time, the relative amounts of monoenes in the multicomponent peak decreased. The high molecular weight of the corresponding adducts prevented the position of the double bonds being

Table 1. Major saturated cuticular hydrocarbons of the sugarcane borer *Diatraea saccharalis.*

Peak[a]	KI	Hydrocarbon[b]	Larva	Pupa	Adult	Diagnostic ions, *m/z*[c]
1	2300	*n*-C23	+	+	++	324
2	2400	*n*-C24	+	+	+	338
3	2500	*n*-C25	+	++	++	352
4	2600	*n*-C26	+	++	+	366
5	2700	*n*-C27	++	+++	++	380
6	2800	*n*-C28	+	++	++	394
7	2900	*n*-C29	++	+++	++	408
8	3000	*n*-C30	+	++	+	422
9	3100	*n*-C31	+	++	++	436
10	3200	*n*-C32	+	+	+	450
11	3300	*n*-C33	+	+	++	464
12	3400	*n*-C34	—	+	+	478
13	3500	*n*-C35	—	+	+	492
14	3745	15,21-DiMeC37	—	—	+	224/225, 351; 252/253, 323; 533
15	3771	13,17,21-TriMeC37	—	—	++	196/197, 393; 267, 323; 252/253, 337; 547
		11,15,21-; 11,17,21-TriMeC37				168/169, 421; 239, 351; 267, 323; 252/253, 337; 547
16	3793	9,13,17,21-TetraMeC37	—	+	+	140/141, 463; 211, 393; 281, 323; 252/253, 351; 561
17	3842	14,18-; 14,20-DiMeC38	—	—	++	210/211, 379; 308/309, 281; 280/281, 309; 547
		3,11,19-TriMeC38				56/57, 547; 183, 421; 294/295, 309; 561
18	3870	12,16,20-; 12,16,22-TriMeC38	—	+	++	182/183, 421; 253, 351; 280/281, 323; 252/253, 351; 561
19	3889	10,14,18,22-TetraMeC38	—	—	++	154/155, 463; 225, 393; 295, 323; 351, 280/281; 575
20	3921	11-; 13- MeC39	—	—	+	168/169, 420/421; 196/197, 392/393; 547
		15-; 17-MeC39				224/225, 364/365; 252/253, 336/337; 547
21	3940	13,17-; 13,19-DiMeC39	—	—	++	196/197, 407; 336/337, 267; 308/309, 295; 561
		15,19-DiMeC39				224/225, 379; 308/309, 295; 561
22	3982	13,17,21-TriMeC39	—	+	+++	196/197, 421; 267, 351; 280/281, 337; 575
		11,15,19-TriMeC39				168/169, 449; 239, 379; 308/309, 309; 575
23	4000	9,13,17,21-TetraMeC39	—	—	++	140/141, 463; 211, 421; 351, 281; 280/281, 323; 589
24	4010	5,11,17,21-TetraMeC39	—	—	+	84/85, 547; 183, 449; 281, 351; 280/281, 351; 589
25	4033	14,18-; 14,20-DiMeC40	—	—	++	210/211, 407; 336/337, 281; 308/309, 309; 575
		14,22-DiMeC40				210/211, 407; 280/281, 337; 575
		3,11,19-; 3,11,21-TriMeC40	—	—	+	56/57, 575; 183, 449; 322/323, 309; 294/295, 337; 589
26	4063	12,16,20-; 12,16,22-TriMeC40	—	+	+++	182/183, 449; 253, 379; 308/309, 323; 280/281, 351; 589
		14,18,22-TriMeC40				210/211, 421; 281, 351; 280/281, 351; 589
27	4076	10,14,18,22-TetraMeC40	—	—	+	154/155, 491; 225, 421; 295, 351; 280/281, 365; 603
		10,16,20,24-TetraMeC40				253, 393; 323, 323; 252/253, 393; 603
28	4103	11-; 13-MeC41	—	—	+	168/169, 448/449; 196/197, 420/421; 575
		15-; 17-MeC41				224/225, 392/393; 252/253, 364/365; 575
		19-; 21-MeC41				280/281; 336/337, 308/309; 575
29	4127	11,17-; 11,19-; 11,23-DiMeC41	—	—	++	168/169, 463; 364/365, 267; 280/281, 351; 589
		13,17-; 13,19-; 13,23-DiMeC41				196/197, 435; 364/365, 267; 280/281, 351; 589
		15,19-; 15,23-DiMeC41				224/225, 407; 280/281, 351; 589
30	4163	13,17,21-; 13,17,23-TriMeC41	—	+	+++	196/197, 449; 267, 379; 308/309, 337; 280/281, 365; 603
		11,15,19-; 11,15,21-TriMeC41				168/169, 477; 239, 407; 336/337, 309; 308/309, 337; 603
31	4171	11,15,19,23-TetraMeC41	—	—	++	168/169, 491; 239, 421; 309, 351; 280/281, 379; 617
32	4227	11,21-; 11,23-DiMeC42	—	—	++	168/169, 477; 322/323, 323; 294/295, 351; 603
		12,20-; 12,24-DiMeC42				182/183, 463; 336/337, 309; 308/309, 365; 603
		13,17-; 13,21-DiMeC42				196/197, 449; 378/379, 267; 322/323, 323; 603
		13,23-DiMeC42				196/197, 449; 294/295, 351; 603
		14,20-; 14,24-DiMeC42				210/211, 435; 336/337, 309; 308/309, 365; 603
33	4254	12,16,20-; 12,16,22-TriMeC42	—	+	++	182/183, 477; 253, 407; 336/337, 323; 308/309, 351; 617
34	4311	13-; 11-MeC43	—	—	++	196/197, 448/449; 168/169, 476/477; 603
		15-; 17-MeC43				224/225, 420/421;252/253, 392/393; 603
		19-;21-MeC43				280/281, 364/365; 308/309, 336/337; 603
35	4346	13,23-; 13,25-DiMeC43	—	—	++	196/197, 463; 308/309, 351; 280/281, 379; 617
36	4365	13,17,21-TriMeC43	—	+	++	196/197, 477; 267, 407; 336/337, 337; 631
		11,15,23-TriMeC43				168/169, 505; 239, 435; 308/309, 365; 631
37	4427	12-; 13-MeC44	—	—	+	182/183, 477; 196/197, 463; 617
		14-;15-MeC44				210/211, 449; 224/225, 435; 617
		21-MeC44				308/309, 351; 617

