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A Silica Gel Based Method for Extracting Insect Surface Hydrocarbons

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Abstract Here, we describe a novel method for the extraction of insect cuticular hydrocarbons using silica gel, herein referred to as "silica-rubbing". This method permits the selective sampling of external hydrocarbons from insect cuticle surfaces for subsequent analysis using gas chromatography–mass spectrometry (GC-MS). The cuticular hydrocarbons are first adsorbed to silica gel particles by rubbing the cuticle of insect specimens with the materials, and then are subsequently eluted using organic solvents. We compared the cuticular hydrocarbon profiles that resulted from extractions using silica-rubbing and solvent-soaking methods in four ant and one bee species: *Linepithema humile*, *Azteca instabilis*, *Camponotus floridanus*, *Pogonomyrmex barbatus* (Hymenoptera: Formicidae), and *Euglossa dilemma* (Hymenoptera: Apidae). We also compared the hydrocarbon profiles of *Euglossa dilemma* obtained via silica-rubbing and solid phase microextraction (SPME). Comparison of hydrocarbon profiles obtained by different extraction methods indicates that silica rubbing selectively extracts the hydrocarbons that are present on the surface of the cuticular wax layer, without extracting hydrocarbons from internal glands and tissues. Due to its surface specificity, efficiency, and low cost, this new method may be useful for studying the biology of insect cuticular hydrocarbons.

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Introduction

Cuticular hydrocarbons (CHCs) form thin hydrophobic layers on the cuticular surface of most insects and other arthropods, and thus play an important role in maintaining water balance and preventing lethal desiccation (Howard and Blomquist, 2005; Blomquist and Bagnères, 2010). However, in many insects and arthropods, CHCs also function as semiochemicals that contain information that is transferred among individuals of the same or different species (Blomquist and Bagnères, 2010). The CHCs of social insects, in particular, have received a great deal of attention because of their role in modulating various types of communication associated with colony membership, hierarchical dominance, fertility status, and task group membership (Blomquist and Bagnères, 2010; Liebig, 2010). The nestmate recognition of some social insects is believed to involve the matching of a "label" (i.e., the chemical profile containing the nestmate cues) with a "template" (i.e., the neural representation of the colony odor stored in the memory) (Vander Meer and Morel, 1998; van Zweden and d'Ettorre, 2010). In such studies, elucidating the CHC profile on the insect cuticular surface has been a crucial step to understand characteristics of the chemical labels that may play important roles in nestmate recognition.

Several different techniques have been developed for the extraction and analysis of insect CHCs, particularly those based on gas chromatography (GC). The most common method of extraction of CHCs involves soaking or rinsing recently killed insects in nonpolar organic solvents, such as

pentane or hexane. The hydrocarbons dissolved in the solvent can be readily separated from polar compounds by silica gel column chromatography (Blomquist, 2010). This method, herein referred to as "solvent-soaking", allows the recovery of relatively large amounts of CHCs from individual insects, which is often necessary for subsequent chemical analyses (e.g., mass spectrometry, chemical derivatization) or bioassays. Several solvent-free extraction methods have been developed for GC analysis. For example, the so-called solid phase injection techniques utilize a pyrolysis unit or sealed glass capillary tubes to insert pieces of insect cuticle directly into the GC injector port, in which the CHCs are thermally vaporized from the insect cuticle (Brill and Bertsch, 1985; Bagnères and Morgan, 1990; Morgan, 1990). Others have adopted solvent-free methods in which the insect cuticular surface is rubbed against adsorptive materials like solid-phase microextraction (SPME) fibers that can be directly inserted into the GC injector for desorption (Moneti et al., 1997; Tentschert et al., 2002). Turillazzi et al. (1998) rubbed the cuticular surface of live insects with a clean piece of cotton wool, which was then washed using organic solvents to recover the surface CHCs. Roux et al. (2009) proposed a water-based technique, in which CHCs were extracted from live insects by placing them into glass vials half-filled with warm water (≈ 34°C) that were vigorously shaken to form an emulsion. After removing the insects, the CHCs were recovered from the emulsion by extracting it with hexane.

Due to their ease of use and efficacy, solvent-soaking and SPME fiber extraction methods are commonly used to study insect CHCs. However, both methods have advantages and disadvantages. For example, solvent-soaking methods may extract compounds that are typically not accessible to the olfactory or gustatory organs of other insects, including internal body lipids and exocrine gland secretions, which may thus "contaminate" the CHC extracts (Monnin et al., 1998; Vander Meer and Morel, 1998; Lacey et al., 2008; Ginzel, 2010). On the other hand, SPME is likely to extract only those chemicals that are potentially accessible to the olfactory and gustatory organs of other insects (Ginzel et al., 2003, 2006). In addition, extraction with SPME fibers is less invasive than solvent-soaking, and insects are likely to survive the extraction procedure, thus allowing sequential examination of chemical profiles across developmental stages (Monnin et al., 1998). Nonetheless, SPME fiber extraction methods have their own limitations. First, SPME fibers extract additional non-CHC compounds along with other CHCs that may co-elute with the CHCs of interest during GC analysis (Tentschert et al., 2002). Second, SPME fibers are expensive and several fibers are required to collect multiple samples simultaneously. Third, SPME fibers require the use of mechanical pressure against the insect's cuticle, thus their use on small and/or fragile insects may not be possible (Turillazzi et al., 1998; Roux et al., 2009). Fourth, samples collected using SPME fibers cannot be stored for extended periods of time, and thus a GC instrument must be readily available (Turillazzi et al., 1998; Roux et al., 2009). Fifth, the compounds extracted via SPME fibers will only include those that are present in the specific area that was rubbed (e.g., thorax, abdomen, or legs) (Liebig et al., 2000; Lenoir et al., 2009). Because some insects have quantitatively different CHC profiles distributed throughout the body (Bagnères and Morgan, 1990; Bonavita-Cougourdan et al., 1993; Lenoir et al., 2009), SPME-based extraction may not include a representative sampling of the overall CHC profiles of the whole body surface. Finally, SPME techniques yield minute quantities of material, typically enough for analysis by GC, CG-MS, and possibly GC-FTIR, but the quantity of extracted compounds is rarely sufficient for behavioral assays (Millar and Sims, 1998).

