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Cuticular hydrocarbons on elytra of the Diaprepes root weevil *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae)

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Abstract 1 External gland openings and associated structures on the elytra of teneral and mature Diaprepes root weevils, *Diaprepes abbreviatus* (L.), were elucidated by scanning electron microscopy (SEM).

2 There were clear differences between teneral, callow adults and fully mature adults. In the field, teneral adults remain in the pupal chamber in the soil until sclerotization of the cuticle is complete or nearly so.

3 Phenotypic variation of the elytra in this species consists of varying patterns and coloration of scaled intervals between a variable number of raised ridges devoid of scales. In addition to being thinner and lighter in colour than fully mature adults, the elytra of teneral adults were devoid of waxy hydrocarbon secretions.

4 External gland openings at the base of each scale were observed on teneral elytra and mature elytra washed with methylene chloride.

5 SEM evidence to document the production of waxy filaments by these glands and partial characterization of these by gas chromatography and mass spectrometry are presented.

Keywords Citrus, *Diaprepes abbreviatus*, hydrocarbons, Insecta, wax glands.

Introduction

Insects secrete a complex mixture of lipids including wax esters, triglycerides, hydrocarbons and other compounds commonly referred to as wax (Waku & Foldi, 1984). The structures associated with production of these compounds range from simple pore canals that produce the epicuticle's outer wax monolayer, to highly modified cuticular structures that give shape and structure to extruded wax, as seen most notably in species of aphids, mealybugs, whiteflies and larvae of lepidopterans and coccinellids (Pope, 1985; Nelson *et al*., 2000).

The Diaprepes root weevil, *Diaprepes abbreviatus* (L.), is a major constraint to production of citrus and ornamentals in Florida and the Caribbean. Since the discovery of this pest in Florida (Woodruff, 1964), it has spread throughout peninsular Florida. Larvae of this species are a primary concern of Florida citrus producers because of the destructive habits of *D. abbreviatus*, and the difficulty of detecting and monitoring soil-inhabiting larvae in general. Larvae and adults are highly polyphagous, feeding on the roots and leaves, respectively, of many wild and cultivated plant species (Simpson *et al*., 1996). Larvae pupate in the soil. When adults emerge from the pupal exuvium, the callow adults remain in the pupal chamber during a teneral period of several days or perhaps weeks until sclerotization of the cuticle is complete (Wolcott, 1936). Wolcott (1936) observed that oviposition commenced at 3–7 days after emergence from the soil and an unknown period in the pupal chamber. Adults are long-lived and oviposition occurs over a prolonged period, probably months.

A trap based on an attractant would be a useful tool for monitoring adult populations and timing management practices for *D. abbreviatus*. Although anecdotal observations indicate that adults aggregate on particular citrus trees in the field, volatile aggregation or mating pheromones have not been identified for this species despite considerable effort. Some insect sex pheromones are similar to hydrocarbons found on insect cuticle (Jurenka & Subchev, 2000). In the German cockroach, for example, Gu *et al*. (1995) showed that hydrocarbon and methyl ketone sex pheromone are synthesized by integumentary tissue, probably oenocytes, and either delivered to the associated epicuticle or transported by lipophorin in the haemolymph

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to epicuticular deposition sites incapable of synthesis. Sex pheromone can be released through areas of the cuticle perforated by tubules, such as occurs in the midge, *Culicoides nubeculosus* (Ismail & Zachary, 1984).

Because we suspect the presence of a contact pheromone in the Diaprepes root weevil, we initiated a survey of external morphology to identify exocrine gland structures that may be associated with aggregation or sexual behaviour. This paper is the first description of the external morphology of the elytra of *D. abbreviatus* using scanning electron microscopy. We present the discovery of previously undescribed glands on the elytra, and preliminary characterization of their hydrocarbon secretions.

Materials and methods

Larvae of *D. abbreviatus* were obtained from a colony maintained at the U.S. Horticultural Research Laboratory (USHRL), Ft. Pierce, Florida. Individuals were reared in cups containing artificial diet as described by Lapointe & Shapiro (1999). A sample of teneral adults was removed from the diet cups within 24 h of adult emergence. Adult weevils were separated by gender and placed in cages with citrus foliage and water until sclerotization of the cuticle was complete and full adult colour developed (approximately 10 days).