Table 1. continued

Peak[a]	KI	Hydrocarbon[b]	Larva	Pupa	Adult	Diagnostic ions, *m/z*[c]
38	4458	12,22-; 12,24-DiMeC44	—	—	+	182/183, 491; 336/337, 337; 308/309, 365; 631
		13,23-; 13,25-DiMeC44				196/197, 477; 322/323, 351; 294/295, 379; 631
		14,22-; 14,24-DiMeC44				210/211, 463; 336/337, 337; 308/309, 365; 631
		15,23-; 15,25-DiMeC44				224/225, 449; 322/323, 351; 294/295, 379; 631
39	4479	12,16,20-TriMeC44	—	—	+	182/183, 505; 253, 435; 364/365, 323; 645
		12,16,22-TriMeC44				182/183, 505; 253, 435; 336/337, 351; 645
		12,18,22-TriMeC44				182/183, 281; 407, 336/337; 351; 645
		14,18,22-TriMeC44				210/211, 477; 281, 407; 336/337, 351; 645
40	4560	13-; 11-MeC45	—	—	+	196/197, 476/477; 182/183, 504/505; 631
		15-; 21-MeC45				224/225, 448/449; 308/309, 364/365; 631
41	4596	13,17-; 13,23-DiMeC45	—	—	++	196/197, 491; 420/421, 267; 336/337, 351; 645
		13,25-DiMeC45				196/197, 491; 308/309, 379; 645
42	4790	13,17-DiMeC47	—	—	+	196/197, 519; 448/449, 267; 673

[a]Numbers correspond to peaks from Fig. 1. The capillary gas chromatography (CGC) peaks are listed in their order of elution. Some minor components may not be evident in Fig. 1.

[b]Straight chain hydrocarbons were identified by comparison of their Kovats index (KI) with that of the corresponding standards. Methyl branching was estimated based on their KI values and their mass fragmentation pattern.

Percentage composition of hydrocarbons was calculated from the integrated area data from CGC-mass spectrometry analysis of groups of 15 insects of each stage: − absent, + < 0.5%, ++ 0.5 −10%, + + + > 10%.

The percentage of the straight chains was 6.7 ± 0.6, 70.7 ± 3.4 and 26.7 ± 0.1, and of methyl-branched chains was 0.0 ± 0.0, 0.3 ± 0.2 and 71.0 ± 0.7, respectively, for larvae, pupae and adults (means ± SD).

[c]Diagnostic ions from chemical ionization mass spectral analysis.

readily determined. Odd-numbered chain mono-unsaturated isomers with the double bond mostly located in position 5-, 7- and 9- were detected; the double bond location predominated in the 5-position in the relatively shorter chains (C23), changing to the 9-position with increasing chain lengths (C35). Monoalkenes represented close to 90% of the unsaturated peaks of 25–27 carbons, similar amounts of mono- and di-unsaturated components were detected between C28 and C31, diminishing to only approximately 8% in the C35 peak (Fig. 1 and Table 2). In this fraction, a series of diene structures with 6,9-arrangement of the double bonds was indicated by the ions *m/z* 67 and 81; the characteristic ions *m/z* 96 and 110 and the occurrence of the $[M\text{-}98]^+$ ion originated from cleavage between carbons 7 and 8, and subsequent hydrogen transfer, as reported by Descoins *et al*. (1986). After DMDS derivatization, the 6,9-arrangement of the double bonds was confirmed by interpretation of the mass spectra of the corresponding DMDS adducts (Carballeira *et al*., 1994; Carballeira & Cruz, 1996). The major components identified were 6,9-C29:2, 6,9-C31:2 and 6,9-C33:2. Figure 2 shows the adducts derived from 6,9-C29:2 with molecular ion = *m/z* 530, the fragmentation pattern showed ions of *m/z* 155, 203 and 327 for adduct 1, and ions of *m/z* 131, 351 and 399 for adduct 2 (Fig. 2). Both adducts also showed the ions of *m/z* 483 and 435 corresponding to mass losses of 47 (CH_3S) and 95 ($CH_3S + CH_3SH$), respectively. The ion *m/z* 155 in adduct 1 came from the loss of a methanothiol group from the ion *m/z* 203 [100%, $C_9H_{15}S]^+$(Fig. 2). In adduct 2, the ions *m/z* 131 and 399 were formed by a cleavage between the carbon atom of the side chain bearing the thiomethyl group and the carbon atom of the ring. Furthermore, elimination of thiomethanol from the ion *m/z* 399 produced the ion *m/z* 351 (Fig. 2). A similar fragmentation pattern [558 (M^+, 5), 511 (22), 463 (75), 379 (33), 355 (48), 203 (27) and 155 (100)] and [586 (M^+, 3), 155 (100), 203 (24), 383 (48), 407 (26), 491(94) and 539 (27)] is shown for the 6,9-C31:2 and 6,9-C33:2 adducts, respectively (Table 2). These fragmentation patterns agree well with the *cis-cis* double bond stereochemistry (Z,Z)-6,9-CXX:2 (Carballeira & Cruz, 1996). Smaller amounts of dienes with larger separation between double bonds, such as 7,19-, 9,19- and 7,21-isomers of C31:2, C33:2 and C35:2 were also detected (peaks 11^U, 16^U and 18^U; Fig. 1 and Table 2). Unsaturated dienes with molecular weight two atomic mass units lower than that of the corresponding alkane, and eluting at a higher KI, were identified as conjugated dienes (Sojak *et al*., 1984) (x,x-C27:2, x,x-C29:2 and x,x-C31:2), corresponding to peaks 5^U, 9^U and 13^U, respectively in Fig. 1; trace amounts prevented establishing the double bond position.

