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ANALYSIS OF INSECT CUTICULAR HYDROCARBONS USING MATRIX-ASSISTED LASER DESORPTION/ IONIZATION MASS SPECTROMETRY

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Abstract—Insect cuticular hydrocarbons (CHCs) were probed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry with a lithium 2,5-dihydroxybenzoate matrix. CHC profiles were obtained for 12 species of diverse insect taxa (termites, ants, a cockroach, and a flesh fly). MALDI spectra revealed the presence of high molecular weight CHCs on the insect cuticle. Hydrocarbons with more than 70 carbon atoms, both saturated and unsaturated, were detected. When compared with gas chromatography/mass spectrometry (GC/MS), MALDI-TOF covered a wider range of CHCs and enabled CHCs of considerably higher molecular weight to be detected. Good congruity between GC/MS and MALDI-TOF was observed in the overlapping region of molecular weights. Moreover, a number of previously undiscovered hydrocarbons were detected in the high mass range beyond the analytical capabilities of current GC/MS instruments. MALDI was shown to hold potential to become an alternative analytical method for insect CHC analyses. The ability of MALDI to discriminate among species varying in the degree of their relatedness was found to be similar to GC/MS. However, neither MALDI-MS nor GC/MS data were able to describe the phylogenetic relationships.

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Key Words—Ants, cockroach, cuticular hydrocarbons, lithium 2,5-dihydroxybenzoate, Nei distance, flesh fly, MALDI-TOF, mass spectrometry, termites.

INTRODUCTION

Hydrocarbons represent universal constituents of the insect cuticle (Wigglesworth, 1965; Blomquist and Dillwith, 1985; Hadley, 1985; Chapman, 1998). Cuticular hydrocarbons (CHCs) are considered to be stable end products of genetically controlled metabolic pathways (Ross et al., 1987; Grunshawn et al., 1990); insects are known to synthesize most hydrocarbons *de novo* by elongation–decarboxylation pathway (review, Blomquist and Dillwith, 1985; Lockey, 1985, 1988; Howard and Blomquist, 2005). The common function of CHCs is to protect against desiccation. In social insects, CHCs are regarded as the main signals responsible for nestmate recognition (review, Blomquist et al., 1998; Howard and Blomquist, 2005; ants, see Lenoir et al., 2001; termites, see Clément and Bagnères, 1998), although this premise has been confirmed only in a few cases (e.g., Takahashi and Gassa, 1995; Lahav et al., 1999). As evidenced by gas chromatography (GC), the hydrocarbon mixture usually contains up to 100 different hydrocarbons (Nelson et al., 1981). The length of hydrocarbon chains usually varies from 23 to 47 carbon atoms (Blomquist and Dillwith, 1985). Species living in dry conditions generally contain longer hydrocarbon chains in comparison to their relatives living in wet conditions (review, Blomquist and Dillwith, 1985; Lockey, 1988). Insect species usually possess complex mixtures of hydrocarbons including *n*-alkanes, branched mono-, di-, or trimethylalkanes, and others (Jackson and Blomquist, 1976). Unsaturated CHCs are also present and the position of the double bond varies within the chain (Howard and Blomquist, 1982; Blomquist and Dillwith, 1985; Lockey, 1985, 1988). Hydrocarbon profiles may serve as fingerprints defining particular species. The composition of CHCs has been extensively studied in social insects, and in termites it is often used in taxonomic discrimination (Watson et al., 1989; Kaib et al., 1991; Bagine et al., 1994; Haverty et al., 1996; Takematsu and Yamaoka, 1997; Haverty and Nelson, 1997) and sibling species recognition (Haverty and Nelson, 1997).

To date, coupled gas chromatography-mass spectrometry (GC/MS) has been the primary tool for CHC analysis. However, both GC and electron ionization MS (EI-MS) have limitations for high molecular weight compounds. For these compounds, special high-temperature GC columns have to be used to withstand the temperatures needed. However, high temperatures can cause samples to decompose. Moreover, EI-MS identification of high-mass hydrocarbons is problematic because the molecular ion is virtually absent. Thus, GC/MS enables detection of only some of the hydrocarbons present on the insect

cuticle. To obtain a more complete picture of the composition of the cuticular waxy layer, other techniques have to be employed. Laser desorption/ionization (LDI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry have been shown to be suitable methods for analyzing hydrocarbons and hydrocarbon polymers. Published methods (Kuhn et al., 1996; Yalcin et al., 1997; Dutta and Harayama, 2001; Pruns et al., 2002) used silver or copper ionization to produce ions that were subsequently detected. Recently, a new method utilizing lithium ions was described (Cvačka and Svatoš, 2003). A simple isotopic pattern of lithium adduct ions greatly simplifies spectrum interpretation. Accurate masses measured by time-of-flight (TOF) mass spectrometry have been used for the assignment of molecular structure.

In this work, the utility of MALDI-MS for characterizing CHCs was demonstrated especially for those of high molecular weight. Our objectives were to learn (1) the range of CHCs' molecular weights, (2) the types of hydrocarbons that form the high molecular weight portion of CHCs, and (3) whether MALDI mass spectra can be used for discriminating among species and for chemotaxonomical purposes. Results obtained by MALDI-MS were compared with data from GC/MS, a well-established method for CHC studies. Most work on CHCs has been done on social insects; therefore, termites were selected as our model group. Species representing different ecological strategies were chosen. In addition to termites, two ant species, the American cockroach, and a flesh fly were selected to represent related and nonrelated, social and nonsocial insects.

METHODS AND MATERIALS

Chemicals and Materials. Reagent grade standards of saturated hydrocarbons (triacontane, hexatriacontane, and tetracontane; ≥97% pure) were obtained from Aldrich; tetratetracontane, pentacontane, and hexacontane from Fluka. Apolane-87 (24,24-diethyl-19,29-dioctadecylheptatetracontane) was from Alltech Associates (Deerfield, MA, USA). Squalane (2,6,10,15,19,23-hexamethyltetracosane) C grade was purchased from the California Corporation for Biochemical Research (Los Angeles, CA, USA), and squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, min. 98%) from Sigma. Polyethylene glycol (PEG) oligomers (PEG 300, PEG 600, and PEG 1000) were purchased from Aldrich. Other hydrocarbon standards, 9-methylheptacosane, nonadecylcyclohexane, and icosylcyclopentane were synthesized and provided by Dr. Karel Stránský (IOCB, Prague, Czech Republic). Solvents used for sample and matrix preparation (chloroform, diethyl ether, hexane, and methanol) were distilled in glass from analytical-grade solvents.

Preparation of Lithium 2,5-Dihydroxybenzoate. Lithium-6 hydroxide monohydrate (0.55 g, 95 at.% ^{6}Li; Aldrich) or lithium-7 hydroxide

monohydrate (0.55 g, 97 at.% ^{7}Li; Aldrich) was dissolved in 6 ml ultrapure water and treated with freshly recrystallized 2,5-dihydroxybenzoic acid (1.50 g, Aldrich) at room temperature. The solution was cooled in a refrigerator, and the resulting white needles were washed with $CHCl_3$ (10 ml) and dried under vacuum. Elemental analyses revealed that both salts (^{6}LiDHB and ^{7}LiDHB) crystallize from water as tetrahydrates. For $C_7H_5O_4{}^{6}Li{\cdot}4H_2O$ calc. 36.36% C, 5.68% H; Found 36.41% C, 5.59% H; for $C_7H_5O_4{}^{7}Li{\cdot}4H_2O$, calc. 36.20% C, 5.65% H; Found 36.29% C, 5.65% H, respectively. A MALDI matrix solution was prepared fresh daily in $CHCl_3$/acetone mixture (1:2, v/v) in a concentration of 7.5 mg/ml.

