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TECHNICAL NOTES

Non-lethal SPME method for insect cuticular analysis by GC-MS

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In the present study a new method involving extraction by SPME fibers and storage in an organic solvent described and tested in an ant species, *Dinoponera quadriceps*. The results demonstrate that the cuticular hydrocarbons trapped by SPME fibers can be efficiently desorbed from fiber to hexane and stored for later analysis. This method can be used as an alternative procedure for the collection of samples in field studies. This technique is an effective non-lethal method for the extraction of cuticular hydrocarbons and was developed specifically for the long-term monitoring of individuals, as well as for situations in which gas chromatography equipment is unavailable at the sampling site.

1. Introduction

In insects, the hydrocarbons on body surface are relatively non-volatile compounds. They play important biological roles as protection of the insect cuticle and chemical communication.[1-3] The cuticular hydrocarbons (CHCs) of many species of solitary insects are species-specific, allowing conspecific individuals to recognize one another after cuticular contact.[4] In a number of species of solitary insects, CHCs are involved in sexual communication, acting as attractants in female houseflies *Musca domestica*[5] and burrowing bees *Amegilla dawsoni*.[6] In social insects, these compounds are important semiochemicals that may indicate caste, gender, age, and reproductive status in stingless bees, honeybees and ants.[7- 12] They also represent important clues for the recognition of nestmates in hymenopterans (in honeybees,wasps and stingless bees[13-16]) and as guides for foragers returning to their nests (ants[17]). A number of non-lethal techniques have been developed in recent years for the collection of cuticular compounds, which provide an important tool for the study of different aspects of insect behavior, such as nestmate recognition, sexual attraction, and fertility signals.

A number of different techniques are available for the extraction of insect cuticular hydrocarbons. Conventional procedures require toxic solvents, such as methanol or hexane, impeding the analysis of live specimens. These techniques impose a number of restrictions on the analysis of phenomena that require the monitoring of individuals over the course of different life stages (e.g. attractiveness of mated and non-mated females) or the study of the reproductive physiology of queens in monogynic colonies.[18-21] A number of other non-destructive techniques have been proposed for living organisms or the successive sampling of the same individual over time. Morgan[18] described a method in which pieces of the insect, such as glands or wings, are placed in micro-capillaries and then injected into a chromatograph system. This technique has been used in several studies of CHCs.[22-23]

Solid phase micro-extraction (SPME) was first described by Berlardi and Pawliszyn and Arthur and Pawliszyn,[24-25] and was developed for applications involving solid, liquid or gaseous samples.[26] A number of studies have shown that the results obtained by using SPME fibers are similar to those from solvent extraction.[27-30] The technique involves exposing a fused silica fiber that has been coated with a stationary phase to a sample containing the compounds to be extracted, according to their chemical affinities. For this, a range of SPME fibers are commercially available. The fiber is then introduced directly into the chromatograph where the compounds are desorbed and analyzed. Polydimethylsiloxane (PDMS) fibers are normally used to capture non-polar (MW 125-600), volatile (MW 60-275), and non-polar semi-volatile compounds (MW 80-500) with high molecular weights, while polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers are used to trap volatiles, amines, and nitro-aromatic compounds (MW 50-300). SPME is an effective tool for the collection of insect cuticular compounds[27,30] but samples must be analyzed relatively rapidly in comparison with other techniques, given that the compounds tend to dissipate rapidly from the surface of the fiber.

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A number of studies have suggested new techniques for the improvement of the storage of samples analyzed by SPME. Turillazzi et al.[28] rubbed the cuticular surface of live insects with a clean piece of cotton wool, which was then washed using organic solvents to obtain the compounds. Crewe et al.[20] proposed a technique where the samples are extracted by SPME but stored in solvents, and suggested the use of silicone tubing treated with bis (trimethylsilyl) trifluoroacetamide as a substitutefor the standard SPME fibers. Rather than heating the silicone tubing, it was washed with solvents, which were injected into the GC-MS. Roux et al.[21] proposed a non-lethal technique for obtaining cuticular compounds from live individuals using tepid water, which formed an emulsion that could be extracted with solvents for analysis by GC-MS. Ferreira-Caliman et al.[30] proposed the extraction of cuticular compounds by SPME using a copolymer (Chromosorb), while Choe et al.[31] recommended using silica gel. The copolymers act as a sorbent with a chemical affinity for non-polar compounds, while the silica gel captures the compounds by physical contact.