Larval hydrocarbons

Very long chain unsaturated components were the predominant structures in the larval epicuticle (93.3 ± 0.6%), with chain length ranging from 37 to 53, and up to four double bonds (Table 2). After derivatization, double bond position was estimated for monoenes of 37 and 39 carbons (peaks 19^U and 21^U; Fig. 1 and Table 2); higher molecular weight components were not volatilized in the chromatographic conditions used, as reported by Carlson *et al*. (1989). Figure 1 shows the three major unsaturated peaks (31^U, 36^U and 27^U of 49, 51 and 47 carbons, respectively). The major component was C49:3 (*m/z* 682) co-eluting with C49:4 (*m/z* 680) and C49:2 (*m/z* 684) in peak 31^U. Peak 36^U comprised mostly C51:3 (*m/z* 710) and C51:4 (*m/z* 708) isomers. Mostly C47:2 (*m/z* 656) and minor amounts of C47:3 (*m/z* 654) components co-eluted in

Table 2. Unsaturated hydrocarbons of *Diatraea saccharalis*.

Peak[a]	KI	Hydrocarbon[b]	Larva	Pupa	Adult	Diagnostic ions m/z[c]
1^U	2285	9-C23:1	—	+	+	322 [173, $243^{P,A}$; 416]
2^U	2465–2488	x,x-C25:2	—	+	—	348^P
		5-;7-C25:1	—	+	—	350 [117, 327^P; 145, 299^P; 444]
		9-C25:1	—	+	+	[173, $271^{P,A}$; 444]
		10-;12-C25:1	—	—	+	[187, 257^A; 215, 229^A; 444]
3^U	2569–2585	5-C26 :1	—	+	—	364 [117, 341^P; 458]
		7-;9-C26:1	—	+	+	[145, $313^{P,A}$; 173, $285^{P,A}$; 458]
		10-;11-;12-;13-C26:1	—	—	+	[187, 271^A; 201, 257^A; 215, 243^A; 229, 229^A; 458]
4^U	2665–2686	x,x,x-C27:3	—	+	—	374^P
		6,9-C27:2	—	+++	+	376 [a1:155, 203, 299, 407, 455^P; 502; a2:131, 323, 371, 407, 455^P; 502]
		5-;7-;9-C27:1	—	+++	+	378 [117, $355^{P,A}$; 145, $327^{P,A}$; 173, $299^{P,A}$; 472]
		10-;12-;13-C27:1	—	—	+	[187, 285^A; 215, 257^A; 229, 243^A; 472]
5^U	2740	x,x-C27:2	—	+	—	376^P
6^U	2766–2787	x,x,x-C28:3	—	+	—	388^P
		6,9-C28:2	—	+	+	390 [a1:155, 203, 313, 421, 469^P; 516; a2:131, 337, 385, 421, 469^P; 516]
		5-;7-;9-C28:1	—	+	+	392 [117, $369^{P,A}$; 145, $341^{P,A}$; 173, $313^{P,A}$; 486]
		10-;11-;12-;13-14-C28:1	—	—	+	[187, 299^A; 201, 285^A; 215, 271^A; 229, 257^A; 243, 243^A; 486]
7^U	2865	x,x,x-C29:3	—	+	—	402^P
		6,9-C29:2	—	++	+	404 [a1:155, 203, 327, 435, $483^{P,A}$; 530; a2:131, 399, 351, 435, $483^{P,A}$; 530]
		5,15-C29:2	—			[117, 243, 255, 381^P; 592]
8^U	2869–2888	5-;7-;9-C29:1	—	+++	+	406 [117, $383^{P,A}$; 145, $355^{P,A}$; 173, $327^{P,A}$; 500]
		10-;12-;13-;14-C29:1[d]	—	—	+	[187, 313^A; 215, 285^A; 229, 271^A; 243, 257^A; 500]
9^U	2947	x,x-C29:2	—	+	—	404^P
10^U	2970–2989	5-;7-;8-C30:1	—	+	—	420 [117, 397^P; 145, 369^P; 159, 355^P; 514]
		9-;10-C30:1	—	+	+	[173, $341^{P,A}$; 187, $327^{P,A}$; 514]
		11-;12-;13-;14-15-C30:1	—	—	+	[201, 313^A; 215, 299^A; 229, 285^A; 243, 271^A; 257, 257^A; 514]
11^U	3045–3069	x,x,x-C31:3	—	+	—	430^P
		6,9-C31:2	—	++	—	432 [a1:155, 203, 355, 379, 463, 511^P; 558; a2:131, 379, 427, 463, 511^P; 558]
		7,19-;9,19-9,21-C31:2	—	++	+	[145, 215, 311, $381^{P,A}$; 620; 173,215, 311, $353^{P,A}$; 620; 173, 187, 339, $353^{P,A}$; 620]
12^U	3074–3088	5-;7-C31:1	—	++	+	434 [117, 411^P; 145, 383^P; 542]
		9-C31:1	—	++	+	[173, $355^{P,A}$; 542]