Insects. Subterranean and dry wood termite species (Isoptera: Rhinotermitidae and Kalotermitidae) used in this study originated from laboratory colonies where they were kept at 24 ± 2°C after being collected. Samples included 20 workers of *Reticulitermes lucifugus* (Rossi, 1792), abbreviated RL, collected in Crocemoschito, 70 km SE of Rome, Italy, 1998; 20 workers of *Reticulitermes* (*lucifugus*) *grassei* (Clément, 1981), abbreviated RG, collected in Forêt de la Coubre, near Bordeaux, France, 1998; 20 workers of *Reticulitermes santonensis* (de Feytaud, 1928), abbreviated RS, collected in Forêt des Saumonards, Ill D'Oleron, France, 1998; 20 workers of *Reticulitermes virginicus* (Banks, 1907), abbreviated RV, collected in Fort Lauderdale, FL, USA, 2003; 20 workers of *Coptotermes formosanus* (Shiraki, 1909), abbreviated CF, from a colony established in 1992 from alates from a colony collected in Hsin-hui, Guandong, China, 1963; 20 pseudergates of *Neotermes castaneus* (Burmeister, 1839), abbreviated NC, collected in Caimito, La Habana, Cuba, 1978; 20 pseudergates of *Neotermes cubanus* (Snyder, 1922), abbreviated NCub, collected in Topes de Collantes, Cuba; and 20 pseudergates of *Cryptotermes declivis* (Tsai and Chen, 1963), abbreviated CD, a colony obtained from the Guandong Institute of Entomology, Guangzhon, China, 1992. Flesh fly, cockroach, and ant species also originated from laboratory cultures where they were reared after being collected: 10 or 100 imagos of gray flesh fly *Neobellieria bullata* (Parker, 1916) (*Diptera: Sarcophagidae*), abbreviated NB; 1 or 10 males of the American cockroach, *Periplaneta americana* (Linnaeus, 1758) (*Blattaria: Blattidae*), abbreviated PA, from laboratory breedings of the Research Institute of Crop Production, Prague, Czech Republic; 7 individuals of the leaf-cutting ant *Acromyrmex echinatior* (Forel, 1899), abbreviated AE, collected in a Paracou field station in French Guiana, 2001; and 6 individuals of *Atta colombica* (Guerin-Meneville, 1844) (*Hymenoptera: Formicidae*), abbreviated AC, collected in Barro Colorado Island, Panama, 1998. The relatively high number of insect individuals in this study was used to optimize experiments; routine measurements require at most a few individuals for successful MALDI experiments.

Sample Preparation. Insects were immobilized in the cold and stored frozen at −18°C. Prior to extraction, they were placed in a dessicator for 1 hr to get rid of surface moisture. CHCs were extracted with $CHCl_3$ (3–6 ml, elution time ca. 5 min) in a small glass column sealed on the bottom with precleaned cotton wool. Solvent from the extract was evaporated almost to dryness under a stream of argon. The crude extract was fractionated on precleaned glass thin layer chromatography plates (TLC, 36 × 76 mm) coated with Adsorbosil-Plus [Applied Science Labs; layer thickness 0.2 mm with gypsum (12%)] using hexane as a mobile phase. Spots were visualized by spraying Rhodamine 6G solution (0.05% in ethanol). To isolate both saturated and unsaturated hydrocarbons, the silica corresponding to a "relative to front'' (R_F) value of 0.22 and 1.0 was scraped off the plate and extracted with 10 ml freshly distilled diethyl ether. The solvent was evaporated to dryness under a stream of argon, and the residues were weighed. The residues were reconstituted in $CHCl_3$ to a concentration of 1% and stored in sealed glass ampoules at −18 °C. Silver ion chromatography was carried out using the same size TLC plates with a silica gel stationary phase impregnated with $AgNO_3$ (20%), and hexane as a mobile phase. To isolate the saturated hydrocarbons, silica around the solvent front was scraped off the plate; unsaturated hydrocarbons were isolated from all the rest. Simultaneously with preparative TLC, an analytical TLC (both normal-phase and silver ion) was run for each sample. Spots were visualized by heating the plates after spraying with conc. sulfuric acid containing ethyl vanillin. A stainless steel MALDI sample target (100 positions, Micromass) was cleaned stepwise with water, acetone, and dichloromethane (sonication for 15 min) to remove possible contamination. Chloroform extract (0.5 μL) was spotted onto a MALDI target, and, after drying, the sample was covered with 0.75 μl LiDHB matrix solution and allowed to dry in air; isotopically pure ^{7}LiDHB was used for routine measurement. For internal calibrations, 0.2 μl of a mixture of PEG oligomers (PEG 300, PEG 600, and PEG 1000, 0.1 mM each in acetone) was added to a sample. Plastic equipment was avoided during all manipulations with the samples and solvents to prevent possible contamination.

Infrared Spectroscopy and Nuclear Magnetic Resonance. IR spectra were taken as KBr pellets using a Bruker Equinox 55/S FT NIR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AVANCE-500 (500.1 MHz for ^{1}H and 125.8 MHz for ^{13}C) FT NMR spectrometer in $CDCl_3$, using Me_4Si as an internal standard at 300 K.

Gas Chromatography. GC/MS experiments were performed using a Fisons 8000 series GC equipped with MD800 quadrupole mass detector. A nonpolar DB-5 MS column (30 m × 0.25 mm i.d., phase thickness 0.25 μm; J&W Scientific, Folsom, CA, USA) was used to separate CHCs. The injector was operated in splitless mode at 220°C. The detector temperature was set to 200°C;

standard 70 eV spectra were recorded at 1 scan/sec. The temperature of the GC oven was programmed as follows: 50°C/min, 10°C/min to 320°C, hold at 320°C for 10 min. Helium was used as a carrier gas at 1 ml/min. The data were analyzed using the MassLab (ver. 1.4) program. CHCs were quantified from chromatograms reconstructed from the *m*/*z* 57 ion; all hydrocarbons eluting after 15 min were integrated.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Matrix-assisted laser desorption/ionization experiments were performed on a TofSpec 2E instrument (Micromass, Manchester, UK) operated in a reflectron mode using delayed extraction (Cvačka and Svatoš, 2003). Desorption and ionization were accomplished using a nitrogen UV laser. Matrix ions were suppressed with a low mass (300 Da or less) cutoff. Scans were accumulated using 20 laser shots and the spectra were averaged from at least 20 consecutive scans. Baseline subtracted data were smoothed, centered, and calibrated with lithium adducts of PEG. Data were collected and analyzed with MassLynx 3.2 software running on a PC workstation.

Statistical Analyses of CHCs. Statistical analysis of data from 12 insect species was carried out to compare the ability of MALDI-MS and GC/MS to discriminate among species. The Nei distances (or the Nei indices of identity) between all possible pairs of species were computed by using their CHC profiles as proposed by Nei (1972) and applied by Gush et al. (1985), Nowbahari et al. (1990), and Kaib et al. (1991). The relative ratios of the 63 most prominent peaks of GC/MS chromatograms and 492 peaks of MALDI-MS spectra were selected for the analysis. The matrices of Nei distances were submitted to cluster analyses using single linkage and UPGMA clustering methods. Hierarchical trees based on this clustering were drawn. The clustering was performed using the Statistica 6.0 program (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Hexane is the most commonly used solvent for extraction of insect CHCs. It dissolves hydrocarbons of low and medium molecular weight, i.e., hydrocarbons that can be analyzed by gas chromatography. However, its ability to extract long-chain hydrocarbons from complex matrices is somewhat limited. Based on our laboratory experiences, we selected $CHCl_3$ for hydrocarbon extractions. It can efficiently dissolve the entire wax layer, including long-chain hydrocarbons and more polar compounds present on the insect cuticle. The hydrocarbon fraction was isolated from the whole extract by TLC on silica, eluting with hexane. Although retention behavior of hydrocarbons and related compounds bearing functional group(s) in TLC is commonly known, we conducted experiments to ensure that very long hydrocarbon chain(s) do not affect

retention of polar compounds considerably. We observed that even very long-chain triacylglycerols, fatty acids, and alcohols stayed at the origin. Wax esters and long-chain ethers were also retained [R_F of behenyl behenate ($C_{44}H_{88}O_2$) was 0.05 and R_F of dioctadecyl ether ($C_{36}H_{74}O$) was 0.08]. Thus, when CHC extracts were separated on silica TLC plates, polar compounds remained at or close to the origin, whereas one to three spots were observed close to the solvent front, with RF values of 0.85–1.00. These were due to hydrocarbons with various structural features, such as branching, double bonds, or rings. For selected samples, the hydrocarbon fraction was further separated on silver ion TLC. Saturated CHCs migrated with or close to the solvent front, whereas unsaturated CHCs formed zone(s) close to the origin (R_F = 0.10–0.26).