All these studies have reinforced the need for the development of non-lethal techniques for the extraction of insect cuticular hydrocarbons. The present study demonstrates the possibility of extracting samples by solid phase micro-extraction (SPME) and storing them for later analysis, without the need for immediate injection into the GC-MS system. The results show that there was no loss of sample quality in comparison with the specimen obtained directly from the SPME fiber.

2. Experimental

2.1. Species

Dinoponera quadriceps (Formicidae: Ponerinae) is a queenless ant which forms small colonies. This species is endemic to the Brazilian tropics, including the Atlantic Forest, Caatinga, and Cerrado biomes.[32] Colonies of *D. quadriceps* normally contain between 30 and 240 workers, of which only one – the gamergate – copulates, determined by a dominance-based linear hierarchy.[33-35] In the present study, an entire colony of *D. quadriceps* was collected in Campo Formoso, Bahia state, Brazil, and transferred to the Laboratory of Animal Behavior and Ecology of at the University of São Paulo. The colony was housed in a plastic box (45×35×10 cm) with internal chambers, which were connected to a foraging arena by a plastic tube. Temperature and humidity were maintained constant, replicating natural conditions (27°C and 60% humidity). Colony was fed with small pieces of fruit and cockroaches four times per week, and water was provided *ad libitum*. In order to avoid variation in individual chemical profiles related to functional roles in the colony,[8,10] samples were collected only from foragers working in the arena. All testswere performedatroomtemperature, that is, at about 25ºC. Samples of cuticular hydrocarbons were obtained from ten individuals using two distinct procedures successively.

2.2. Extraction of cuticular hydrocarbons

In the first procedure, the cuticular hydrocarbons of each ant were extracted by SPME using a polydimethysiloxane (PDMS) fiber (100 μm). The ants were immobilized with the aid of two clamps and then the PDMS fiber was rubbed gently against the cuticle (thorax and abdomen) for 30 seconds. Immediately after extraction, the fiber was introduced into the GC-MS injection oven port for 4 minutes to desorb the compounds. In the second procedure, the same SPME extraction technique was used, but the fibers were placed in 50 μL glass inserts containing 20 μL of hexane Mallinckrodt (n-Hexanes, 95.0%) during 30 seconds to allow the absorption of the compounds by the solvent. The solid phase micro extraction (SPME) process is based on the creation simultaneous balances in multiphase systems. Thus, in contrast with a conventional headspace system, the hydrocarbons were desorbed from the SPME fiber to the solvent. As the fiber has a limited absorption surface, this procedure was repeated five times for each ant, in order to increase the amount of compound in the solvent. After the fifth repetition, the fiber was washed in 10 mL of hexane for 1 minute, following which it was ready for the extraction of the next sample. To prevent evaporation of the hexane, the samples were kept in a refrigerator at between 6ºC and 10ºC for two days.

2.3. Validation tests

We used a solution (5 μl/mL of hexane) containing *n*-pentacosane, *n*-octacosane, *n*-dotriacontane and *α*-cholestane (internal standard) to confirm the effectiveness of the technique (analytical standards Sigma-Aldrich). Six individual ants were used as biological matrix and washed three times in a glass vial with 10ml of hexane. Before the validation test, we analyzed the ants using a PDMS fiber (100 μm) to confirm the absence of chemical compounds in the cuticle. We dropped 5 μL of the standard solution on to the abdomen and the PDMS fiber (100 μm) was rubbed against the cuticle (abdomen) for 30 seconds. The fibers used on three ants were introduced sequentially into the GC-MS injection oven port for 4 minutes to desorb the compounds. The fibers from the other three ants were subjected to the same SPME extraction technique, but in this case, the fibers were placed in 100 μL glass inserts containing 60 μL of hexane (Mallinckrodt 95.0%) during 30s to permit the absorption of the compounds by the solvent. This procedure was repeated five times for each ant.