Here, we describe a novel extraction technique for the analysis of insect CHCs. Silica gel possesses excellent adsorptive properties, which are ideal for extracting a wide range of compound classes. Fine-granule particles of silica gel (a granular, vitreous, highly porous form of silicon dioxide) and diatomaceous earth (composed of >90% silicon dioxide) have been used as insecticidal agents for their capacity of removing lipid layers from insect cuticles primarily by adsorption mechanisms (Ebeling, 1961, 1971; Cook et al., 2008). If insects contact these adsorptive dust particles, they lose a significant portion of their lipid layer from the cuticular surface, and thus become susceptible to rapid desiccation. Because silica dust particles are chemically stable and clean, it is possible to use organic solvents to recover these adsorbed lipid compounds for subsequent analyses. For example, Cook et al. (2008) successfully recovered cuticular compounds of mites (hydrocarbons and fatty acids) for GC-MS analysis by extracting diatomaceous earth that had been exposed to the mites for several hours. Chen (2007) reported that CHCs and other venom alkaloids were isolated from silica gel powder that was used as the nesting material by the red imported fire ant, *Solenopsis invicta* Buren. In the present study, we test whether CHCs can be extracted from insects using a novel extraction method: "silica-rubbing". The CHCs are first adsorbed onto the silica gel particles, and are then selectively eluted with a nonpolar solvent for subsequent GC-MS analysis. We validate the silica-rubbing method by comparing it with other two common extraction methods: solvent extraction and SPME.

Methods and Materials

Insects Chemical extractions were conducted using four species of ants and one species of bee. For the ant species, we used workers of the Argentine ant [*Linepithema humile* (Mayr) (Dolichoderinae)], *Azteca instabilis* (Smith) (Dolichoderinae),

the Florida carpenter ant (*Camponotus floridanus* (Buckley)) (Formicinae), and the red harvester ant [*Pogonomyrmex barbatus* (Smith) (Myrmicinae) (Hymenoptera: Formicidae)]. We used males of the solitary / semi-social orchid bee, *Euglossa dilemma* Eltz & Bembé (Hymenoptera: Apidae). The workers of *L. humile* were obtained from an outdoor foraging trail at Berkeley, CA in October 2010. Workers of *A. instabilis* were collected at the Finca Irlanda, Chiapas, Mexico in July 2010 (K. A. Mathis, University of California, Berkeley). The workers of *C. floridanus* were obtained from a laboratory colony collected at the Archbold Biological Station, FL in August 2009. The workers of *P. barbatus* were obtained from a laboratory colony collected in the field near Rodeo, NM in 2006 (S. Sturgis, Stanford University, Palo Alto). The colonies of *L. humile, A. instabilis,* and *C. floridanus* were fed with 25% (wt/vol) sucrose water, protein solution, and scrambled eggs three times a week. The colony of *P. barbatus* was provided with water, seeds (Wild Bird Food, Priority Total Pet Care, Pleasanton), apples, and crickets *ad libitum*. All ant colonies were maintained at room temperature. Males of the orchid bee *E. dilemma* (Eltz et al., 2011) were collected at chemical baits in Ft. Lauderdale, FL in February 2011, and kept in a temperature and humidity controlled insectary room at the University of California, Berkeley (see Ramírez et al., 2010 for details).

Extraction by Silica-Rubbing Live insects were anesthetized with CO_2 and subsequently killed by freezing in dry ice for 1 min. We placed the freeze-killed specimen under a fume hood for 5–10 min to remove any moisture that may have condensed on the surface of specimens while thawing. Surface lipids were extracted by placing the thawed insects in a 2-ml glass vial or small test tubes (10×75 or 13×100 mm) with 0.1~0.15 g of silica gel (70–230 mesh, Fisher Scientific), and subsequently vortexing it for 30 s. The silica gel in the vial or test tube was previously washed with 300–500 μl of hexane and dried under constant N_2 flow until individual gel particles moved freely without clumping. We extracted from single individuals, except for *L. humile*, for which we used a total of 30 ants per extraction. After vortexing, the insects were carefully removed with clean forceps, and the silica gel was extracted with hexane. The dried (with Na_2SO_4) silica extracts were subjected to flash liquid chromatography (0.4-cm diam×1.5-cm long column packed with 70–230 mesh silica gel) by elution with hexane. The volume of hexane used for the extraction and elution varied among species depending on the amount of silica gel used for initial extraction (Table 1). Both *A. instabilis* and *C. floridanus* have polymorphic workers, and thus only major workers were used for extraction.

Extraction by Hexane-Soaking Live insects were anesthetized using CO_2 and subsequently killed by freezing on dry ice for 1 min. Cuticular lipids were extracted by soaking the thawed insects in hexane for 10 min. A single insect was used in each replicated extraction, except for *L. humile*, for which we used a total of 30 ants per extraction in order to obtain enough CHCs for subsequent GC-MS analysis. The dried extracts were subjected to flash liquid chromatography by elution with hexane. The volume of hexane used for the extraction and elution varied among species, depending on the amount of hexane required to completely submerge the insects (Table 1).

Solid-Phase Microextraction (SPME) Using the same individuals of male *E. dilemma,* we compared the chemical profiles obtained via SPME with those obtained via silica-rubbing and hexane-soaking techniques. *Euglossa dilemma* was chosen because of its relatively large body size. A single SPME fiber (Supelco Inc.) coated with a 65-μm polydimethylsiloxane / divinylbenzene (PDMS / DVB) stationary phase was rubbed on the cuticle of an anaesthetized (CO_2) bee (three times on both the thoracic and the gastric tergites). The fiber was immediately injected into a GC-MS. We first extracted each individual bee using SPME, and subsequently applied the silica-rubbing and solvent-soaking techniques, in that order.

Efficacy of Silica-Rubbing To determine the efficacy of silica-rubbing in removing the cuticular lipid layer, we compared the rates of water loss of extracted and non-extracted workers of *L. humile* and *C. floridanus.* The rate of water loss was estimated by measuring weight loss over time under ambient conditions [20–28°C, 22–34% relative humidity (RH)]. A group of insects killed by freezing were subjected to silica-rubbing. Another group of insects was killed by freezing but not subjected to extraction, which thus served as control. To determine rates of water loss, the weights of extracted and control insects were measured immediately after extraction and 5 h (for *L. humile*) or 24 h (for *C. floridanus*) after extraction. An individual worker (*C. floridanus*) and a group of 30 workers (*L. humile*) were used to obtain each replicate measurement.

In *E. dilemma*, the efficacy of silica-rubbing was determined by comparing the total amount of CHCs extracted (via hexane-soaking) from insects that had been previously extracted by silica-rubbing to the amount of CHCs recovered by hexane-soaking without prior silica-rubbing. The CHC extracts were prepared using the method previously described in hexane-soaking. Because volumes of the extracts examined via GC-MS (1 μl) were identical throughout the GC-MS analysis, we directly compared the total integrated peak areas of the selected CHCs between the insects previously extracted via silica-rubbing and non-extracted controls.