		12-;13-;14-;15-C31:1[d]	—	—	+	[215, 313^A; 229, 299^A; 243, 285^A; 257, 271^A; 528]
13^U	3146	x,x-C31:2	—	+	—	432^P
14^U	3170	x,x,x-C32:3	—	+	—	444^P
		x,x-C32:2	—	+	—	446^P
		7-;8-;9-;11-;12-C32:1	—	+	—	448 [145, 397^P; 159, 383^P; 173, 369^P; 201, 341^P; 215, 327^P; 542]
15^U	3240	x,x,x-C33:3	—	++	—	458^P
16^U	3245–3270	6,9-C33:2	—	++	+	460 [a1:155, 203, 383, 407, 491, 539^P; 586; a2:131, 407, 455, 491, 539^P; 586]
		7,21-; 9,19-9,21-C33:2	—	++		[145, 215, 339, 409^P; 648; 173, 243, 311, 381^P; 648; 173, 215, 339, 381^P; 648]
17^U	3275–3290	5-;7-;9-C33:1	—	+	—	462 [117, 439^P; 145, 411^P; 173, 383^P; 556]
		13-;14-;15-;16-C33:1[d]	—	—	+	[229, 327^A; 243, 313^A; 257, 299^A; 271, 285^A; 556]
18^U	3450	x,x,x-C35:3	—	+	—	486^P
		9,21-C35:2	—	+	+	488 [173, 243, 409, 339^P; 676]
		9-C35:1	—	+	—	490 [173, 411^P; 584]
19^U	3643	15-;16-;17-;18-C37:1	+	—	—	518 [257, 355^L; 271, 341^L; 285, 327^L; 299, 313^L; 612]
20^U	3847	x,x-C39:2	++	—	—	544^L
21^U		11-;13-;14-;15-C39:1	+	—	—	546 [201, 439^L; 229, 411^L; 243, 397^L; 257, 383^L; 640]
		16-;17-;18-;19-C39:1	+	—	—	[271, 369^L; 285, 355^L; 299, 341^L; 313, 327^L; 640]
22^U	nd	x,x-C41:2	+	—	—	572^L

Table 2. continued

Peak[a]	KI	Hydrocarbon[b]	Larva	Pupa	Adult	Diagnostic ions m/z[c]
23[U]	nd	x,x-C43:2	+	—	—	600[L]
24[U]	nd	x,x,x-C45:3	+	—	—	662[L]
25[U]	nd	x,x-C45:2	++	—	—	628[L]
26[U]	nd	x,x,x-C47:3	++	—	—	654[L]
27[U]	nd	x,x-C47:2	++	—	—	656[L]
28[U]	nd	x,x,x-C48:3	++	—	—	668[L]
29[U]	nd	x,x-C48:2	++	—	—	670[L]
30[U]	nd	x,x,x,x-C49:4	+	—	—	680[L]
31[U]	nd	x,x,x-C49:3	+++	—	—	682[L]
32[U]	nd	x,x-C49:2	+++	—	—	684[L]
33[U]	nd	x,x,x-C50:3	++	—	—	696[L]
34[U]	nd	x,x-C50:2	++	—	—	698[L]
35[U]	nd	x,x,x,x-C51:4	++	—	—	708[L]
36[U]	nd	x,x,x-C51:3	+++	—	—	710[L]
37[U]	nd	x,x-C51:2	++	—	—	712[L]
38[U]	nd	x,x-C52	++	—	—	726[L]
39[U]	nd	x,x,x-C53	++	—	—	738[L]

[a] Numbers correspond to peaks from Fig. 1. The capillary gas chromatography (CGC) peaks are listed in their order of elution. Some minor components may not be evident in Fig. 1.
[U] Unsaturated hydrocarbon peaks.
[b] Unsaturated hydrocarbons were identified based on their Kovats index (KI), mass fragmentation pattern and diagnostic ions after dimethyl disulphide (DMDS) derivatization. x- indicates an unidentified double bond position.
Percentage composition of hydrocarbons was calculated from the integrated area data from CGC-mass spectrometry analysis of 15 insects from each stage: − absent, + < 0.5%, ++ 0.5, − 10%, + + + > 10%.
[c] Ions in parenthesis are from the dithiomethyl ether derivatives. a1 and a2 correspond to diagnostic ions after DMDS derivatization of adducts derived from hydrocarbons with a 6,9-arrangement of the double bonds. [A,P,L] Corresponding to the isomer detected in each insect stage (adult, pupa and larva, respectively).
[d] Peaks 8[U], 12[U] and 17[U] indicate isomers with the double bond in the middle of the chain and the shortest retention time. These trace components were only detected in female.
The percentage of unsaturated chains was 93.3 ± 0.6%, 29.0 ± 3.5% and 2.3 ± 0.5% for larvae, pupae and adults, respectively (means ± SD).