Weevil elytra from teneral and fully mature adults were excised at their base from live weevils and prepared according to standard techniques for scanning electron microscopy. Elytra were dried in an analytical oven at 35 °C for 24 h before being sputtered with gold in a Technics Hummer II and examined with a JEOL 6400 Visions scanning electron microscope (SEM).

To determine the time required for sexual maturation after emergence, teneral adults were caged in pairs and provided with citrus foliage ('Valencia' orange), water and wax paper strips for oviposition (Wolcott, 1933). Teneral females were caged with fully mature males, and teneral males were caged with fully mature females. As controls, mature females were caged with mature males, and teneral females were caged with teneral males. Cages were observed three times daily for mounting behaviour and copulation, and the wax paper strips were checked daily for oviposition. To estimate the preoviposition period, virgin females were held in a cage ($10 \times 10 \times 10$ cm) and placed in an environmental chamber at 26 °C, and a photoperiod of LD 14 : 10 h.

To compare genders and developmental stages, elytra from individual teneral (24-h-old) and mature (> 10-day-old) adult weevils were excised and placed in 1 mL of methylene chloride (CH_2Cl_2) for 15 min. Extractions were replicated five times (five individual weevils for each gender and age combination for a total of 20 insects). Controls included solvent blanks and extract of the artificial diet used to rear the larvae to adult (Lapointe & Shapiro, 1999). To test the possibility that compounds from the diet were contaminating our samples, we took samples of fresh diet and fed-on diet and mixed each with enough anhydrous sodium sulphate to convert the mix into a free-flowing material. We triturated the dried diet samples with 50 mL of methylene chloride, concentrated the solution to 1.0 mL and then examined the concentrated diet extracts under the same gas chromatography conditions used for elytral extracts.

We examined the extracts by gas chromatography/mass spectrometry (GC/MS) using a HP 5971 Mass Selective Detector (MSD) coupled to a HP 5890 series II gas chromatograph equipped with a cold on-column injection system and a direct capillary inlet to the MSD. The MS was set to scan mass-to-charge ratios (m/z) in the range 50–450 at autotune conditions.

Results and discussion

Teneral males and females did not engage in sexual behaviour, either mounting or copulation. Mature males and females did not attempt to copulate with teneral adults of the opposite gender until the teneral period had passed. No oviposition was observed in cages containing teneral adults. Mating of mature adults is prolonged and easily observed. Copulating pairs and oviposition were abundant in the cages containing mature males and females. The preoviposition period lasted approximately 7 days.

The elytra of fully sclerotized adults are black and incompletely covered with scales (Fig. 1). Scales at the margins of scaled areas were narrow and pointed, whereas the majority of scales in the interior of scaled areas had the shape illustrated in Fig. 3(B–D). Of these, we observed two types: one recumbent alternating with a second, erect type (Fig. 3B). Scales were 40 μm wide at their widest point, 25 μm wide at the apex, with a pedicel (5.8 μm wide) inserted into a circular socket (5 μm diameter) on the elytron (Fig. 2). The erect scales are usually found inclined at an angle to the elytral surface with the superior lamella bearing longitudinal ridges at 5–8 μm intervals. Overall, the shape and manner of insertion of scales are similar to that of lepidopterans except that scales are not deciduous, being firmly anchored to the elytron.

In addition to scales, elytra were observed to have regularly spaced circular strial pits (50 μm diameter) readily visible without magnification (Fig. 3A). These were evenly spaced in longitudinal rows at 200 μm intervals. No wax production or other function was observed associated with these strial pits. The scaled dorsal areas of elytra of teneral adults were colourless and free of waxy secretions (Fig. 1A, C and 3A, B). Tubercle-like, gland ducts (5–8 μm inside diameter, 10 μm outside diameter) were observed at the base of each erect scale (Fig. 3). Observations of elytra in varying stages of development confirmed that the ducts were the source of the secretions (e.g. Fig. 1C, D and Fig. 3B–D). Mature elytra that had obtained full mature coloration (orange in this case) had abundant secretions consisting of a mass of spaghetti-like rods (0.8 μm diameter) of varying length that adhered closely to the upper ridged lamellae of the scales and obscured the external duct openings (Fig. 3B). The presence of gland ducts was confirmed