Table 1 Amounts of hexane used for initial extraction and elution of hydrocarbons during flash liquid chromatography

Method		*L. humile*	*A. instabilis*	*C. floridanus*	*P. barbatus*	*E. dilemma*
Silica-rubbing	Extraction	200 µl	200 µl	300 µl	200 µl	1 ml
	Elution	200 µl	200 µl	100 µl	200 µl	-
Hexane-soaking	Extraction	200 µl	200 µl	200 µl	300 µl	1 ml
	Elution	200 µl	200 µl	300 µl	200 µl	1 ml

Chemical Analyses Prior to conducting GC-MS analyses, all CHC extracts were examined viathin-layer chromatography (TLC) for purity and concentration. TLC plates were developed with 100% hexane, and spots were visualized by spraying 5% (wt/vol) phosphomolybdic acid in ethanol, followed by heating with a heat gun. The concentration of CHC extracts was estimated based on the intensity of the dark spots. When needed, samples obtained via either silica-rubbing or hexane-soaking were concentrated under constant N_2 flow or diluted by adding clean hexane to make within-species concentrations similar. To minimize the loss of target compounds through volatilization, most samples were analyzed via GC-MS immediately after preparation, but some were stored in sealed vials at −20°C for<24 h prior to the analysis. For GC-MS, electron impact mass spectra (70 eV) were acquired with an Agilent 5975 C mass selective detector interfaced to a Agilent 7890A gas chromatograph fitted with an DB-5 column (30-m×0.32-mm i.d., Agilent Technologies). Extracts were analyzed in a splitless mode, with a temperature program that started at 100°C for 1 min which then increased by 15°C min^{-1} until it reached 300°C. Injector and transfer line temperatures were kept at 300°C (250°C for SPME) and 280°C, respectively. Individual hydrocarbon peaks were identified by comparing retention times and mass spectra with those of synthetic standards, matching with previously published spectra, and studying fragmentation patterns.

Statistical Analyses Automatic peak integration of chromatograms was conducted using the software Chemstation vE.02.00 (Agilent Technologies). We selected major peaks that were consistently present across samples on each species. Minor peaks with inconsistent integration results were excluded from the analyses. For each individual, CHC profiles were quantified by dividing the peak area of each compound by the total area of peaks selected for the analysis. The relative proportions of individual peaks were compared across extraction methods using Wilcoxon rank-sum test or Kruskal-Wallis ANOVA. To compare CHC profiles within and between different extraction methods, the relative areas of the selected CHC peaks were subjected to the non-metric Multidimensional Scaling (nMDS), an ordination technique where a predetermined number of axes of variation are chosen, and non-metric distances are fitted to those dimensions. We calculated a triangular distance matrix between samples (individuals) using the Bray-Curtis index of dissimilarity. We computed 2-dimensional MDS plots (50 iterations per run) using the software package *ECODIST v1.2.2* (written in R). We ran each analysis 10 times, and visually checked for convergence between solutions. To statistically assess whether CHC profiles exhibit greater dissimilarity between methods of extraction than within methods of extraction, we conducted Analysis of Similarity (ANOSIM) tests, as implemented in the software package *VEGAN v1.15-4* (written in R). We also estimated the relative contribution of individual compounds to the observed ordinal dissimilarities using the the Similarity Percentage (SIMPER) method, as implemented in the software package *PRIMER v6* (Clarke and Gorley, 2006). All other calculations, plots, and statistical tests were performed using basic R packages (http://cran.r-project.org).

Results

Linepithema humile Representative chromatograms of CHCs obtained with silica-rubbing and hexane-soaking are shown in Fig. 1a, with compounds numbered in order of elution (retention time). Thirteen hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C27 to C37 were selected for comparisons between extraction methods (Table 2). The total areas of thirteen CHC peaks obtained by silica-rubbing and hexane-soaking were $(13.2\pm3.4)\times10^7$ and $(8.5\pm1.0)\times10^7$, respectively (mean±SEM, N=9 for silica-rubbing and N=10 for hexane-soaking).

The two different methods of extraction produced qualitatively similar GC-MS profiles for *L. humile*. However, quantitative differences were observed in some hydrocarbons (Fig. 1b and c; Table 2). The most conspicuous differences between extraction methods were the significantly greater proportions of three saturated alkanes (n-C_{27}, n-C_{29}, and n-C_{32}) in silica-rubbing extracts, and greater proportions of three methyl-branched alkanes (dimethyl-C_{35}, trimethyl-C_{35}, and trimethyl-C_{37}) in hexane-soaking extracts (Fig. 1c; Table 2).

The nMDS analysis showed a strong central tendency separation by extraction method (Fig. 1d). This pattern was further supported by the ANOSIM results, where a greater dissimilarity among CHC profiles was found between extraction methods than within them (R=0.419, P=0.001).