Cuticular Hydrocarbons Analyzed by FT-IR, ^1H NMR, and ^{13}C NMR. To obtain basic information on CHC mixtures extracted using $CHCl_3$, a TLC-separated hydrocarbon fraction was examined by IR and NMR spectroscopy. Because these methods require relatively large amounts of samples, the experiments were performed only with the extract of *N. bullata*, which was available in sufficient quantity. The goal of these experiments was to learn more about the structural features of hydrocarbons present in the sample and to confirm that no compounds containing polar functionalities remained in the hydrocarbon fraction. The IR spectrum showed intense methyl and methylene C–H stretching and bend absorptions, methylene (–$(CH_2)_n$–) rocking absorptions, and bend absorption of methyl group. Alkene C–H stretching absorption gave weak signals in the spectrum. No frequency that can be attributed to polar functional groups was found in the spectrum. In the ^1H NMR spectrum, signals of hydrogens in methylene groups and in terminal methyls were recorded. Allylic protons were also observed. ^{13}C NMR revealed the presence of methylene groups of long-hydrocarbon chains and terminal and nonterminal methyls. Carbons of methylene groups next to methyl branching or next to quarternary carbon atoms were also detected. As in ^1H NMR, signals indicating the presence of isolated double bonds on a long hydrocarbon chain were observed. No signals that would suggest the presence of six-membered rings or saturated polycyclic structures were found. Based on the IR and NMR spectra, it was concluded that *N. bullata* CHC fractions contained only saturated and unsaturated long-chain hydrocarbons, some of which were branched.

Cuticular Hydrocarbons Analyzed by Gas Chromatography. CHC fractions of $CHCl_3$ extracts were analyzed by GC/MS using a conventional nonpolar capillary column, and individual hydrocarbons were identified based on their mass spectra (Doolittle et al., 1995) and retention behavior (Katritzky et al., 2000). In the samples, *n*-alkanes, methylalkanes, or alkadienes (*P. americana*) were the main components; di- and trimethylalkanes and alkenes were also present (Table 1). The hydrocarbon profile was noticeably different in the *A. colombica* sample, in which long-chain CHCs (>30 carbon atoms) formed

TABLE 1. INSECT CUTICULAR HYDROCARBONS IDENTIFIED BY GC/MS[a]

Compound	CN	*R. lucifugus*	*R. virginicus*	*R. grassei*	*R. santonensis*	*C. formosanus*	*N. cubanus*	*N. castaneus*	*C. declivis*	*P. americana*	*N. bullata*	*A. colombica*	*A. echinatior*
Pentadecane	15	0.6	–	–	–	–	0.1	0.2	–	–	–	1.1	7.7
Hexadecane	16	0.3	–	–	–	0.4	0.2	0.2	–	–	–	1.6	8.7
Heptadecane	17	1.2	0.3	0.4	0.4	1.3	0.4	0.4	–	–	–	2.6	14.6
Octadecane	18	0.7	–	0.2	0.2	0.8	0.2	0.2	–	–	–	1.4	6.2
Unknown		0.5	–	0.2	–	0.5	0.1	0.2	–	–	–	0.6	4.0
Nonadecene	19	–	–	–	0.1	–	–	–	–	–	–	1.2	–
Nonadecane	19	0.5	–	0.2	0.2	1.4	0.2	0.2	–	–	–	0.9	5.8
Eicosane	20	0.7	0.2	0.3	0.2	1.5	0.2	0.2	–	–	–	0.8	3.9
Heneicosane	21	0.9	0.4	0.4	0.3	1.7	0.2	0.8	–	–	–	0.8	4.3
Docosane	22	1.6	0.6	0.6	0.5	1.9	0.4	0.8	–	–	–	–	3.5
Tricosane	23	2.2	11.5	0.8	7.5	2.1	14.4	41.9	0.1	–	0.1	0.9	12.0
11-Methyltricosane	24	0.6	0.8	–	1.0	0.5	9.2	0.4	–	–	0.1	–	0.7
2-Methyltricosane	24	–	1.6	–	2.2	–	4.6	2.2	0.3	–	–	–	0.4
3-Methyltricosane	24	–	0.9	–	0.3	–	0.3	2.4	–	–	–	–	0.6
Tetracosane	24	1.5	4.7	1.0	5.9	2.6	3.1	3.0	–	–	0.2	–	1.9
Unresolved 11-Methyltetracosane and 12-Methyltetracosane	25	0.4	0.9	–	1.9	–	–	–	–	–	–	–	–
2-Methyltetracosane	25	–	17.4	–	25.8	–	2.3	7.6	1.4	–	–	–	–
3-Methyltetracosane	25	–	0.3	–	0.6	–	0.3	0.9	–	–	–	–	–
Pentacosane	25	9.0	22.5	1.8	24.7	4.0	12.9	31.7	23.7	11.9	16.6	5.9	12.1
Unresolved 13-Methyl and 9-Methylpentacosane	26	–	–	–	–	–	–	–	–	–	4.1	–	–
5-Methylpentacosane	26	4.5	–	0.1	1.6	–	39.6	–	–	–	2.1	–	–
2-Methylpentacosane	26	0.9	3.7	0.3	7.6	10.6	2.1	–	3.4	–	–	–	–
3-Methylpentacosane	26	–	7.6	–	13.0	2.0	3.6	3.4	6.5	16.9	1.4	–	–

Hexacosane	26	2.5	1.2	2.7	1.2	3.8	0.6	0.6	5.4	–	1.1	–	–
Unknown	27	0.4	–	2.1	0.3	–	1.4	–	–	–	–	–	–
2-Methylhexacosane	27	2.8	0.6	0.8	0.6	2.7	–	–	7.7	–	–	–	–
6,9-Heptacosadiene	27	–	–	–	–	–	–	–	–	71.3	–	–	–
Heptacosene	27	0.3	–	0.5	0.2	0.4	–	–	10.1	–	0.3	–	–
Heptacosane	27	26.8	3.1	29.5	1.6	7.2	2.8	2.7	33.2	–	26.4	7.1	8.7
Unresolved 13-Methyl and 9-Methylheptacosane	28	4.9	3.1	14.5	0.3	23.9	0.6	–	2.2	–	2.3	–	–
7-Methylheptacosane	28	–	–	–	–	–	–	–	–	–	1.8	–	–
5-Methylheptacosane	28	7.6	–	2.1	–	–	–	–	1.1	–	0.4	–	–
2-Methylheptacosane	28	–	3.0	15.8	–	13.6	–	–	–	–	–	–	–
3-Methylheptacosane	28	2.2	1.3	–	–	6.9	–	–	0.7	–	5.1	–	–
Octacosane	28	3.0	0.5	4.8	0.5	2.1	0.3	–	0.9	–	0.8	1.0	1.2
Unknown		–	–	4.5	–	–	–	–	0.5	–	–	–	–
Nonacosane	29	4.4	–	7.1	–	2.3	–	–	–	–	14.0	11.4	3.7
Unknown		–	0.9	–	0.7	–	–	–	1.9	–	–	–	–
Unresolved 13-Methyl-, 9-Methyl-, and 7-Methylnonacosane	30	1.9	0.8	3.4	–	5.6	–	–	–	–	8.0	–	–
5-Methylnonacosane	30	7.3	1.5	–	–	–	–	–	–	–	0.8	–	–
2-Methylnonacosane	30	3.8	–	–	–	–	–	–	–	–	–	–	–
3-Methylnonacosane	30	–	–	–	–	–	–	–	–	–	9.0	–	–
Unknown		–	–	–	–	–	–	–	–	–	1.1	–	–
Unknown		0.4	–	–	0.3	–	–	–	0.8	–	1.0	0.8	–
Unresolved 13-Methyl-, 9-Methyl- and 7-Methylhentriacontane	31	–	–	–	–	–	–	–	–	–	2.0	–	–
3-Methylhentriacontane	31	–	–	–	–	–	–	–	–	–	1.3	–	–

TABLE 1. (CONTINUED)

Compound	CN	*R. lucifugus*	*R. virginicus*	*R. grassei*	*R. santonensis*	*C. formosanus*	*N. cubanus*	*N. castaneus*	*C. declivis*	*P. americana*	*N. bullata*	*A. colombica*	*A. echinatior*
11,18-Dimethyl triacontane	32	2.2	1.9	1.6	–	–	–	–	–	–	–	–	–
11,16-Dimethyl hentriacontane	33	1.5	2.5	2.2	–	–	–	–	–	–	–	–	–
8,12-Dimethyl dotriacontane	34	1.8	2.8	1.1	–	–	–	–	–	–	–	–	–
Unknown		–	0.8	–	–	–	–	–	–	–	–	–	–
11-Methyltritriacontane	34	–	2.5	0.9	0.4	–	–	–	–	–	–	–	–
3,7,11-Trimethyl hentriacontane	34	–	–	–	–	–	–	–	–	–	–	12.9	–
4,8,12-Trimethyl dotriacontane	35	–	–	–	–	–	–	–	–	–	–	8.3	–
Unknown		–	–	–	–	–	–	–	–	–	–	4.8	–
3,7,11-Trimethyl tritriacontane	36	–	–	–	–	–	–	–	–	–	–	17.8	–
Unknown		–	–	–	–	–	–	–	–	–	–	0.6	–
Unknown		–	–	–	–	–	–	–	–	–	–	2.2	–
4,8,12- Trimethyl tetratriacontane	37	–	–	–	–	–	–	–	–	–	–	7.3	–
Unknown		–	–	–	–	–	–	–	–	–	–	0.9	–
Unknown		–	–	–	–	–	–	–	–	–	–	2.9	–
3,7,11- Trimethyl pentatriacontane	38	–	–	–	–	–	–	–	–	–	–	2.3	–
Unknown		–	–	–	–	–	–	–	–	–	–	1.0	–
Unknown		–	–	–	–	–	–	–	–	–	–	1.0	–