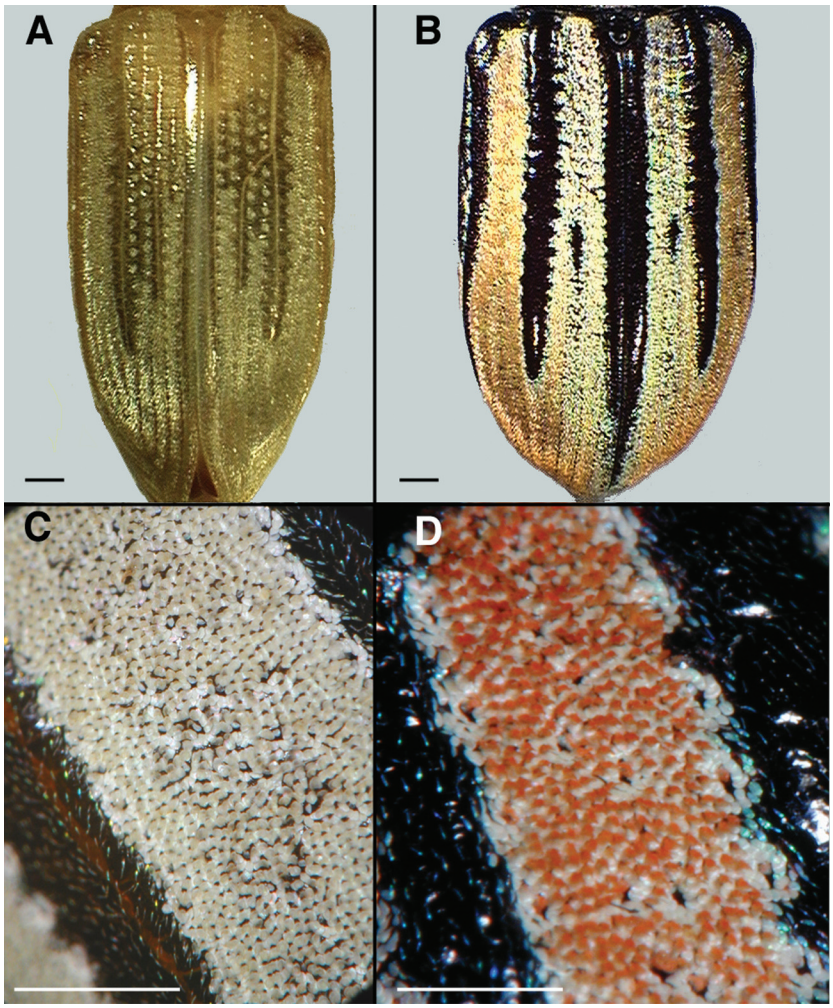

Figure 1 Elytra of a teneral (A and C) and a fully mature (B and D) adult Diaprepes root weevil. Bars are 1 mm.

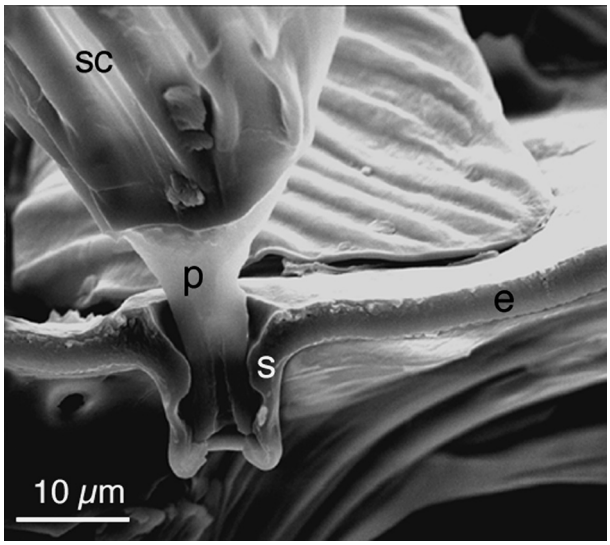

Figure 2 Cross-section SEM of a scale (sc) showing insertion ofthe pedicel (p) into a socket (s) on the elytral cuticle (e) of a teneral Diaprepes root weevil.

on mature elytra by SEM observation of mature elytra washed in methylene chloride.