2.4. Chemical analyses

The analyses were conducted with a Shimadzu QP2010 GC-MS. Separation was achieved in a Rtx-5ms column (30 m) using helium as the carrier gas at 1.0 mL min^{-1}. The oven temperature was initially set to 50ºC (held for 1 min), and increased by 10ºC min^{-1} until it reached 300ºC, for 15 min. Analyses were conducted in the splitless mode. The mass spectra were obtained by 70 eV ionization. The SPME fibers were mounted directly in the CG-MS injection oven port for 4 minutes to desorb the compounds. The hexane extracts were placed in the GC-MS system in batch mode, and set to inject 1 μL of solution.

2.5. Data analyses

The data were analyzed with GC-MS Postrun Analysis for

Windows (Shimadzu Corporation) and the chemical compounds were identified based on their mass spectra by comparison with the NIST Library data and with standard alkane solutions for compounds with 9 to 25 and with 21 to 40 carbon atoms (Fluka). The branched alkanes were identified based on comparisons with mass spectral data from Monnin et al.[27] and Carlson et al.[36,37]

The relative abundance of each compound was estimated from the proportion of the peak area of the total ion chromatograms. The amount of each compound in each sample was grouped in n-alkanes, alkenes, and branched alkanes, and their average proportions were compared between treatments (fiber *vs.* fiber and hexane). For this analysis, the values for each single peak area (expressed as a percentage of each compound) were analyzed by an Analysis of Variance (ANOVA) and the F test. The F test was used to assess the statistical significance of the differences between means. All statistical tests were run in Statistica for Windows 7.0 (Statsoft, Inc.).

3. Results and Discussion

The efficiency of recovery recorded in this study indicated that the relative proportions obtained after the proposed treatment were similar to those found in the samples analyzed using the PDMS fiber alone. The mean proportions of *n*-pentacosane, *n*-octacosane, *n*-dotriacontane and α-cholestane in the SPME fiber samples were 38.7% (SD=1.0), 25.5% (SD=0.7), 14.8% (SD=5.3) and 21.0% (SD=6.3), respectively (Figure 1). In the hexane extract of SPME fiber, the mean values recorded for these compounds were 34.9% (SD=7.85), 26.9% (SD=1.13), 16.34% (SD=5.7) and 21.82% (SD=4.38), respectively.

The analysis of the cuticular waxes of the *Dinoponera quadriceps* workers using both injection techniques (SPME fiber placed directly into the GC-MS and the hexane extract of the SPME fiber revealed 40 hydrocarbon peaks (Table 1). The compoundsvaried between 23 and 35 atoms of carbon and were classified as linear alkanes, linear alkenes, and branched chain hydrocarbons (methyl and dimethyl alkanes). The most abundant group of compounds was the branched alkanes, followed by alkanes and alkenes. The branched alkanes, alkanes, and alkenes accounted for 27, 10, and 3 chromatographic peaks, respectively.

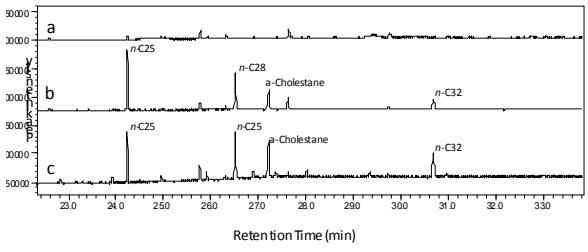

Figure 1 The relative proportions of *n*-pentacosane, *n*-octacosane, *n*-dotriacontane and α-cholestane in the biological matrices recorded for *D. quadriceps*. a) hydrocarbon-free matrix, b) hexane extract of the SPME fiber and c) SPME fiber inserted directly into the GC-MS.

Table 1 Relative proportions (mean±standard deviation) of cuticular hydrocarbons obtained from *Dinoponera quadriceps* workers using two different techniques: SPME fiber directly into the GC-MS and the hexane extract of the SPME fiber extract (N=10).