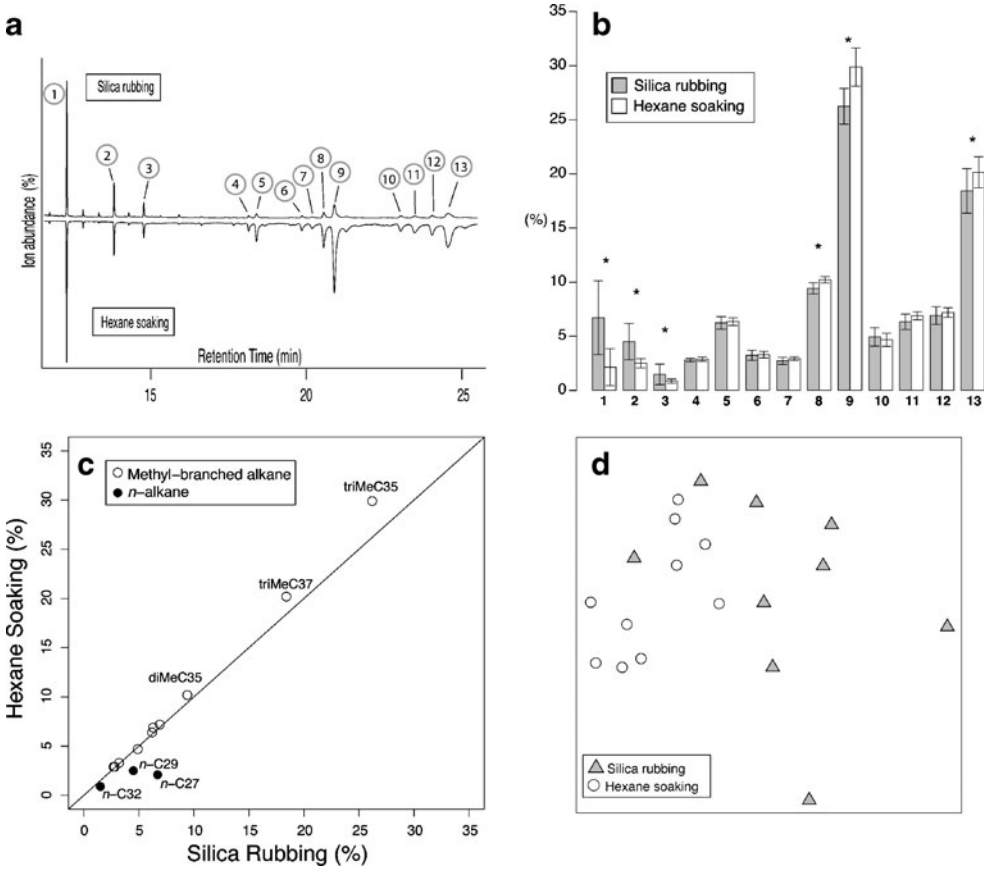

Fig. 1 Analyses of cuticular hydrocarbons (CHCs) from workers of *Linepithema humile*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

The SIMPER analysis revealed that three peaks (peaks 1, 9, and 13) jointly contributed to >60% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Table 2 Cuticular hydrocarbons of *Linepithema humile* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	*n*-C_{27}	6.7±3.4*	2.1±1.7
2	*n*-C_{29}	4.5±1.7*	2.5±0.4
3	*n*-C_{32}	1.5±1.0*	0.9±0.2
4	5,15- and 5,17-diMeC_{33}	2.8±0.2	2.9±0.2
5	5,15,19-triMeC_{33}	6.2±0.6	6.4±0.4
6	MeC_{35}	3.2±0.5	3.3±0.3
7	15,19-diMeC_{35}	2.7±0.3	2.9±0.2
8	5,15- and 5,17-diMeC_{35}	9.4±0.5	10.2±0.3*
9	5,13,17- and 5,15, 19-triMeC_{35}	26.2±1.7	29.9±1.8*
10	13- and 15- and 17- and 19-MeC_{37}	4.9±0.8	4.7±0.6
11	15,19-diMeC_{37}	6.3±0.7	6.9±0.4
12	5,15- and 5,17-diMeC_{37}	6.9±0.82	7.2±0.4
13	5,15,19- and 5,13, 17-triMeC_{37}	18.4±2.1	20.2±1.4*

[a] Value with asterisk is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05)

Azteca instabilis Representative chromatograms of CHCs obtained via silica-rubbing and hexane-soaking are shown in Fig. 2a. Thirteen hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C23 to C29 were selected to compare the different extraction methods (Table 3). The total areas of thirteen CHC peaks obtained by silica-rubbing and hexane-soaking were $(13.1\pm1.6)\times10^8$ and $(8.0\pm0.6)\times10^8$, respectively (mean±SEM, N=10 for each treatment).

The two different methods of extraction produced qualitatively similar GC-MS profiles for *A. instabilis*. However, we found substantial quantitative differences for most hydrocarbons (Fig. 2b and c; Table 3). The most conspicuous differences between extraction methods were the significantly greater proportion of most methyl-branched alkanes (monomethyl and dimethyl alkanes) in silica-rubbing extracts, and the greater proportion of three saturated alkanes (*n*-C_{25}, *n*-C_{26}, and *n*-C_{27}) and a single methyl-branched alkane (3-MeC_{27}) in hexane-soaking extracts (Fig. 2c; Table 3).

The nMDS analysis showed a strong clustering by extraction method (Fig. 2d). This pattern was further supported by the ANOSIM analysis, suggesting a greater dissimilarity

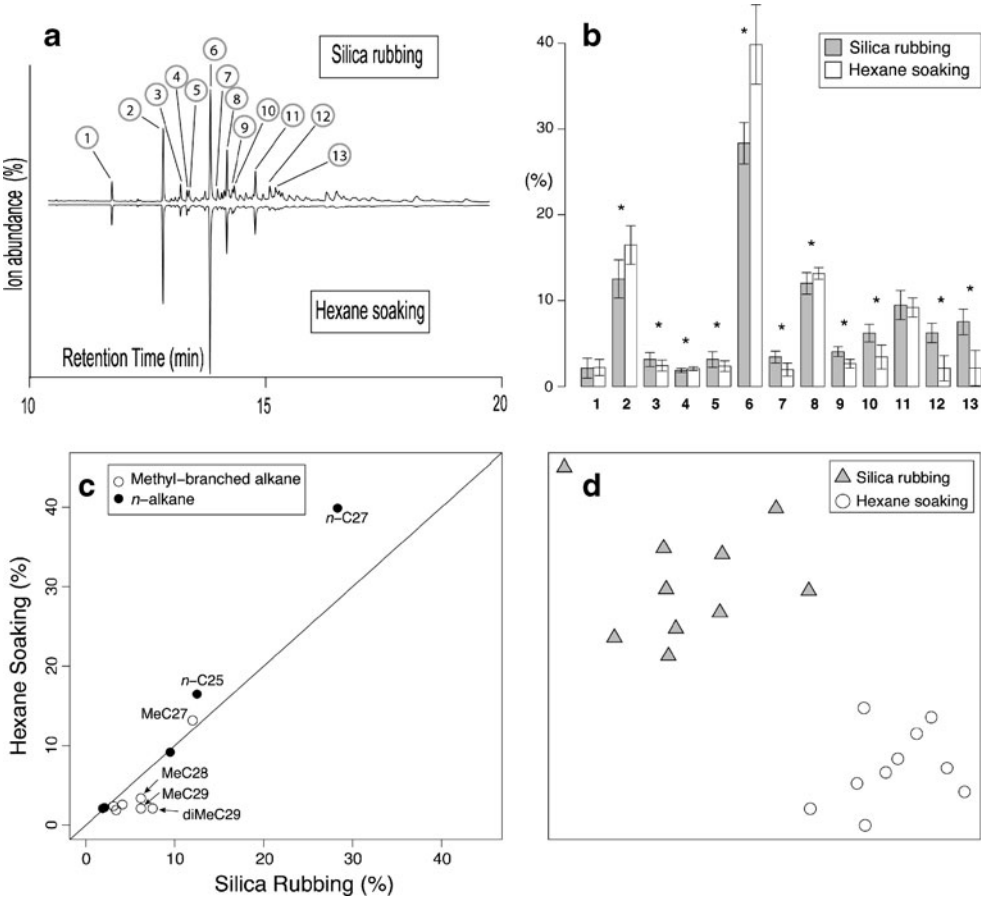

Fig. 2 Analyses of cuticular hydrocarbons (CHCs) from workers of *Azteca instabilis*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