The total ion chromatogram (TIC) developed from the initial methylene chloride extracts of mature adults contained only aliphatic hydrocarbons (Fig. 4). These comprised four groups of homologous compounds: a group of normal hydrocarbons, $C_{23}H_{48}$ to $C_{32}H_{66}$; a group of monomethyl-branched alkanes with backbones of 25–31 carbon atoms; a group of dimethyl-branched alkanes with backbones 27–31 carbon atoms; and two alkenes, $C_{27}H_{54}$ and $C_{29}H_{58}$ (Table 1). In the TIC, the percent areas of each group were 20.4%, 73.1%, 3.4% and 3.06%, respectively. We established exact branch positions by applying the interpretation techniques developed for examination of cuticular hydrocarbons (Carlson & Brenner, 1988; Carlson *et al.*, 1998).

None of the compounds detected in the sample extracts was found in the extraction blank prepared and analysed under the same experimental conditions. The chromatogram of the fresh diet extract contained only one peak after the solvent peak, which eluted close to the retention time of dotriacontane. The chromatogram of the fed-on diet

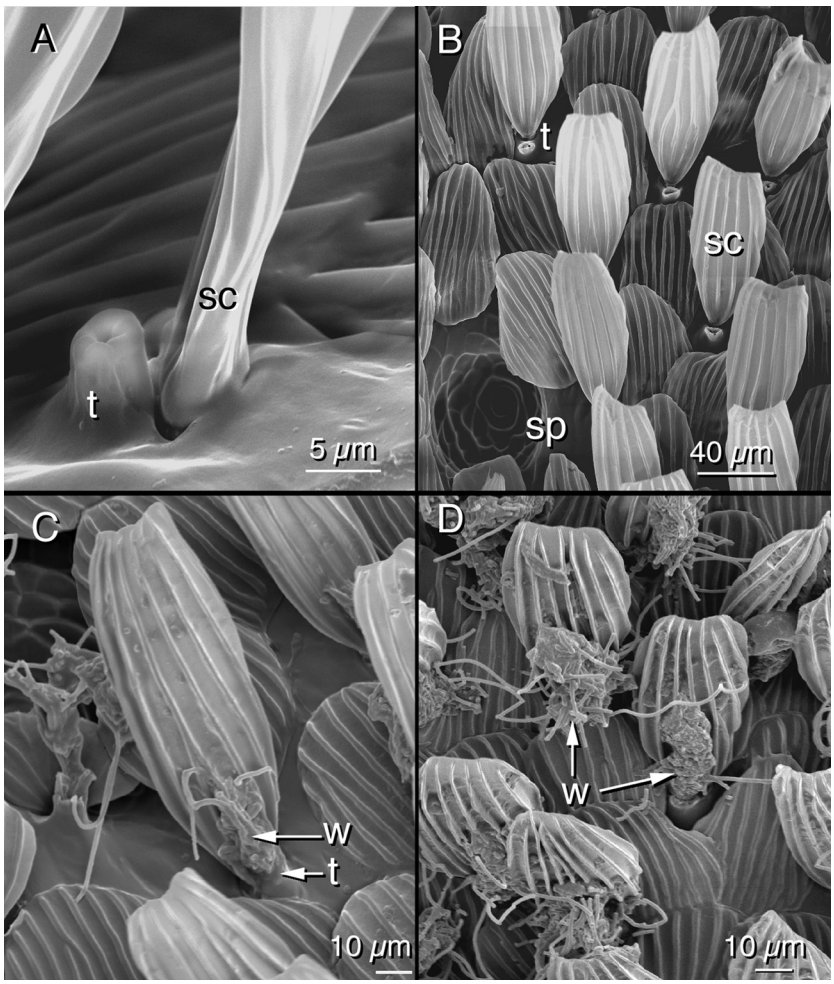

Figure 3 Views of teneral (A and B) and mature (C and D) adult Diaprepes root weevils. (A) Lateral view of a secretory duct (t) at the base of a scale (sc). (B) Dorsal view of scales and associated secretory ducts (t) near a strial pit (sp) of a teneral adult. (C and D) Dorsal views of 'wax' (w) extrusion from secretory ducts on a maturing elytron.

extract contained most of the peaks that eluted from the elytral extracts. These findings indicate that compounds extracted from elytra contaminated the fed-on diet, but that the diet did not contaminate the elytra.