[a] Relative composition in percent based on the peak areas integrated in reconstructed chromatograms based on the *m/z* 57 ion.
CN = carbon number.

about 60% of the total peak area. We compared our GC/MS results with literature data; reports for *R. grassei*, *R. santonensis*, *C. formosanus*, *A. colombica*, *P. americana*, and *N. bullata* were available (Martin and MacConnell, 1970; Jackson, 1972; Jackson et al., 1974; Armold and Regnier, 1975; Haverty et al., 1990, 2000; Vauchot et al., 1998). We found a good correlation between published data and our results with all the main compounds being detected. Minor differences were observed for unsaturated CHCs, where some alkenes were not found, probably due to coelution with other hydrocarbons. The integration of reconstructed chromatograms based on the *m*/*z* 57 ion also underestimated unsaturated hydrocarbons. Although direct comparison of the extraction using $CHCl_3$ and the common method with hexane was not performed, the agreement with literature data suggests that both extraction methods are comparable. As expected, silver ion TLC efficiently separated saturated CHCs from unsaturated CHCs. In *N. bullata*, almost all CHCs detected by GC/MS were saturated, with only a few tiny peaks due to unsaturated compounds being detected. Using GC/MS, we observed CHCs with up to 35 carbons. Higher injector and analysis temperatures would probably shift the CHC profile toward the hydrocarbons of slightly higher molecular weights, but it is clear that GC/MS discriminates against higher molecular weight components, if they are detected at all.

Cuticular Hydrocarbons Analyzed by MALDI-TOF. When irradiated with a laser pulse, the LiDHB matrix produced a mixture of matrix ions. Spectra of ^{7}LiDHB measured without ion suppression showed intense lithium cation signals at *m*/*z* 7.0 and an intense ion series at *m*/*z* 161.0, 167.1, and 173.1, corresponding to protonated ^{7}LiDHB and to protonated ^{7}LiDHB, with one and two hydrogen atoms replaced with ^{7}Li, respectively. An ion at *m*/*z* 143.0 was formed from loss of water from protonated ^{7}LiDHB. For a short time at the beginning of irradiation, we observed two more ion series representing clusters of two and three molecules of LiDHB with a various number of hydrogens replaced by lithium. These ions had the general structures $C_{14}H_{10-n}Li_{2+n}O_8$ and $C_{21}H_{15-n}Li_{2+n}O_{12}$, where $N = 0$–4; they disappeared quickly after several laser shots. Another lithiated ion series separated by 28 mass units at *m*/*z* 409.3, 437.3, and 465.3 was observed in the spectra. These ions were probably not related to the matrix and came from contamination; however, we were not able to trace the source of the contamination. The relatively small number of matrix ions enabled spectra to be recorded starting from *m*/*z* 200. However, because compounds with low molecular weights are somewhat volatile, compounds with molecular weights of <400 Daltons were not analyzed. Thus, with the exception of the matrix and contamination peaks mentioned above, clean, featureless blank matrix spectra were routinely obtained.

Using the ^{7}LiDHB matrix, lithium adduct ion formation was studied for several hydrocarbon standards. Representatives of diverse hydrocarbon classes were analyzed, including long-chain saturated hydrocarbons (triacontane, hexa-

triacontane, tetracontane, tetratetracontane, pentacontane, and hexacontane), branched saturated hydrocarbons (9-methylheptacosane, squalane, Apolane-87), and unsaturated and cyclic hydrocarbons (squalene, nonadecylcyclohexane, icosylcyclopentane). The sample (0.5 μg) was spotted onto a MALDI target. For all these compounds, single signals at *m*/*z* $[M + {}^{7}Li]^{+}$ with the expected isotope pattern were observed in the spectra. No cluster ions (e.g., $[2M + Li]^{+}$) were observed for any of the compounds or for mixtures. At this concentration range, no dehydrogenation was observed; however, at 10 times the concentration, a minor peak two mass units lower than that of $[M + {}^{7}Li]^{+}$ was detected. In no case did the relative intensity of this peak exceed 10% of the adduct ion. The mechanism of formation of these dehydrogenated ions is unclear. However, a tentative explanation could be laser-induced hydrocarbon dehydrogenation, which may be more pronounced at low matrix/hydrocarbon ratios. The alkenes formed likely have more affinity for complexing with the Li ion and therefore appear more abundant. Different ionization efficiencies of diverse hydrocarbon classes (saturated vs. unsaturated) were observed but not quantified because of the lack of sufficiently nonvolatile standards.

When CHC samples were subjected to MALDI-TOF analysis, distinctive profiles were observed for individual insect species (Figures 1–3). The most intense ions were observed roughly between *m*/*z* 500 and 700. In some samples, a second series of hydrocarbons also appeared around *m*/*z* 800–850. The largest hydrocarbons observed in the spectra had more than 70 carbon atoms. In the low-mass region of MALDI-TOF spectra, ions that correspond to hydrocarbons measured by GC/MS were observed. An example of the low-mass region of *R. lucifugus* spectrum is shown in Figure 4. When compared with GC/MS results (see Table 1), the peaks of high intensity in the MALDI-TOF spectrum can be assigned as follows: *m*/*z* 387.4 corresponds to the sum of heptacosane and 2-methylhexacosane; *m*/*z* 401.4 can be explained by a sum of methylheptacosanes and octacosane; *m*/*z* 415.4 represents nonacosane; and *m*/*z* 429.4 is the sum of methylnonacosanes. In the MALDI-TOF spectrum, there were also peaks that were not seen in the GC/MS traces. These ions might be unsaturated or cyclic derivatives of heptacosane (*m*/*z* 383.4) and nonacosane (*m*/*z* 409.4; *m*/*z* 411.4; and *m*/*z* 413.4). There are two possible explanations for why these compounds were observed in MALDI but not in GC/MS traces. First, these compounds form cationic adducts efficiently in MALDI experiments. Second, these compounds may decompose in GC injectors. In order to characterize ions observed in the MALDI spectra more precisely, the samples were mixed with an internal calibrant (PEG) and mass spectra were recorded. Because the nominal mass of methylene group is the same as the nominal mass of 14 hydrogens, one can easily confuse a hydrocarbon having seven double bonds or rings with a hydrocarbon that is one methylene group shorter. The difference (CH_2-14H) is 0.094 u, which can be reliably measured with instruments having a resolution in