Table 3 Cuticular hydrocarbons of *Azteca instabilis* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	*n*-C_{23}	2.1±1.2	2.2±0.9
2	*n*-C_{25}	12.5±2.2	16.5±2.2*
3	3-MeC_{25}	3.1±0.8*	2.4±0.6
4	*n*-C_{26}	1.9±0.2	2.1±0.2*
5	10- and 12- and 14-MeC_{26}	3.1±0.9*	2.4±0.6
6	*n*-C_{27}	28.3±2.4	39.9±4.6*
7	11- and 13-MeC_{27}	3.4±0.7*	1.9±0.8
8	3-MeC_{27}	12.0±1.3	13.2±0.7*
9	6,16- and 8,15-diMeC_{27}	4.1±0.6*	2.6±0.5
10	10- and 12- and 13- and 14-MeC_{28}	6.2±1.0*	3.4±1.4
11	*n*-C_{29}	9.5±1.7	9.2±1.1
12	MeC_{29}	6.2±1.1*	2.1±1.5
13	7,15- and 7,17-diMeC_{29}	7.5±1.5*	2.1±2.1

[a] Value with asterisk is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05)

of CHC profiles between extraction methods than within them (R=0.979, P=0.001). The SIMPER analysis revealed that three peaks (peaks 6, 12, and 13) jointly contributed to >50% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Pogonomyrmex barbatus Representative chromatograms of CHCs obtained by silica-rubbing and hexane-soaking are shown in Fig. 3a. Ten hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C23 to C31 were selected for the comparison between extraction methods (Table 4). The total areas of ten CHC peaks obtained via silica-rubbing and hexane-soaking were (2.0±0.3)×10^7 and (2.3±0.2)×10^7, respectively (mean±SEM, N=10 for each treatment).

The two different methods of extraction produced qualitatively similar, but quantitatively different GC-MS profiles. Proportional differences were observed in 9 of the 10 hydrocarbons selected for the analysis (Fig. 3b and c; Table 4). Seven peaks of methyl-branched alkanes (monomethyl and dimethyl alkanes) were found in greater proportions in silica-rubbing extracts. On the other hand, two saturated alkanes (*n*-C_{25} and *n*-C_{27}) were found in greater proportions in hexane-soaking extracts (Fig. 3c; Table 4).

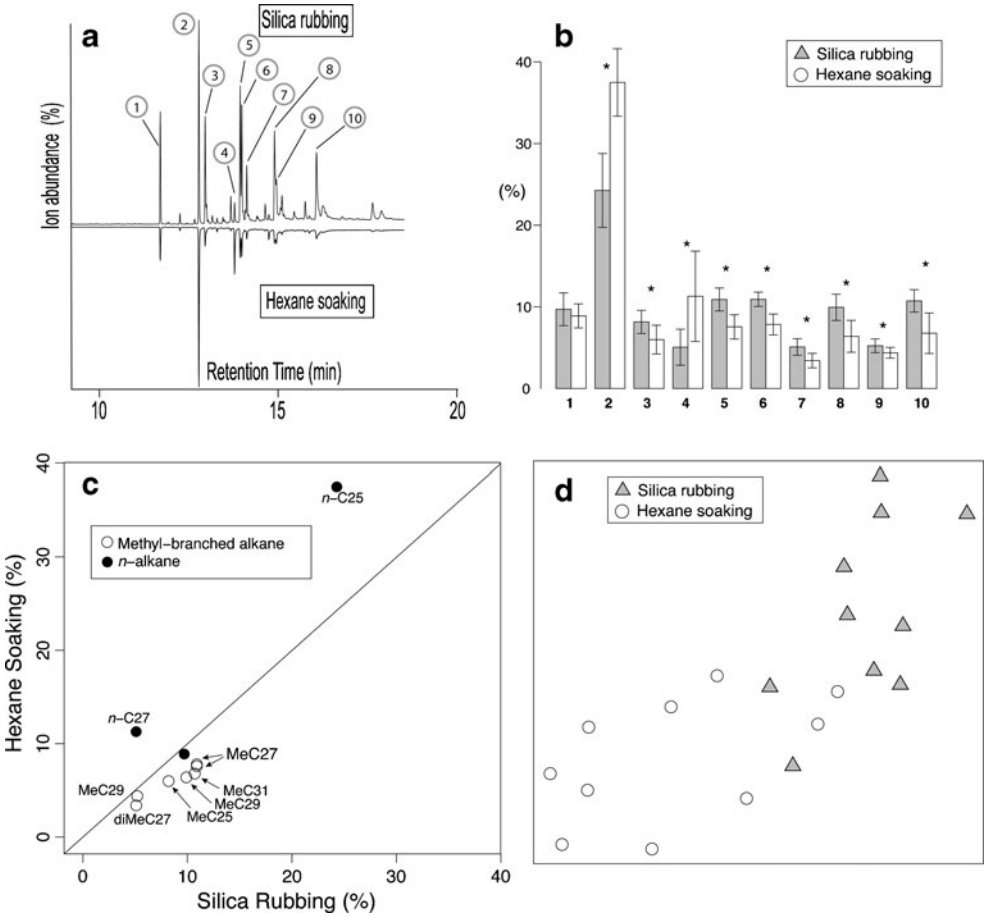

Fig. 3 Analyses of cuticular hydrocarbons (CHCs) from workers of *Pogonomyrmex barbatus*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

The nMDS analysis indicated a strong clustering by extraction method (Fig. 3d). This pattern was further supported by the ANOSIM analysis, where a greater dissimilarity among CHC profiles was found between extraction methods than within them (R=0.7, P=0.001). The SIMPER analysis revealed that a single compound (peak 2: n-C_{25}) contributed to >50% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Table 4 Cuticular hydrocarbons of *Pogonomyrmex barbatus* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	n-C_{23}	9.7±2.0	8.9±1.5
2	n-C_{25}	24.3±4.5	37.5±4.1*
3	13-MeC_{25}	8.2±1.4*	6.0±1.8
4	n-C_{27}	5.1±2.2	11.3±5.5*
5	13-MeC_{27}	10.9±1.4*	7.6±1.5
6	7-MeC_{27}	10.9±0.9*	7.8±1.3
7	7,13-diMeC_{27}	5.1±1.0*	3.4±0.9
8	15-MeC_{29}	9.9±1.6*	6.4±2.0
9	7- and 9-MeC_{29}	5.2±0.8*	4.4±0.7
10	11- and 13- and 15-MeC_{31}	10.7±1.4*	6.8±2.5

[a] Value with underline is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05)

Camponotus floridanus Representative chromatograms of CHCs obtained by silica-rubbing and hexane-soaking are shown in Fig. 4a. Eighteen hydrocarbons (saturated and methyl-branched alkanes) with chain lengths ranging from C30 to C33 were selected for comparison between extraction methods (Table 5). The total areas of eighteen CHC peaks obtained via silica-rubbing and hexane-soaking were $(10.5\pm0.9)\times10^8$ and $(11.0\pm1.3)\times10^8$, respectively (mean± SEM, N=10 for each treatment).