The n-alkanes (C23–C32) were identified by GC/MS. We matched the retention times of the peaks in the extract chromatogram and their spectra with the corresponding peaks in the chromatogram of a paraffin wax standard. Once identified, we used the retention times of the extracted n-alkanes as embedded standards to calculate the Kovats Index (KI) (Kovats, 1965) of the remaining peaks.

The most abundant group of compounds in the TIC was the monomethyl-branched hydrocarbons (73.1%). Of this group, interiorly monomethyl-branched alkanes account for nearly two-thirds of the total peak area. These compounds have KIs in the regions XX25 to XX35, in close agreement with data from other insect cuticular hydrocarbons (Carlson *et al*., 1998). Many of the interiorly-branched monomethylalkanes coeluted. Convoluted spectra generated alkene daughter ions that excluded the possibility of a single monomethyl-branched isomer. Alkene daughter ions arise typically by cleavage at the methyl branch, in which one large alkyl radical and a hydrogen atom are lost (Carlson *et al*., 1984).

The mass spectrum (Fig. 5) of the largest chromatographic peak (23.9%, retention time 40.94 min, KI = 2735) displayed one molecular ion at m/z 394. It also had ions at m/z 393 $[M\text{-}H]^+$ and 392 $[M\text{-}2H]^+$ that were larger than the molecular ion. These ions, resulting from the loss of hydrogen atoms from the molecular ion, are always found in branch-chained hydrocarbons. This spectrum also contained prominent ions at m/z 196 and m/z 224, which corresponded to the molecular ions of tetradecene and hexadecene. This peak consisted primarily of 13-methylheptacosane, the only aliphatic hydrocarbon with molecular weight 394 that decomposes to m/z 196 and 224. The mass sprectrum also contained smaller ions at m/z 168, 182, 210 and 238. These ions indicate that the chromatographic peak contained lesser amounts of 11-methylheptacosane, and much smaller amounts of 12-methyl and 14-methyl heptacosanes (Carson & Brenner, 1988). Other peaks in the chromatogram with KI = XX31 ± 5 consist solely of coeluting