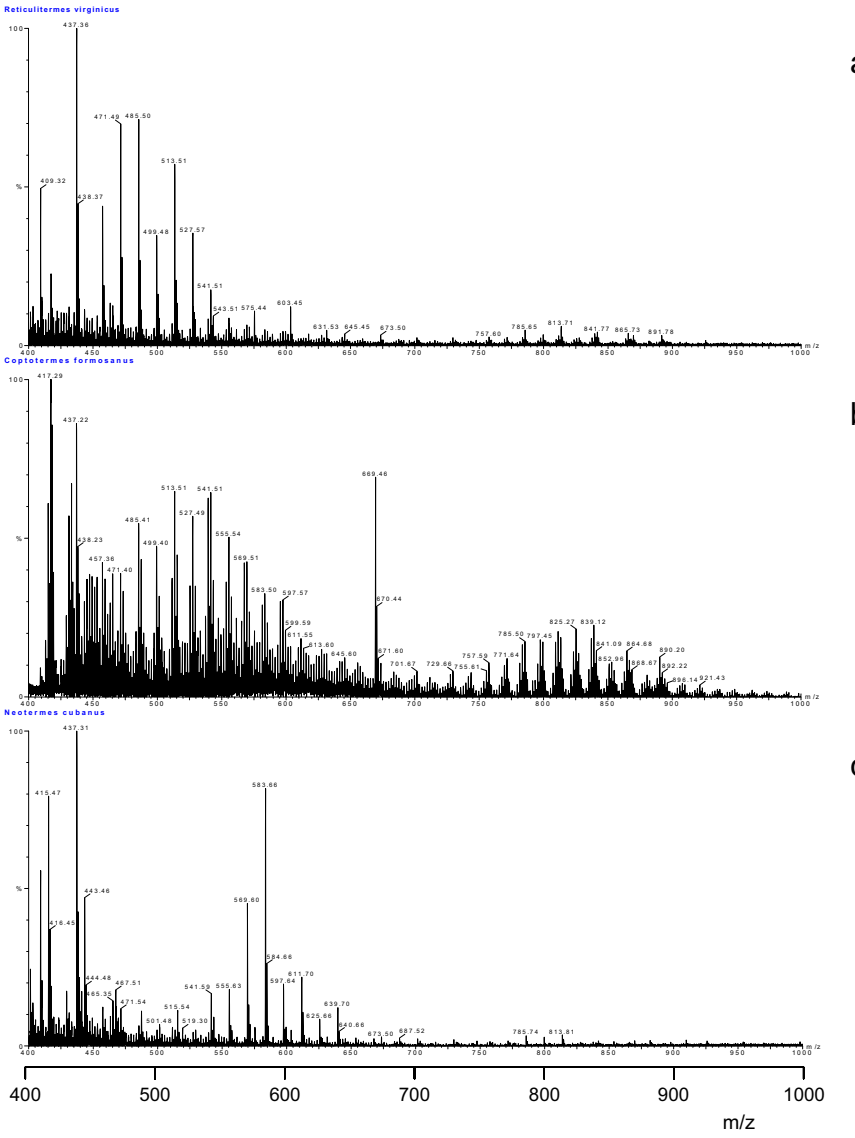

FIG. 1. MALDI-TOF mass spectrum of cuticular hydrocarbons of (a) *Reticulitermes virginicus*, (b) *Coptotermes formosanus*, and (c) *Neotermes cubanus*. ^{7}LiDHB matrix ions were eliminated by a using low mass (300 Da) cutoff, scans were accumulated from 20 laser shots, and spectra shown are averages of at least 20 consecutive scans. Baseline subtracted data were smoothed, centered, and *m*/*z* scale calibrated using lithium ions of polyethylene glycol standards.

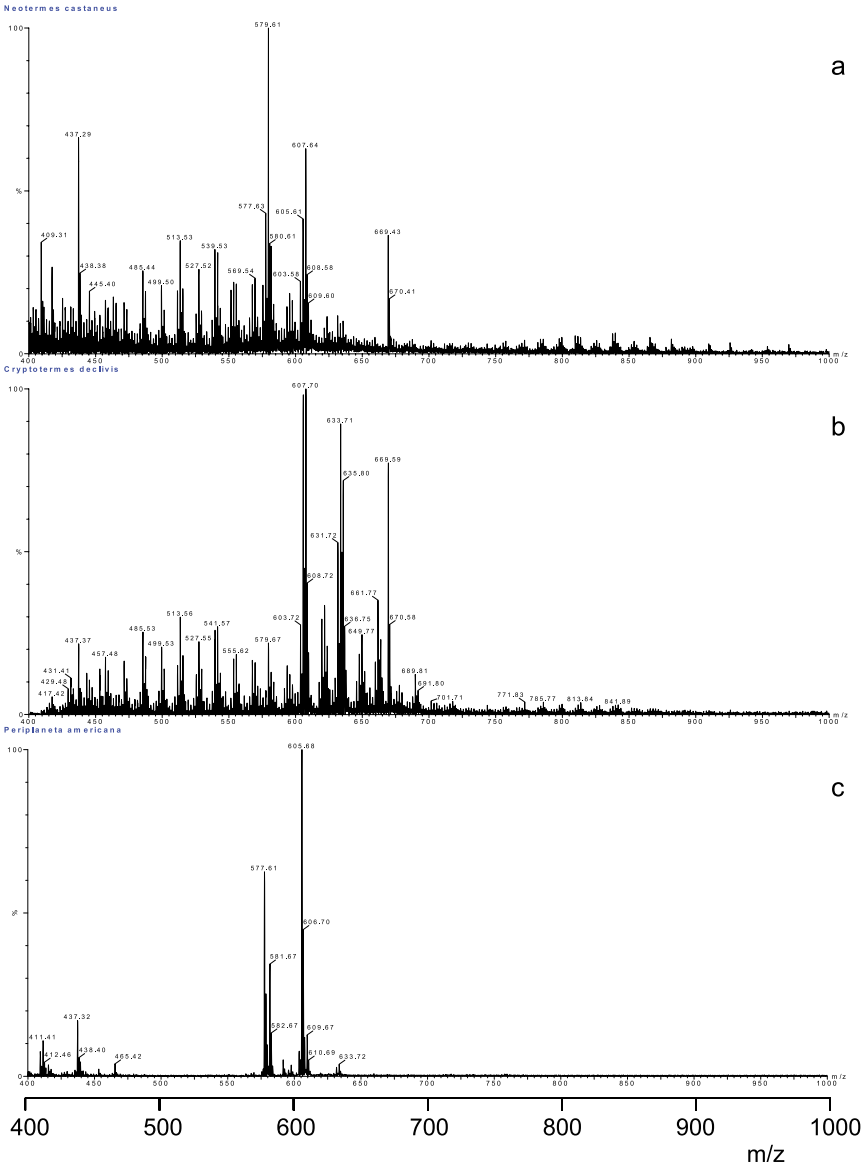

FIG. 2. MALDI-TOF mass spectrum of cuticular hydrocarbons of (a) *Neotermes castaneus*, (b) *Cryptotermes declivis*, and (c) *Periplaneta americana*. For details, see Fig. 1.

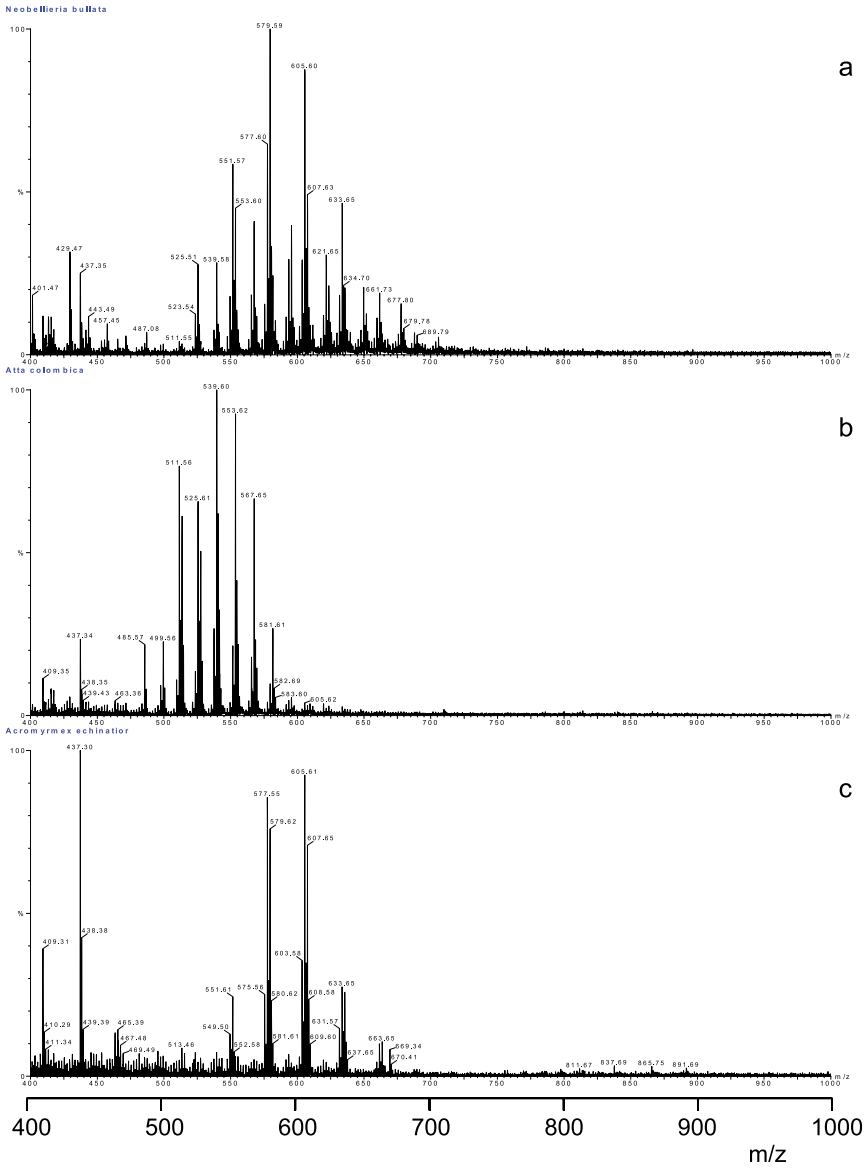

FIG. 3. MALDI-TOF mass spectrum of cuticular hydrocarbons of (a) *Neobellieria bullata*, (b) *Atta colombica*, and (c) *Acromyrmex echinatior*. For details, see Fig. 1.