Even if two different extraction methods produced qualitatively similar GC-MS profiles, we found quantitative differences in 5 of 18 hydrocarbons (Fig. 4b and c; Table 5). The most conspicuous differences between extraction methods were the significantly greater proportions of three peaks of methyl-branched alkanes (dimethyl and trimethyl alkanes) in silica-rubbing extracts, and greater proportions of one saturated alkane (n-C_{31}) and three co-eluting dimethylhentriacontanes (5,9- and 5,11- and 5,13-diMeC_{31}) in hexane-soaking extracts (Fig. 4c; Table 5).

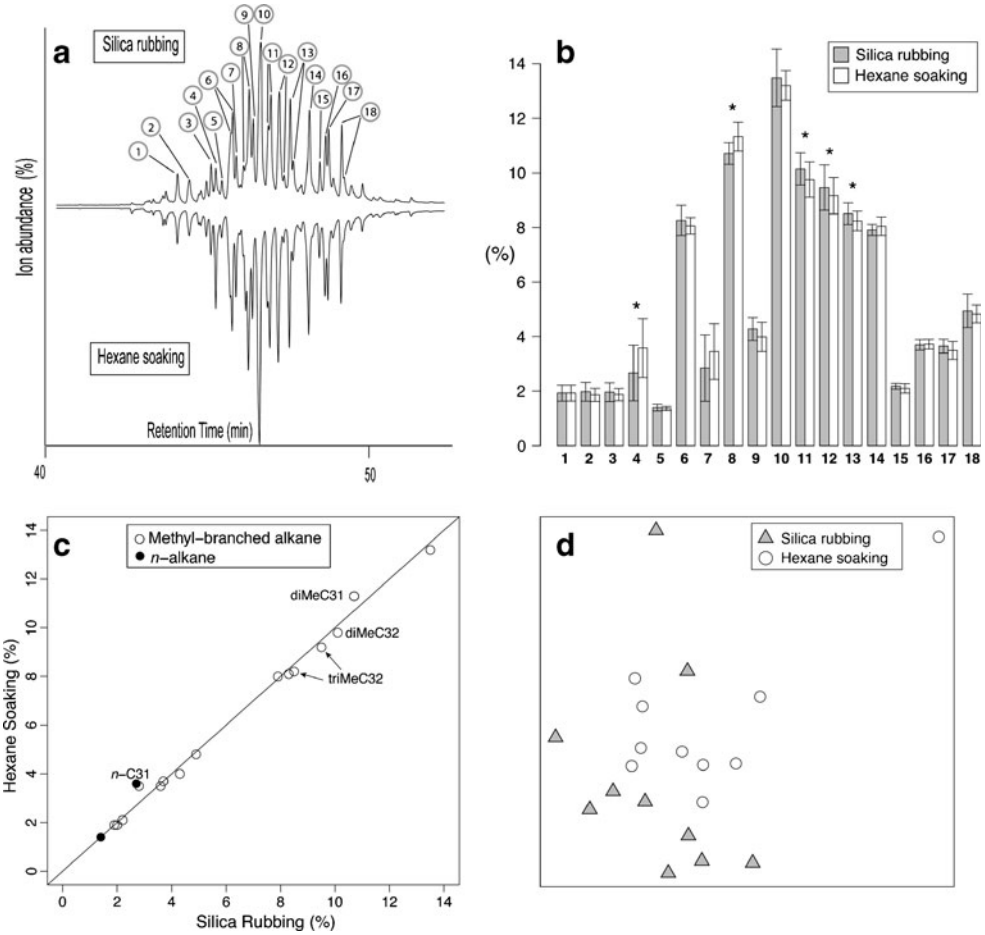

Fig. 4 Analyses of cuticular hydrocarbons (CHCs) from workers of *Camponotus floridanus*. (**a**) Representative total ion chromatograms of CHCs extracted by silica-rubbing (top) and hexane-soaking (bottom). (**b**) Proportional abundance (mean ±SD) of the selected peaks. Bars with asterisk indicate that the proportional areas of the peak were significantly different between two extraction methods (Wilcoxon rank-rum test: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of two different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

The nMDS analysis revealed a moderate clustering by extraction method (Fig. 4d). Likewise, the ANOSIM analysis indicated a marginally greater dissimilarity of CHC profiles between extraction methods than within them (R=0.2, P=0.014). The SIMPER analysis revealed that five peaks (peaks 4, 7, 8, 10, and 12) jointly contributed to >50% of the observed chemical dissimilarity between silica-rubbing and hexane-soaking extracts.

Euglossa dilemma Representative chromatograms of CHCs obtained by SPME, silica-rubbing, and hexane-soaking are shown in Fig. 5a. Five hydrocarbons (three *n*-alkanes and two alkenes) with chain lengths ranging from C23 to C27 were selected to compare extraction methods (Table 6). The total areas of five CHC peaks obtained via SPME, silica-rubbing, and hexane-soaking were $(12.0\pm1.7)\times10^7$, $(8.9\pm0.6)\times10^7$, and $(6.2\pm0.4)\times10^7$, respectively (mean±SEM, N=9 for SPME and N=10 for the rest).

Three different methods of extraction (i.e., SPME, silica-rubbing, and hexane-soaking) produced qualitatively similar but quantitatively different GC-MS profiles for three of five hydrocarbons (Fig. 5b and c; Table 6). Two alkene compounds (C_{25}:1 and C_{27}:1) were present in greater proportions in hexane-soaking extracts than either in SPME or silica-rubbing extracts (Fig. 5b and c; Table 6). However, one saturated alkane (n-C_{27}) was found in smaller proportions in the hexane-soaking extracts compared to either SPME or silica-rubbing extracts (Fig. 5b and c; Table 6).

The nMDS analysis showed a strong clustering by extraction method (Fig. 5d). This pattern was supported by the ANOSIM analysis, which showed a greater dissimilarity of CHC profiles between methods than within them (R=0.4, P=0.001). The SIMPER analysis revealed that two peaks (peaks 2 and 4 for SPME vs. silica-rubbing; peaks 3 and 4 for SPME vs. hexane-soaking and silica-rubbing vs. hexane-soaking) jointly contributed >50% of the observed chemical dissimilarity between two different extraction methods.