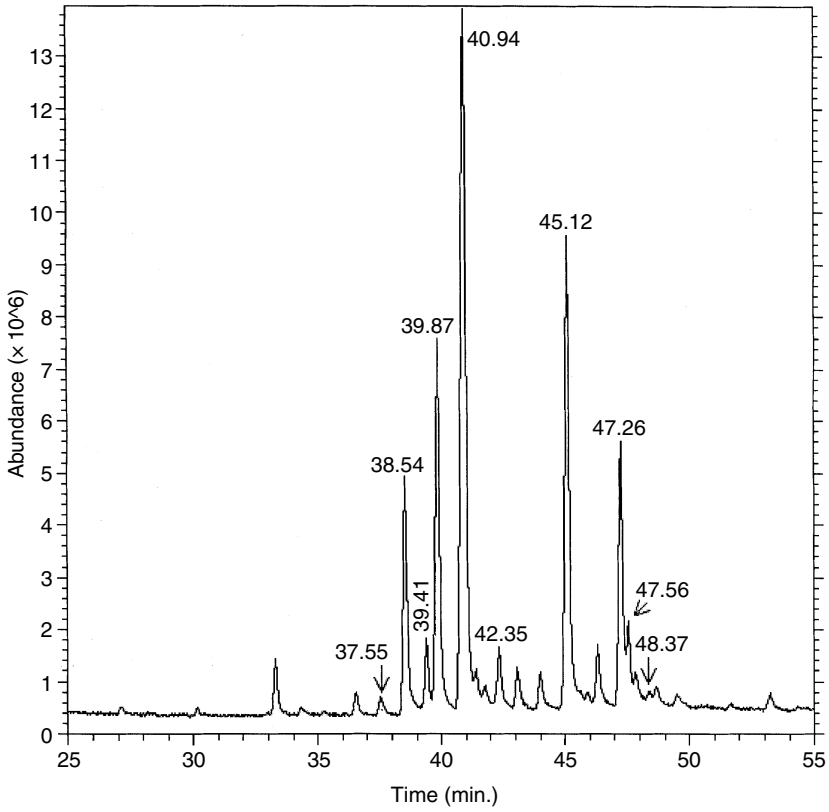

Figure 4 Total ion chromatogram of a methylene chloride extract of elytra from a single mature male Diaprepes root weevil. GC conditions: 0.2 μL injection volume; 30 m × 0.25 mm × 0.75 μm film DBBlend (20% diphenyl/80% dimethyl silicone) column; H2 carrier gas at constant flow of 1 mL/min; injector temperature tracked the column oven. Oven temperature program was: $T_{init}(1) = 35\,^{\circ}C$ for 4 min; rate = 20 °C/min; $T_{final}(1) = 210\,^{\circ}C$ for 1 min; rate(2) = 2 °C/min; T_{final} (2) = 280 °C for 10 min.

monomethyl-branched alkanes. Other monomethyl-branched alkanes, in which the methyl group is located closer to the end of the carbon chain than position 10, elute somewhat later and can be separated easily from this peak.

The extract contained eight dimethyl-branched alkanes compared with 28 monomethyl-branched alkanes and 10 normal alkanes. In most dimethyl-branched cases, the branching pattern contained one interior methyl branch and one closer to the end of the carbon chain with nine and 11 methylene groups between the methyl branches. We are uncertain of the exact substitution pattern of two coeluting dimethyltriacontanes that were detected.

We detected two alkenes, $C_{27}H_{54}$(KI2686) and $C_{29}H_{58}$(KI2888), in this extract. The proposed alkene structures were based on the occurrence of molecular ions at m/z 378 and 406, the fact that $C_nH_{2n-1}^+$ ion intensities were greater than those of the $C_nH_{2n+1}^+$ ions in the low mass regions, and the lack of anomalously large m/z 69 or m/z 83 which would indicate a cycloalkyl moiety. Neither the position nor stereochemistry of the double bond have been determined. However, we compared the mass spectra that we recorded for these peaks with the mass spectra of terminal and interiorly unsaturated alkene spectra ($C_{20}H_{40}$ and larger) in the Wiley and NIH spectral libraries. The terminal alkene library spectra contain a homologous series of even mass peaks near the molecular ion that are much larger than the same peaks in the isomeric interiorly unsaturated alkenes. Therefore, these alkenes are probably not terminal alkenes.

We examined extracts from adult males and females from one-day-old to fully mature adults. The chromatograms appeared qualitatively and quantitatively similar. All chromatograms are dominated by the interior substituted methylheptacosane peak eluting at KI2734, followed by methylnonacosane peak at KI2930. The major differences in the chromatograms involved the n-alkanes, which were more concentrated in the extracts from the fully mature insects regardless of gender.

Aggregation or sex pheromones have not been identified for the Diaprepes root weevil. The means of communication by this weevil that would explain observations of adult aggregations on host plants (Wolcott, 1936) have remained mysterious. We have shown that teneral adults do not elicit mating behaviour from mature adults of the opposite gender. Although the compounds that we have characterized from the wax glands on elytra of *D. abbreviatus* are not volatile at ambient temperatures, the possibility of contact pheromones has not been explored for this species. We have described a new gland from the Diaprepes root weevil and have described its principal products although other lipid classes not amenable to analysis by GC may be present. We will endeavour to describe differences in the chemical composition of waxes from male and female Diaprepes root weevil elytra.