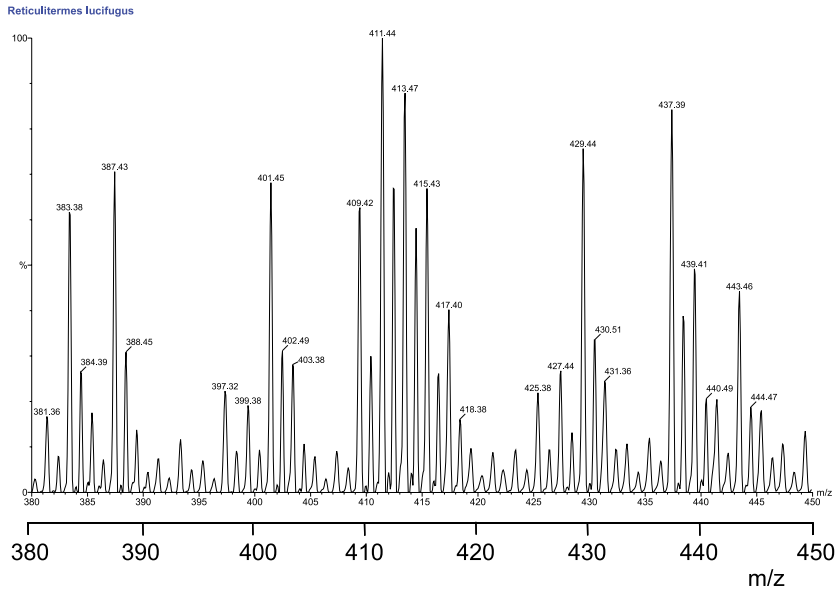

FIG. 4. Low mass region of MALDI-TOF mass spectrum of *Reticulitermes lucifugus*. For details, see Fig. 1.

the range of 5000–10,000. Such resolution is possible among current TOF analyzers. The mass precision achieved with our instrument was affected by a relatively low sampling rate (500 MHz) giving roughly 8 data points per spectrum peak, but internal calibration provided mass precision of measurements to about 75 ppm. The ions observed in the spectra were identified with the help of a software (available upon request) that calculates the possible hydrocarbon adduct ions of the general structure C_xH_yLi and scores the hits based on the mass error. Hits with the best matches were considered positively identified.

The mass spectra of *Reticulitermes* termite samples indicated the presence of saturated acyclic hydrocarbons up to roughly C45 (e.g. *R. virginicus*, Figure 1a). These compounds were the main constituents and were found in all samples of this genus studied in this work. Differences between species were in the intensity of particular hydrocarbons and in the presence of other hydrocarbons. *Reticulitermes* species also showed a second series of lithium adduct ions corresponding to highly unsaturated or cyclic C55–C65 hydrocarbons with 18–19 sites of unsaturation. The CHCs of *C. formosanus* (Figure 1b) were quite complex. The first series reached its maximum around C36–C38, and the second around C60. A surprisingly high number of sites of unsaturation (27) was

calculated for an ion at *m*/*z* 669.43 (C51). However, the presence of such large numbers of double bonds or rings in both the *Reticulitermes* species and *C. formosanus* remains to be confirmed by other methods. Mass spectra of CHCs two *Neotermes* species differed greatly from one another. The *N. cubanus* sample (Figure 1c) contained mainly saturated acyclic hydrocarbons up to C46. A few peaks of highly unsaturated or cyclic C55–C65 CHCs were also present. The mass spectrum of *N. castaneus* CHCs (Figure 2a) showed groups of ions with 0–4 sites of unsaturation. As with *C. formosanus*, a distinct peak at *m/z* 669.45 as well as a second series around C60–C70 was observed. A MALDI mass spectrum of *C. declivis* is shown in Figure 2b. Internal calibration for this sample was not performed because of insufficient sample amounts. Thus, the interpretation was done based on external calibration data only. Both saturated and unsaturated or cyclic hydrocarbons were identified. The spectrum showed distinct intense peaks interpreted as C43 and C45 with 3–4 sites of unsaturation. The MALDI spectrum of *P. americana* (Figure 2c) showed two groups of hydrocarbons (C41 and C43) with 1 and 3 sites of unsaturation. A mass spectrum of flesh fly *N. bullata* CHCs (Figure 3a) showed a distribution of C36–C50 hydrocarbons. Lithium adduct ions formed groups corresponding to acyclic saturated hydrocarbons and unsaturated or cyclic CHCs with 1–4 sites of unsaturation. Hydrocarbons with an odd number of carbon atoms were more intense compared to those with an even number of carbons. In the mass spectrum of a leaf-cutting ant *A. colombica* (Figure 3b), a distribution of C34–C42 CHCs was found. Adduct ions formed groups corresponding to saturated hydrocarbons and those having 1–2 sites of unsaturation. The sample from another leaf-cutting ant, *A. echinatior* (Figure 3c), contained almost exclusively odd-numbered CHCs (C41–C49). Lithium adduct ions in the spectra corresponded to compounds with 1–4 sites of unsaturation.

To obtain more in-depth insight into CHCs, fractions of saturated and unsaturated hydrocarbons were analyzed separately. Figure 5 shows the MALDI spectrum of saturated (Figure 5a) and unsaturated (Figure 5b) hydrocarbons of *P. americana*. The most intense signals in the spectrum of saturated CHCs give alkanes with 25 and 26 carbons; they were identified by GC as pentacosane and 3-methylpentacosane (Table 1, Jackson, 1972). Less abundant higher hydrocarbons, C27 and C42, were previously characterized as heptacosane and 3-methylhentetracontane (Jackson, 1972). Few other saturated CHCs (C29, C40, and C44) were observed in the MALDI spectrum. The spectrum of unsaturated hydrocarbons from *P. americana* (Figure 5b) showed alkenes with 1–3 sites of unsaturation. An ion at *m*/*z* 383.43 is a lithium adduct of (*Z*,*Z*)-6,9-heptacosadiene, previously identified from this cockroach (Jackson, 1972; Suiter et al., 1996). Two clusters of CHCs with 41 and 43 carbons probably contain trienes, dienes, and monoenes. Trienes had not previously been described, whereas monoenes were identified as *cis*-15-hentetracontene and