Efficacy of Silica-Rubbing The insects extracted using the silica-rubbing method lost more water via evaporation than did the non-extracted control specimens. During the first 5 h after the extraction was conducted, the *L. humile* workers lost 62.7±0.4% (mean±SEM, N=5) of their body weight, whereas the control ants lost 20.8±0.5% (N=3) (two-sample t-test: t=65.5, df=6, P<0.001). The initial weights of the extracted and control insects were 11.3±0.1 and 12.8±0.2 mg, respectively (mean±SEM). In the case of *C. floridanus*, the extracted worker ants lost 51.3±1.9% of their body weight compared to

Table 5 Cuticular hydrocarbons of *Camponotus floridanus* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]	
		Silica-rubbing	Hexane-soaking
1	10- and 12- and 14-MeC_{30}	1.9±0.3	1.9±0.3
2	8,16- and 10,16-diMeC_{30}	2.0±0.3	1.9±0.2
3	Methyl-branched C_{30}	2.0±0.8	1.9±0.2
4	*n*-C_{31}	2.7±1.0	3.6±1.1*
5	Methyl-branched C_{31}	1.4±0.1	1.4±0.1
6	7- and 9- and 11- and 13-MeC_{31}	8.3±0.6	8.1±0.3
7	5-MeC_{31}	2.8±1.2	3.5±1.0
8	5,9- and 5,11- and 5, 13-diMeC_{31}	10.7±0.4	11.3±0.5*
9	7, 11, 15-triMeC_{31}	4.3±0.4	4.0±0.5
10	10- and 12-MeC_{32}	13.5±1.1	13.2±0.5
11	8,12- and 10,14-diMeC_{32}	10.1±0.6*	9.8±0.6
12	5,9,13-triMeC_{32}	9.5±0.8*	9.2±0.7
13	8,12,16-triMeC_{32}	8.5±0.4*	8.2 ±0.4
14	4,8,12,16-tetraMeC_{32}	7.9±0.2	8.0±0.3
15	7,11-diMeC_{33}	2.2±0.1	2.1±0.2
16	5,9- and 5,11- and 5, 13-diMeC_{33}	3.7±0.2	3.7±0.2
17	7,11,15-triMeC_{33}	3.6±0.3	3.5±0.3
18	5,9,13,17-tetraMeC_{33}	4.9±0.6	4.8±0.3

[a] Value with asterisk is significantly larger than a corresponding value in the other extraction method (Wilcoxon rank-sum test: α=0.05).

5.3±1.2% in the control ants (mean±SEM, N=10 for each treatment) during the first 24 h after the extraction (two-sample t-test: t=20.8, df=18, P<0.001). The initial weights of the extracted and control insects were 26.6±1.7 and 27.7±1.5 mg, respectively (mean±SEM).

Study with *E. dilemma* also indicated that silica-rubbing technique was effective in removing significant amount of CHCs from surface of the insects (i.e., > 50% of total amount extractable with solvent). The total peak area of five CHCs obtained by soaking the insects previously extracted via silica-rubbing [$(6.2\pm0.4)\times10^7$] was≈60% smaller than the total peak area of the CHCs obtained from control insects without previous extraction with silica-rubbing [$(15.6\pm1.4)\times10^7$] (mean±SEM, N=10 for each treatment) (Wilcoxon rank-sum test: z=3.7, P<0.001).

Discussion

Sampling of cuticular hydrocarbons (CHCs) by extracting dead insects with solvent has been an established method in chemical ecology of insects (Howard and Blomquist, 2005). However, such method has the disadvantage of extracting additional compounds from internal glands or deep wax layers that may not be readily available to olfactory or gustatory organs of other individual insects. The relatively recent introduction of SPME fibers alleviated this problem (Moneti et al., 1997), although it remains expensive and difficult to scale up for multiple individual extractions. Our study demonstrates that insect CHCs can be extracted quickly and easily using fine silica gel particles. Using a vortex mixer, cuticular surfaces of freeze-killed insects are rubbed with a small amount of granular silica gel. The adsorbed compounds are subsequently eluted with nonpolar organic solvents for further chemical analyses. Our results suggest that silica-rubbing is powerful enough to remove a significant portion of the lipid layer of insects.

The CHC profiles obtained via silica-rubbing and hexane-soaking were qualitatively similar, with all CHCs being present in extracts from both methods. However, the chemical profiles were quantitatively different. In particular, we found marked differences in the relative proportions of saturated alkanes, methyl-branched alkanes, and alkenes. For example, in the Argentine ant *L. humile* and the orchid bee *E. dilemma*, all or some of the saturated alkanes examined exhibited higher proportions in silica-rubbing samples than hexane-soaking samples. On the other hand, several methyl-branched alkanes (in *L. humile*) or alkenes (in *E. dilemma*) were relatively more abundant in hexane-soaking samples. In the other three ant species (*A. instabilis, P. barbatus,* and *C. floridanus*), the proportion of the most abundant saturated alkanes (i.e., *n*-C_{27}, *n*-C_{25}, and *n*-C_{31} for *A. instabilis*, *P. barbatus*, and *C. floridanus*, respectively) was consistently higher in hexane-soaking samples. On the other hand, several methyl-branched alkanes exhibited relatively higher concentrations in silica-rubbing samples.

Similar quantitative discrepancies between solvent extraction and SPME techniques have been previously reported in other insect species. For example, SPME samples of a cerambycid beetles cuticle contained greater proportions of methyl-branched alkanes and smaller proportions of saturated alkanes than the whole-body hexane extracts (Ginzel et al., 2003; Lacey et al., 2008). A female contact sex pheromone of a cerambycid beetle *Megacyllene robiniae*, (Z)-9-pentacosene, was present in hexane extracts of male insects, but represented in negligible proportions when the male insect cuticle was extracted using SPME fiber (Ginzel et al., 2003; Ginzel, 2010). Furthermore, in a queen of *Vespa* sp. wasp, the proportion of the most abundant saturated alkane was higher in hexane-soaking samples, whereas the proportion of the most abundant methyl-branched alkane was higher in SPME samples (Moneti et al., 1997). Monnin et al. (1998) noted that SPME sampling of ponerine ants underestimated the presence of shorter-chain hydrocarbons while overestimating the concentration of longer-chain hydrocarbons when it compared to pentane extracts of cuticle.