Table 1 Retention time, Kovats Index (KI), peak area, and compound identification from methylene chloride extracts of elytra of the Diaprepes root weevil by combined GC/MS analysis

Retention time (min)	KI	Molecular formula	Area (%)	Identification
27.15	2300	$C_{23}H_{48}$	0.18	tricosane
30.16	2400	$C_{24}H_{50}$	0.20	tetracosane
33.30	2500	$C_{25}H_{52}$	1.67	pentacosane
34.34	2533	$C_{26}H_{54}$	0.41	11- and 13- methylpentacosanes
35.23	2561	$C_{25}H_{52}$	0.14	3-methylpentacosane
36.55	2600	$C_{26}H_{54}$	0.69	hexacosane
37.54	2631	$C_{27}H_{56}$	0.65	10, 11-, 12- and 13- methylhexacosanes
38.54	2661	$C_{27}H_{56}$	7.88	2-methylhexacosane
39.41	2686	$C_{27}H_{54}$	2.42	heptacosene
39.88	2700	$C_{27}H_{56}$	12.39	heptacosane
40.94	2734	$C_{28}H_{58}$	29.31	13-, 11-, 14-, 12-methylheptacosanes
41.41	2749	$C_{28}H_{58}$	1.55	5-methylheptacosane
41.77	2760	$C_{28}H_{58}$	1.03	2-methylheptacosane
42.36	2778	$C_{29}H_{60}$	2.24	5,15- and 5,17- dimethylheptacosanes
43.08	2800	$C_{28}H_{58}$ & $C_{29}H_{60}$	1.18	octacosane and 3,15-dimethylheptacosane
44.00	2829	$C_{29}H_{60}$	1.24	14-, 13- and 12- methyloctacosanes
45.13	2864	$C_{29}H_{60}$	15.85	2-methylctacosane
45.94	2888	$C_{29}H_{58}$	0.64	nonacosene
46.32	2900	$C_{29}H_{60}$	2.42	nonacosane
47.26	2930	$C_{30}H_{62}$	8.39	14- and 13-methylnonacosane
47.56	2940	$C_{30}H_{62}$	2.62	7-, 8- and 6-methylnonacosanes
47.85	2949	$C_{30}H_{62}$	1.52	5-methylnonacosane
48.37	2965	$C_{31}H_{64}$	0.33	7,17-dimethylnonacosane
48.68	2975	$C_{30}H_{62}$	1.01	3-methylnonacosane
49.51	3000	$C_{30}H_{62}$ & $C_{31}H_{64}$	0.48	triacontane & 5,17- and 5,15-dimethylnonacosanes
51.65	3058	$C_{31}H_{64}$ & $C_{32}H_{66}$	0.25	2-methyltriacontane and x,18- and y,16-dimethyltriacontanes
53.24	3100	$C_{31}H_{64}$	0.81	hentriacontane
54.34	3126	$C_{32}H_{66}$	0.24	15- and 13-methylhentriacontane
57.66	3200	$C_{32}H_{66}$	0.52	dotriacontane

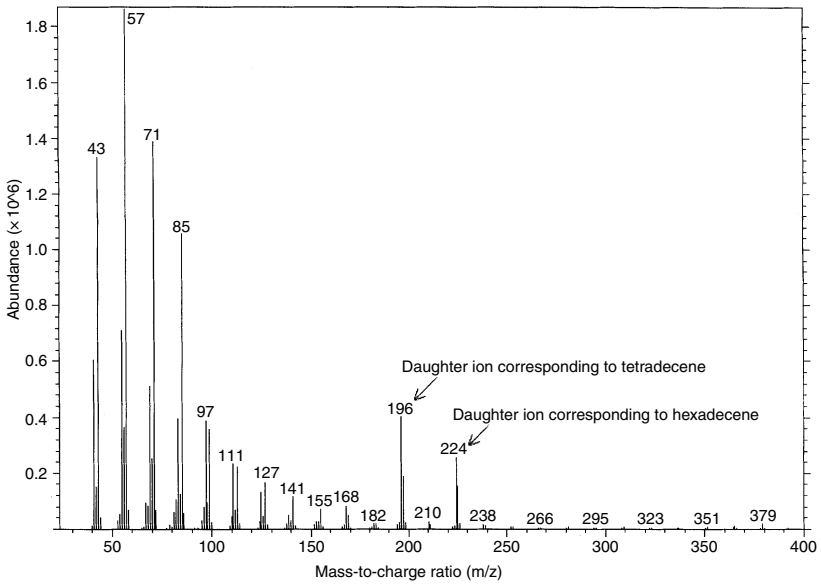

Figure 5 Mass spectrum of 13-methylheptacosane, a major component of a methylene chloride extract of elytra of the Diaprepes root weevil.

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