peak 27[U] (Figs 1 and 3). The saturated hydrocarbon fraction of larvae (6.7 ± 0.6%) mostly comprised *n*-alkanes of 23–33 carbon atoms, with *n*-C27 being the major component (Fig. 1 and Table 1); only trace amounts of branched components were detected in some samples (data not shown).

Discussion

The order Lepidoptera is one of the most species-rich orders, including some major forest and agronomy pests, among them larvae of the families Geometridae, Lymantriidae, Tortricidae, Noctuidae and Pyralidae. Nelson & Buckner (1995) describe the cuticle hydrocarbon components of larvae of the tobacco budworm *Heliothis virescens* and corn earworm *Helicoverpa zea*, with predominant mono- and dimethyl-branched components up to 35 carbon atoms, together with smaller amounts of branched components up to 53 carbons in the straight chain backbone; less than 5% of the alkene 31:1 is found only in *H. zea*. By contrast, in the surface hydrocarbons of the diapausing pupae of the tobacco hornworm *Manduca sexta*, Coudron & Nelson (1981) report alkenes, alkadienes and alkatrienes of 23–44 carbons, together with straight chain saturated hydrocarbons of 21–41 carbons and branched components with 1–3 methyl groups inserted in carbon backbones ranging from 23 to 43 carbons. The cuticular hydrocarbons of the cabbage looper *Trichoplusia ni* are shown to vary with development. The major components of the larval and pupal hydrocarbons are 2-methyl- and internally branched mono-, di- and tri-methylalkanes of 31–42 carbons. After the adult transition, the methylalkane relative amounts diminish, accompanied by increasing amounts of straight chains, as shown from the incorporation of radioactive precursors into the hydrocarbons, which switches from almost exclusive synthesis of methylalkanes during the larval stages to the production of only *n*-alkanes as adults (de Renobales & Blomquist, 1983). In the southern armyworm *Spodoptera eridania*, *n*-alkanes, mono- and dimethylalkanes ranging from 23 to 35 carbons are reported during larvae and pupae development. Hydrocarbons are shown to be produced at specific stages during larval stadia and accumulated internally, possibly to be transported to the surface of the next stage (Guo & Blomquist, 1991). Interestingly, Howard & Baker (2004) report major developmental changes in the cuticle of the pyralid moth *Plodia interpunctella*, a stored product pest. The adult cuticle contains mostly saturated straight and methyl-branched components together with few monoene isomers of C27:1 and C29:1. Dramatic changes are revealed in the larvae and pupae cuticles with almost no hydrocarbons (other than small amounts of straight chain components); however, the presence of large amounts of 2-acyl-1,3-cyclohexanediones is postulated to mimic the physical