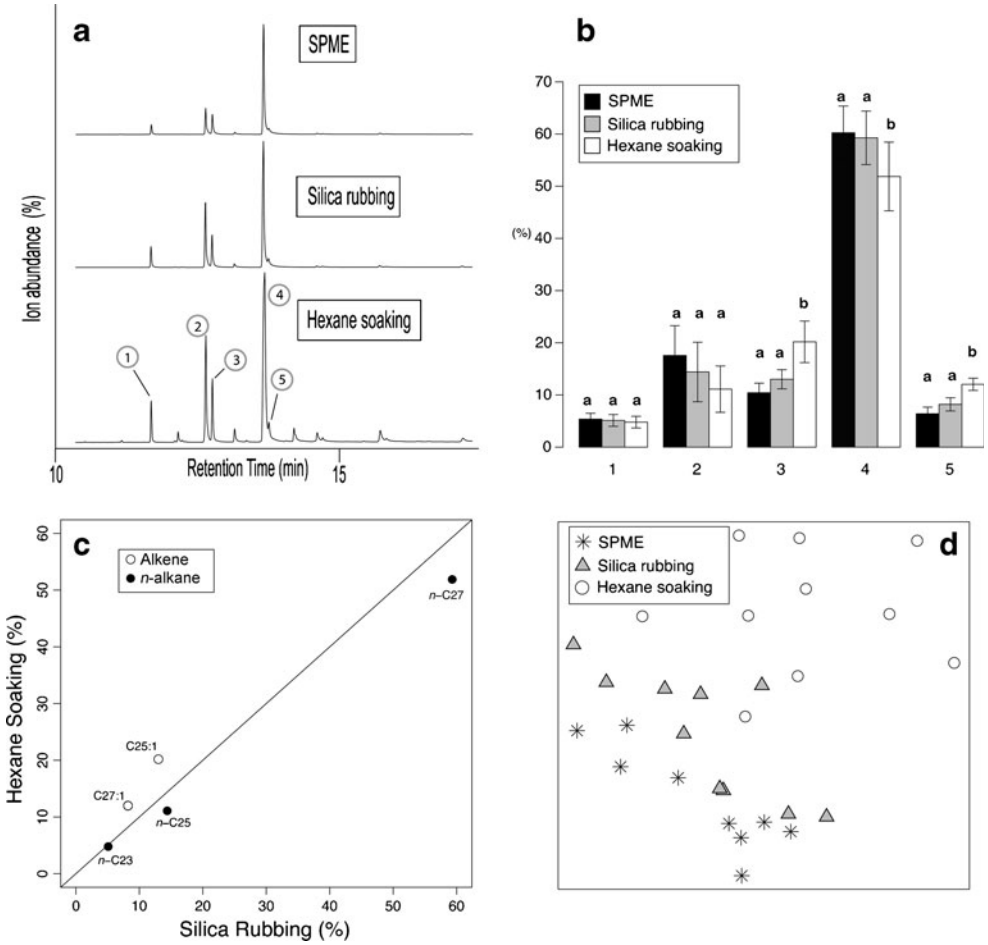

Fig. 5 Analyses of cuticular hydrocarbons (CHCs) from males of *Euglossa dilemma*. (**a**) Representative total ion chromatograms of CHCs extracted by SPME (top), silica-rubbing (middle), and hexane-soaking (bottom). (**b**) Proportional abundance (mean ±SD) of the selected peaks. Bars with the same letter within a peak are not significantly different (Kruskal-Wallis ANOVA followed by all-pairwise comparisons of mean ranks: α=0.05). (**c**) Scatter plot of proportional abundances (mean) of the selected peaks in silica-rubbing versus hexane-soaking. The sold line is the 45° perfect fit reference. (**d**) Separation of three different extraction methods based on non-metric Multidimensional Scaling (nMDS) of relative proportions of the selected peaks

Our study showed that the CHC profiles obtained via silica-rubbing and SPME fibers were more similar to each other than either one was to the profiles obtained via hexane-soaking (in *E. dilemma*). Thus, it is likely that the quantitative discrepancies between whole-body solvent extraction and surface wiping techniques result from the extraction of non-cuticular hydrocarbons (particularly the contents of internal glands) during whole-body solvent extraction. High selectivity is known to be involved in the synthesis, transportation, and deposition of insect hydrocarbons (Blomquist and Bagnères, 2010), resulting in different CHC profiles in different parts of body (e.g., cuticular body surface, glands, and alimentary tract). For example, Bagnères and Morgan (1991) reported substantial proportional differences between CHC profiles of cuticle and postpharyngeal gland of queen and worker *Myrmica rubra* L., where alkanes were relatively more abundant in the cuticle, and methyl-branched alkanes were more abundant in the gland. In some ants, colony-specific hydrocarbon mixtures are stored in the postpharyngeal gland, and lipids may be distributed from there throughout the body surface by grooming (Hefetz et al., 2001; Bagnères and Blomquist, 2010). Extraction of such glandular contents is likely to occur to a greater degree during solvent extraction than during silica-rubbing or SPME. Therefore, the silica-rubbing technique may be more appropriate than whole-body solvent extraction in studies that investigate the chemical ecology of cuticular substances.

Our study demonstrates that the silica-rubbing method can be effectively used as an alternative to SPME for sampling insect CHCs. Even though both methods target surface-

Table 6 Cuticular hydrocarbons of *Euglossa dilemma* and their relative proportions

Peak No.	Components	Composition (%, mean±SD)[a]		
		SPME[b]	Silica-rubbing[b]	Hexane-soaking[b]
1	*n*-C_{23}	5.4±0.8a	5.1±1.1a	4.8±1.1a
2	*n*-C_{25}	17.6±5.2a	14.4±5.7a	11.1±4.4a
3	C_{25}:1	10.4±1.2a	13.0±1.8a	20.2±4.0b
4	*n*-C_{27}	60.2±5.1a	59.3±5.1a	51.9±6.6b
5	C_{27}:1	6.4±0.7a	8.2±1.3a	12.0±1.2b

[a] Values followed by the same letter within each row are not significantly different (Kruskal-Wallis ANOVA followed by all-pairwise comparisons of mean ranks: α=0.05)

[b] Data were obtained from same individuals, which were extracted first by SPME and subsequently by silica-rubbing and hexane-soaking

specific compounds, silica-rubbing is advantageous because it allows extracting compounds from the entire body surface. Also, unlike SPME fibers, our silica gel method does not require the use of several extraction devices for simultaneously extracting multiple samples, and thus the total expenses are significantly reduced. Furthermore, extractions using silica-rubbing may permit the recovery of significant quantities of CHC that could be used in subsequent bioassays. Lastly, because only small quantities of non-toxic silica-gel are required, this technique is particularly suited for field studies, thus eliminating the need to transport highly flammable solvents. The silica-rubbing technique may yield a more representative CHC profile that is actually encountered by other insects' sensory organs, and thus it may facilitate the discovery and identification of biologically important components which are potentially meaningful in chemical communication. We suggest that silica-rubbing technique might be employed as a routine method for insect CHC analyses.

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