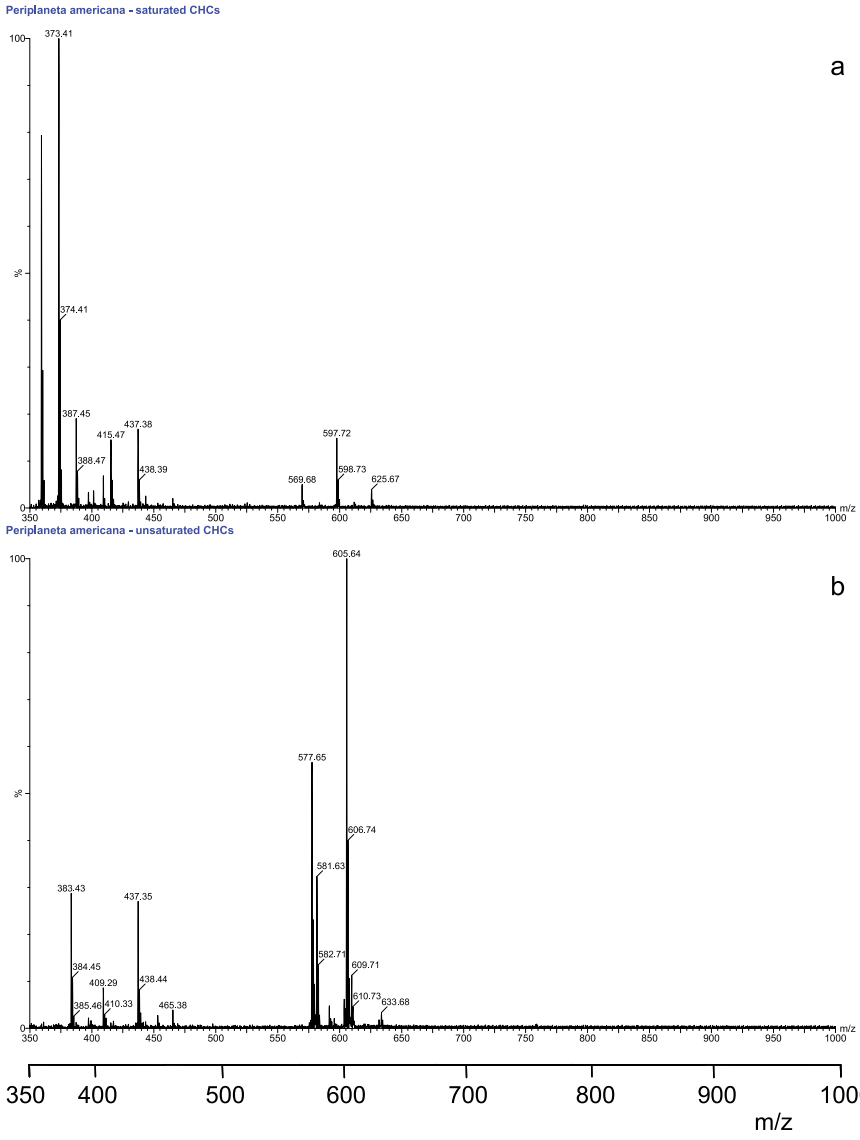

FIG. 5. MALDI-TOF mass spectrum of (a) saturated and (b) unsaturated CHCs of *Periplaneta americana*. For details, see Fig. 1.

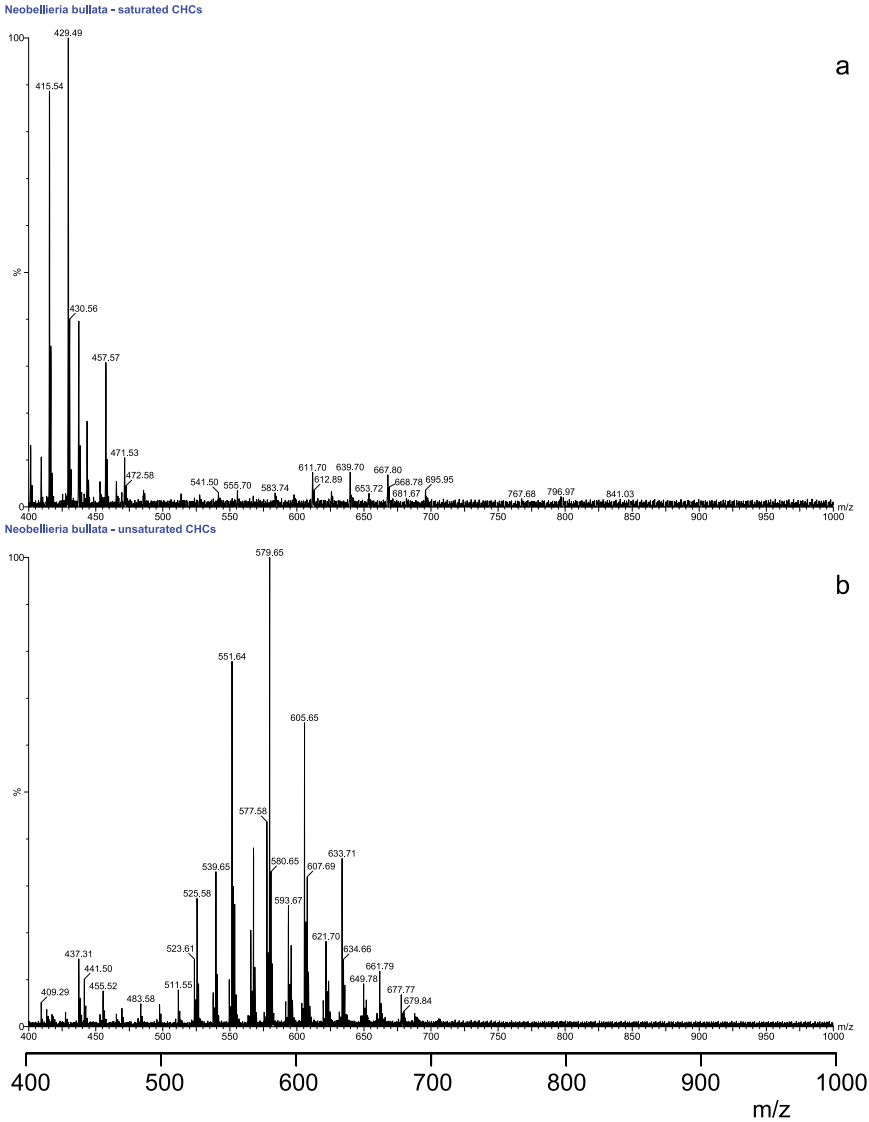

FIG. 6. MALDI-TOF mass spectrum of (a) saturated and (b) unsaturated CHCs of *Neobellieria bullata*. For details, see Fig. 1.

cis-15-tritetracontene (Jackson, 1972). In the MALDI spectra, low-intensity signals of CHCs with 42 and 45 carbons and 3 sites of unsaturation were also detected. Thus, our MALDI spectra was in agreement with published data. In addition, hydrocarbons with 3 sites of unsaturation, which are present in significant amounts, were newly revealed. Another example of separately analyzed saturated and unsaturated CHCs (*N. bullata*) is given in Figure 6a and b. The spectrum of saturated CHCs shows alkanes with up to 47 carbons. The literature (Jackson et al., 1974; Armold and Regnier, 1975) and our results from GC/MS identified these peaks as nonacosane (C29), methyl nonacosanes (C30), hentriacontane (C31), methyl hentriacontane and dotriacontane (C32), and tritriacontane (C33), whereas the others had not been described previously. When Figure 6b is compared with Figure 3a, it is apparent that almost all MALDI peaks in *N. bullata* are due to the unsaturated CHCs. As described above, CHCs with up to 4 sites of unsaturation were detected, with those having an odd number of carbons being most abundant. We regard the presence of rings as unlikely (no evidence exists for rings from NMR experiments) and consider these structures to be mono-, di-, tri,- and tetraenes.

The spectra of all CHCs were a simple sum of the spectra of saturated and unsaturated CHCs because unsaturated compounds more effectively compete for lithium ions, resulting in saturated CHCs being less pronounced in the composite spectra. Therefore, crude extracts should be fractionated into saturated and unsaturated hydrocarbons by silver ion chromatography before analysis of complex hydrocarbon mixtures. Importantly, silver ions do not appear to contaminate CHC fractions during separation, because no silver ion adducts were observed in the MALDI spectra. To summarize, distinctive CHC profiles were recorded for all tested species using MALDI-TOF analysis. Saturated hydrocarbons, as well as hydrocarbons with varying degrees of unsaturation, were found. It is likely that polyunsaturated CHCs readily ionize, increasing their intensities in the spectra. Also, the exact structures of the unsaturated hydrocarbons could not be determined from the MALDI-TOF spectra.