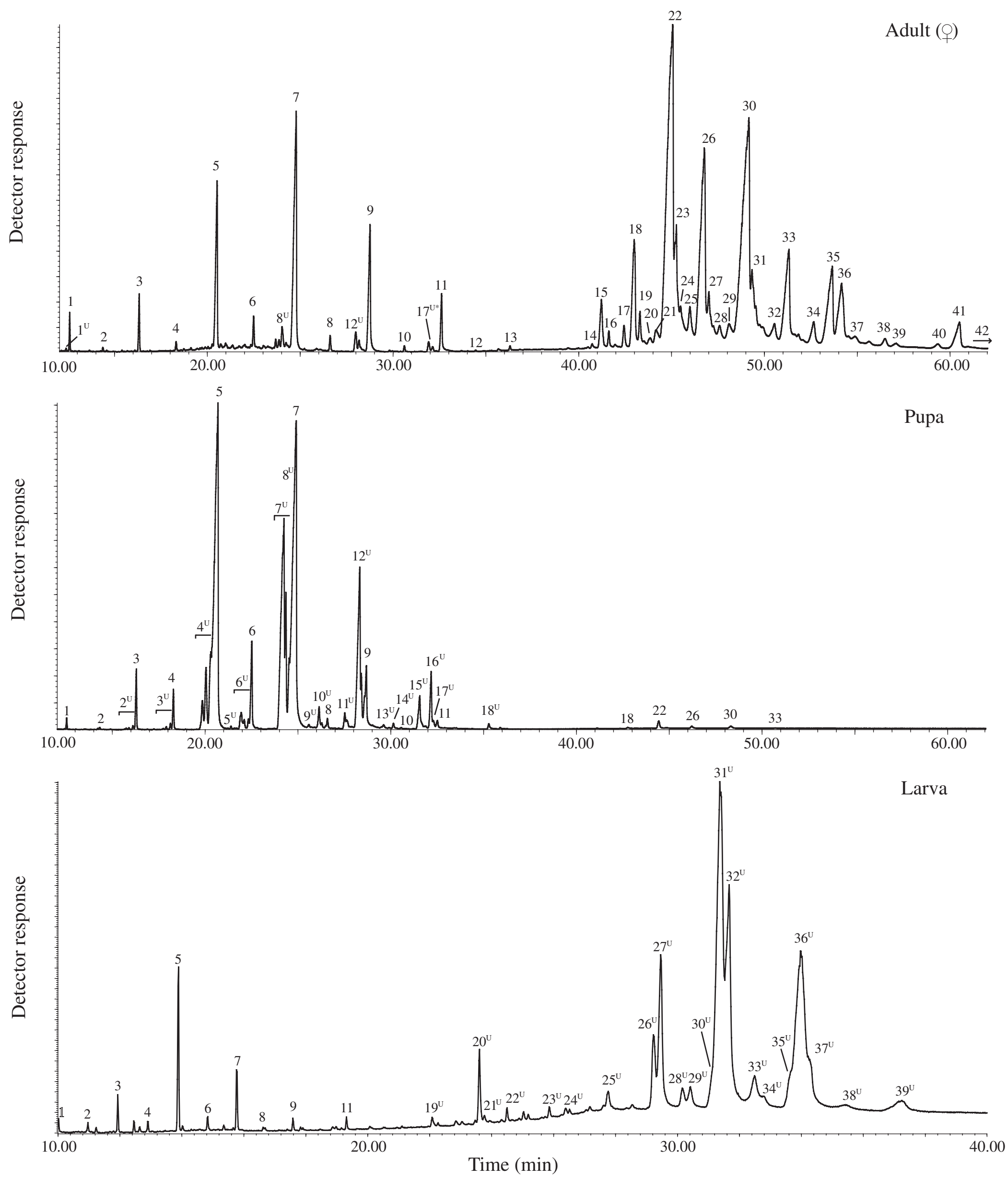

Fig. 1. Chromatographic hydrocarbon profiles of adult, pupae and larvae of *Diatraea saccharalis*. Adult and pupal hydrocarbons were analyzed using a HP-5MS column, with injector and oven temperatures set at 280 and 300 °C, respectively. Larval hydrocarbons were analyzed using a ZB-5HT Inferno column; oven and injector temperatures were set at 360 °C, as detailed in the Materials and methods. Numbers indicating each hydrocarbon peak correspond to peak numbers from Tables 1 and 2.

properties of cuticle hydrocarbons. Unsaturated hydrocarbons are much less common than saturated hydrocarbons in insect cuticles, although they might account for more than 70% of the total hydrocarbon components in the hymenopteran *Pikonema alaskensis* (Bartelt & Jackson, 1984). Mostly unsaturated chains (97%) are reported in *M. sexta* pupae, with chain lengths between C31 to C44 (major components of C39, C41 and C37) (Coudron & Nelson, 1981). Insect alkenes very often occur as mixtures of positional isomers (Blomquist *et al*., 1987; de Renobales *et al*., 1991; Buckner *et al*., 2009). Usually, a few monoalkenes are the predominating components, as in the termites *Reticulitermes flavipes* and *Zootermopsis* sp. (*n*-pentacosene and *n*-tricosene plus *n*-hentriacontene, respectively) (Howard *et al*., 1978; Haverty *et al*., 1988), although, in *Macrotermes subhyalinus*, close to 20 alkenes correspond to 65% of the total cuticular hydrocarbons (Kaib *et al*., 2004). Most of these mixtures contain monoalkenes; with alkadienes and alkatrienes being much less common. Martin & Drijfhout (2009) review the cuticular hydrocarbon structures of 78 species of ants. Of these, 73% show alkenes;

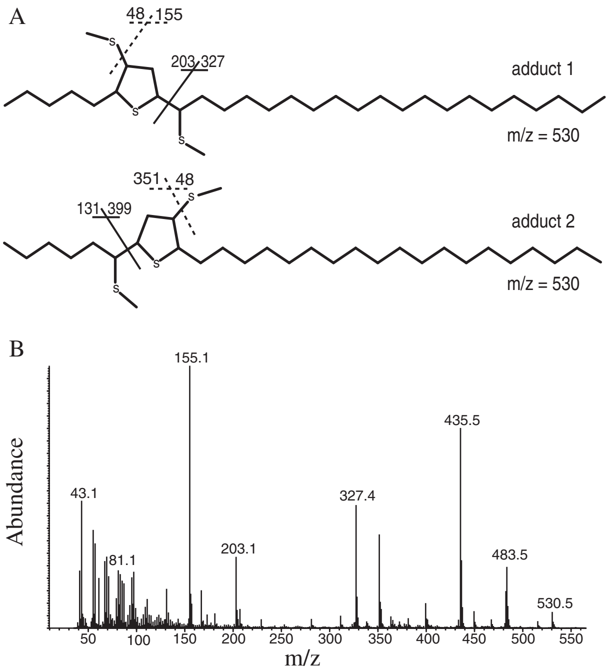

Fig. 2. (A) Molecular structure of adducts resulting from the reaction of (Z,Z)-6,9-C29:2 with dimethyldisulphide. (B) Electron ionization mass spectra of the adduct 1 ($[M]^+$, m/z 530).

alkadienes are detected in 23%, and only one alkatriene is reported. In the sugarcane borer *D. saccharalis*, the present study identifies straight and multiple branched chains, together with a large variety of unsaturated components of different chain lengths and unsaturation. Major developmental changes are detected from the larvae to the adult stage; the straight chain fraction, mostly *n*-C25, *n*-C27, *n*-C29 and *n*-C31, increases from 6.7 ± 0.6% in the soft-body larval stage, up to 70.7 ± 3.4% in pupae, declining to 26.7 ± 0.1% in adults. Branched hydrocarbons are almost undetectable in larvae and only trace amounts are evident in the pupae (0.3%); however, a large variety of branched components comprise 71.0 ± 0.7% of the total hydrocarbon fraction in the adult stage, with a prevalence of trimethylderivatives of 37–44 carbons, usually with methylene bridge interruptions (I) between CH_3 groups at I = 3/3 or 3/5. The alkene fraction represents 93% of the total cuticular hydrocarbons in larvae, showing a high diversity of hydrocarbon pattern. A group of very long chain hydrocarbons (37–53 total carbons) with from two to four unsaturations is detected, with three major peaks of 49 carbons (with a prevalence of C49:3 and C49:2 components), 51 carbons (C51:3) and 47 carbons (C47:2), together with smaller amounts of different isomers. The unsaturated fraction of the pupae shows quite distinct chain length components, suggesting a shutdown of the corresponding elongating enzymes (Juárez, 2004; Wicker-Thomas *et al*., 2009). Chain lengths (27–35 carbons) similar to those of the major saturated components and with one to three double bonds are detected in the unsaturated fraction of the pupae (29.0 ± 3.5%). A small amount of conjugated dienes are also detected, although trace amounts prevented further estimation of double bond location. In adults, the unsaturated fraction represents only 2.3 ± 0.5% of the total hydrocarbons, with double bond locations similar to those of the pupae (5-, 7-, 9-position). These major changes might be caused by inactivation of the desaturase activity that are characteristic of the younger stages. Small amounts of monounsaturated isomers of C29:1, C31:1 and C33:1 are only detected in females, with the predominant double bond location in the middle of the chains. No reports are available on very long chain unsaturated hydrocarbons above 45 carbons using classic gas chomatography-mass spectrometry techniques (Nelson & Leopold, 2003). However, there is growing evidence that the apparent limit of the chain length of insect hydrocarbons is a result of limitations on the usual analytical techniques (gas chomatography-mass spectrometry) rather than on nature. Saturated and unsaturated hydrocarbons of chain lengths over 70 carbons are reported for a number of insect species using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Cvačka *et al*., 2006). Also, external chemical ionization-tandem mass spectrometry was used for the determination of double-bond positions alkenes and alkadienes without prior derivatization (Kroiss *et al*., 2011). Highly unsaturated or cyclic C55–C65 hydrocarbons with 18–19 sites of unsaturation are revealed in termites *Reticulitermes* spp.; cuticular hydrocarbons with 42–45 carbons and three sites of unsaturation are reported in the flesh fly *Neobellieria bullata*; and two groups of unsaturated C43 and C45 components with one to three sites of unsaturation are reported in the American cockroach *Periplaneta americana*. Thus, the growing evidence of the existence of very-very-long chains emphasizes the need to develop new tools to elucidate the chemical structure of these compounds, and to explore the potential complementation of both analytical techniques (Blomquist & Bagnères, 2010). Multiple unsaturations of hydrocarbons raise yet another major technical challenge and, very importantly, reinforce the need to gain an understanding of their functionality and role in insect fitness through the concerted action of the corresponding biosynthetic enzymes, as well as their activation/inactivation at specific life stages.

Developmental changes and functionality

The development from the larval to the pupal stage is usually accompanied by significant changes in the surface hydrocarbons. In the lepidopteran pests *H. zea* and *H. virescens* (Nelson & Buckner, 1995; Buckner *et al*., 1996), and in *M. sexta* (Buckner *et al*., 1984), major developmental changes are reported in the surface lipids. In the larval stages, hydrocarbons are the major components, although oxygenated lipids are predominant in pupae. By contrast, in *D. saccharalis*, both larvae and pupae show important amounts of hydrocarbons and oxygenated lipids. However, the major compositional differences are detected in the unsaturation type and chain length of the hydrocarbons, and might suggest that, in larvae, very long chain alkenes contribute to regulating cuticle permeability. The relative amount of saturated components increases to