The MALDI technique was also tested for reproducibility and capacity for quantitatively measuring CHCs, for its possible use in chemotaxonomy. Although LDI and MALDI are not techniques generally considered suitable for quantitation, their usefulness in this field has been demonstrated (Dutta and Harayama, 2001). Pruns et al. (2003) applied principal component analysis to LDI-TOF mass spectra to characterize paraffin oils and petrolatum samples. In our experiment, spectra of a model sample (paraffinum liquidum, 5.0 μg) spotted in triplicates onto two MALDI targets were recorded. Averaged spectra from 20 to 120 scans were compared, and similar-looking spectra differing slightly in peak intensities were obtained. To quantify the reproducibility of the measurement, relative standard deviations (RSDs) were calculated for ratios of selected peaks. Low RSDs (<10%) were calculated for ratios of peaks differing

in 14 mass units (adjacent alkanes). However, the reproducibility of ratios of peaks differing by more than 100 mass units was worse, with RSDs between 20 and 50% being obtained. Several factors affect the reproducibility of the signals in MALDI experiments. First, the homogeneity of sample deposition and uniform incorporation of analyte molecules into the matrix is critical. The LiDHB matrix is polar and finding a suitable solvent system that allows the dissolution of both the sample and the matrix is not straightforward. Nonuniform samples with variable concentrations of sample components across the sample well on the MALDI target can result. Uniform samples can be achieved using solvent-free preparation methods, but a substantially larger amount of sample is needed. Another method to obtain homogenous MALDI samples is electrospray sample deposition (Hanton et al., 2004). Second, lower molecular weight hydrocarbons under the low pressure in the ion source may tend to volatilize off the target. Therefore, the time between the introduction of the target plate into the vacuum and sample measurement should be controlled. Third, a substance-dependent time delay between the beginning of the irradiation of the sample and the appearance of the signal in the spectrum was observed, with long-chain saturated hydrocarbons taking a longer time (or laser shots) to appear in the spectrum. Therefore, the relative intensities of peaks in the spectra depend on the acquisition time. Thus, precise control of experimental parameters will increase reproducibility. The reproducibility that exists in GC/MS measurements cannot be achieved but the quality of the MALDI data is sufficient for statistical processing and discriminating among species. The work described below summarizes results from two sets of experiments (including extraction steps) that were performed consecutively over 6 months. Comparable CHC profiles were obtained for all model species.

Statistical Analysis. Nei distances proved to be an appropriate tool for evaluating phenotype distances within CHCs among studied species. The clustering of Nei distances among CHC profiles (Figure 7) showed a high potential for discriminating with both GC/MS and MALDI-MS data. The consistency between hierarchical classifications calculated by two different methods (UPGMA and single linkage) was similar for both MALDI-MS and GC/MS. The position of a single species (*C. formosanus* in MALDI-MS and *N. cubanus* in GC/MS) was the only difference between the two statistical methods used (the interchange of *P. americana* and *C. declivis* in Figure 7d and e is due to the different insertion of *C. formosanus*). When compared with GC/MS, MALDI-MS data showed similar efficiency in discriminating among particular species (compare Figure 7a–d). With both techniques, some related species were repeatedly grouped together (e.g., *R. santonensis* and *R. virginicus* in GC/MS, the whole genus *Reticulitermes* in MALDI), whereas others were consistently separated (both ant species, both *Neotermes* species). The CHC profile of an unrelated species, the cockroach *P. americana*, measured by GC/

MS, differed quite notably from all others. It consisted primarily of three CHCs (Table 1). Moreover, the major compound (6,9-heptacosadiene, 71%) was unique to this species, which set it apart from all others (Figure 7a,b). In the MALDI spectrum of *P. americana* CHCs, all 25 peaks (the average number is 199 for the other species studied) had the same mass as peaks in other species, and therefore the cockroach was placed deeply inside the tree (Figure 7c,d).

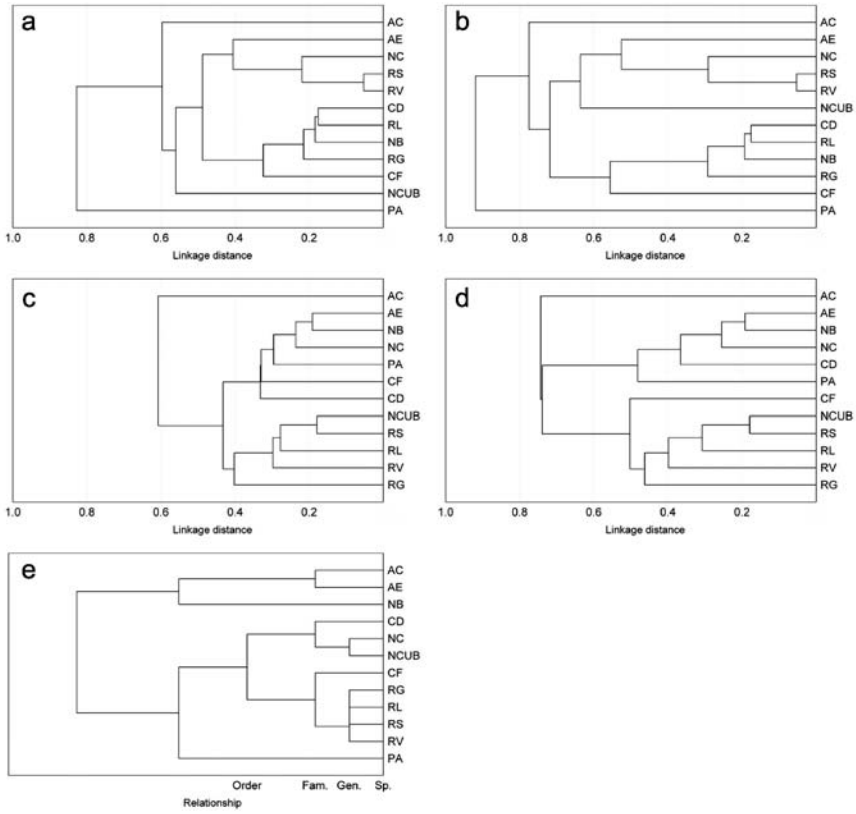

FIG. 7. Hierarchical tree made by single linkage clustering of Nei distances among cuticular hydrocarbon profiles analyzed by GC/MS (a), made by UPGMA clustering of Nei distances among cuticular hydrocarbon profiles analyzed by GC/MS (b), made by single linkage clustering of Nei distances among cuticular hydrocarbon profiles analyzed by MALDI MS (c), made by UPGMA clustering of Nei distances among cuticular hydrocarbon profiles analyzed by MALDI MS (d), hierarchical tree representing the general phylogenetic relationships among the 12 analyzed species (e). Abbreviations of insect species are listed in Insects; Sp.: species, Gen.: genus, Fam.: family.

Conversely, because few peaks in both the GC/MS and MALDI spectra of *A. colombica* CHCs are shared with other species, this ant species was always placed apart from the others (Figures 7a–d).

The primary function of CHC, water regulation, may be largely independent of their composition. Therefore, CHCs may be good candidates for phylogenetic characters. However, this selection neutrality may also enable rapid changes in CHC profiles during evolution, which could conceal the real relatedness even in closely related species. Support for this hypothesis is provided by the considerable differences found in CHC profiles of many closely related species (Watson et al., 1989; Kaib et al., 1991; Golden et al., 1992; Bagine et al., 1994; Haverty et al., 1996; Haverty and Nelson, 1997; Takematsu and Yamaoka, 1997). On the other hand, CHCs were shown to reliably indicate the relatedness on the lowest levels, i.e., at the (sub)species, population, or colony level (Kutnik et al., 2004). Not surprisingly, neither GC/MS nor MALDI-MS was able to figure out the phylogeny of the studied species. Contrary to our expectations, none of the groups of closely related species were separated from nonrelative species in any of the tests. On the other hand, MALDI-MS data grouped together all four species from the genus *Reticulitermes*, but, unfortunately, also with *N. cubanus*. Nevertheless, MALDI-MS of CHCs may have some utility in phylogenetic and chemotaxonomic studies.

In conclusion, MALDI-TOF mass spectrometry with a lithium 2,5-dihydroxybenzoate matrix revealed the presence of high molecular weight hydrocarbons on insect cuticles, including saturated hydrocarbons, and highly unsaturated and/or cyclic compounds. A detailed investigation of *N. bullata* CHC extracts using IR and NMR confirmed the presence of structural features commonly observed in GC/MS analyses, such as long hydrocarbon chains with some methyl branching and double bonds, but no evidence of rings. MALDI-MS spectra for the same samples corroborated the presence of hydrocarbons with 0–4 double bonds. The samples of other species, however, gave composite spectra possibly consistent with a considerably higher number of double bonds and/or rings. However, no independent proof of such hydrocarbons has been obtained yet. Both MALDI mass spectra and GC/MS chromatograms were able to discriminate among the species studied. Therefore, MALDI-MS has the potential to become an alternative or complementary analytical technique for chemotaxonomic studies, enabling us to extend the range of molecular weights of compounds studied. Further instrumentation developments may provide even more information about analyzed samples, for example, by interfacing of MALDI with high-performance liquid chromatography (Esser et al., 2000) or supercritical fluid chromatography (Planeta et al., 2002). Furthermore, excellent quality data (mass accuracy, resolution, and sensitivity) can be obtained with MALDI Foudrier-transform ion cyclotron resonance mass spectrometry, and MS experiments might help to elucidate structures of unknown CHCs. Further research is needed

to elucidate complete structures of the high molecular weight hydrocarbons found in this study, and subsequently, to determine their possible biological activity.

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