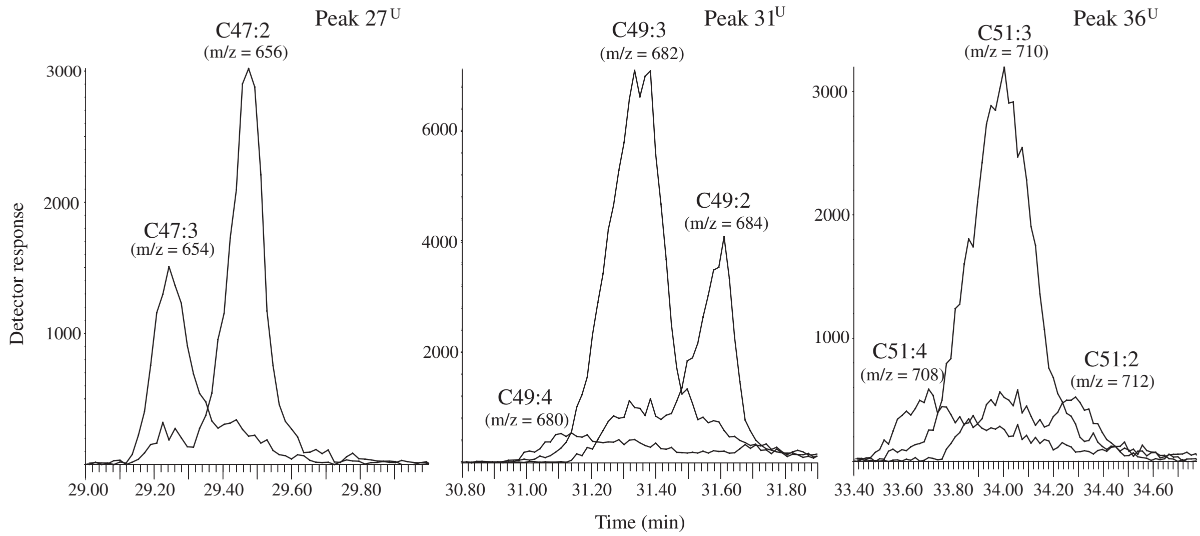

Fig. 3. Ion extracted chromatogram corresponding to peaks 27^{U}, 31^{U} and 36^{U} with chain lengths of C47, C49 and C51, respectively (Fig. 1 and Table 2) from the larvae cuticle. The relative abundance of dienes, trienes and tetraenes co-eluting in each hydrocarbon peak, as well as their corresponding m/z, is shown.

70.7 ± 3.4% in pupae, although the most significant change is observed in the composition of the unsaturated fraction (29.0 ± 3.5%), with relatively shorter chains (23–35 carbon atoms) predominating isomers of C27, C29, and C31 monoalkenes; furthermore, isomer unsaturation location varies with chain length (i.e. 5- > 7- ≫ 9-, for C27: 1; 9- > 5- ≫ 7- for C29:1 and C31:1). Other than nonpolar *n*-alkanes, a relatively polar mix is suggested to help provide a semi-fluid matrix to facilitate the deposition and embedding of different surface lipids to provide protection against abrasion, among other environmental aggressions. Toolson (1982) reports that, in the larva–pupa transition of *Drosophila pseudoobscura*, higher water loss is associated with a higher ratio of shorter hydrocarbon chains, whereas lower permeability correlates well with a greater abundance of longer carbon chains, either branched and/or unsaturated. These dramatic structural changes (chain length, unsaturation and branching) during development provide further support for the hypothesis that hydrocarbons are nonreusable, being newly formed upon each moulting cycle, as suggested by experimental evidence of quantitative hydrocarbon loss through ecdysis, together with exclusive synthesis of *n*-alkanes in adults of *T. ni* (De Renobales & Blomquist, 1983; Dwyer *et al*., 1986), and by the similarity in the composition of the larvae cuticle and the corresponding exuvium in the blood-sucking hemipteran *Triatoma infestans* (Juárez & Brenner, 1985). These metabolic changes are possibly associated with hormonal regulation. The reported 10-fold reduction in water loss during the transition from larva to pupa in *M. sexta* may be influenced by juvenile hormone titre (Jungreis *et al*., 1982). Thus, it is possible to speculate on a hormone-related mechanism that may regulate the formation of different chain lengths, as suggested in the flesh fly *Sarcophaga bullata* (Armold & Regnier, 1985), the house fly, German cockroach *Blattella germanica* and ants (Chase *et al*., 1992; Blomquist *et al*., 1994; Lengyel *et al*., 2007).

Physicochemical properties

With regard to their physicochemical properties, hydrocarbons of chain lengths longer than 16 carbons are solid and should have increasingly higher melting temperature (T_m) because T_m values are related to the number of C-C bonds. However, Gibbs (2011) postulates that chain length is the least important factor affecting T_m; the insertion of a *cis* double bond disrupts the crystalline structure and melting can occur at approximately 50 °C lower. The introduction of a methyl group internally can lower T_m by as much as 30 °C. However, the T_m of a specific cuticle lipid mixture can also be affected by the ability of the molecules to become self-packed, and will depend on characteristics such as saturation, double bonds, branching and length of the hydrocarbon and oxygenated lipid chains (Gibbs & Pomonis, 1995). Thus, the ubiquitous straight and monomethyl-branched chain insect alkanes are usually combined with lower T_m hydrocarbon components to conform unique species-specific blends to provide the required plasticity and permeability (Morgan, 2010). Larvae of *D. saccharalis* larvae possess an unusual mixture of very long chain alkenes of 37–53 carbons, with a predominance of 47, 49 and 51 alkenes, mostly tri- and di-unsaturated. This complex hydrocarbon mix should be fluid at ambient temperature or below, and might help protect larvae from abrasion during feeding and stalk tunnelling activities. Carbon chains up to C70 with varying degrees of unsaturation are reported by Cvačka *et al*. (2006) in adults of termites and ants. The present study is the first report of unusual very long chain alkenes in an insect larva, and many questions need yet to be answered. Further instrumental developments and/or combinations may be required to elucidate the structure of these unsaturated components, and to re-analyze the hydrocarbon composition in species of interest. The precise physiological role of these components has yet to be established. There are also questions about which precursors and biosynthetic pathways are involved, as well as their regulation at specific developmental stages. Are they formed by the usual fatty acid elongation mechanism or by some other means?

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