Peak	*Hydrocarbon*	*SPME Fiber*	*Hexane extract of SPME fiber*
1	*n*-C_{23}	1.99 ± 1.00	0.72 ± 0.51
2	3-MeC_{23}	0.08 ± 0.06	0.03 ± 0.01
3	*n*-C_{24}	0.26 ± 0.16	0.32 ± 0.19
4	*n*-C_{25}	6.30 ± 1.77	4.04 ± 3.49
5	13-,11-MeC_{25}	0.25 ± 0.15	0.08 ± 0.03
6	3-MeC_{25}	0.41 ± 0.20	0.14 ± 0.03
7	*n*-C_{26}	1.32 ± 0.38	0.62 ± 0.19
8	3-MeC_{26}	0.20 ± 0.11	0.19 ± 0.20
9	*n*-C_{27}	14.35 ± 3.1	10.16 ± 4.0
10	13-,11-,9-MeC_{27}	2.19 ± 0.63	1.02 ± 0.17
11	7-MeC_{27}	0.35 ± 0.10	0.10 ± 0.03
12	5-MeC_{27}	0.68 ± 0.22	0.22 ± 0.08
13	??-diMeC_{27}	1.04 ± 0.24	0.43 ± 0.13
14	3-MeC_{27}	9.02 ± 1.15	5.00 ± 0.86
15	*n*-C_{28}	1.74 ± 0.32	1.45 ± 0.50
16	13-,11-,9-MeC_{28}	1.69 ± 0.70	0.85 ± 0.19
17	5-MeC_{28}	0.44 ± 0.12	0.25 ± 0.10
18	3-MeC_{28}	0.81 ± 0.54	0.37 ± 0.14
19	*Z*-?-C_{29}	0.78 ± 0.33	1.15 ± 0.78
20	*n*-C_{29}	4.89 ± 0.99	3.57 ± 1.99
21	13-,11-,9-MeC_{29}	16.64 ± 3.1	15.62 ± 2.1
22	7-MeC_{29}	1.13 ± 0.73	0.33 ± 0.34
23	5-MeC_{29}	2.35 ± 0.38	1.58 ± 0.62
24	11,15-; 13,17-diMeC_{29}	1.99 ± 0.51	2.22 ± 0.91
25	3-MeC_{29}	1.48 ± 0.61	1.17 ± 1.07
26	*n*-C_{30}	3.77 ± 0.74	3.23 ± 0.74
27	11-,12-,13-,14-MeC_{30}	0.33 ± 0.14	0.53 ± 0.18
28	*Z*-?-C_{31}	1.30 ± 0.41	1.07 ± 0.50
29	*n*-C_{31}	11.93 ± 2.3	17.82 ± 3.2
30	15-,13-,11-,9-MeC_{31}	2.22 ± 0.99	4.66 ± 1.39
31	*n*-C32	1.09 ± 0.49	2.20 ± 0.71
32	14-,12-MeC_{32}	0.31 ± 0.09	0.17 ± 0.06
33	*Z*-?-C_{33}	0.32 ± 0.25	0.39 ± 0.22
34	17-,15-,13-,11-MeC_{33}	3.07 ± 0.67	7.04 ± 1.62
35	15,19-diMeC_{33}	1.39 ± 0.33	3.57 ± 2.16
36	13,17-diMeC_{33}	0.57 ± 0.24	0.55 ± 0.16
37	11,15-diMeC_{33}	0.19 ± 0.05	0.72 ± 0.23
38	9,11-diMeC_{33}	0.21 ± 0.06	0.98 ± 0.74
39	17-,15-,13-,11-MeC_{35}	0.51 ± 0.26	2.35 ± 1.08
40	9,13-;11,15-;13,17-; 15,19-diMeC_{35}	0.42 ± 0.23	3.11 ± 0.82

The compounds with the highest percentage areas were hentriacontane, heptacosane, 3- methyl heptacosane, pentacosane, nonacosane and triacontane. In addition, two chromatographic peaks (22 and 32) indicated a large number of isomers of the C_{29} and C_{31} branched alkanes (Figure 2).

The two methods used to analyze of the cuticular hydrocarbon profile of ants yielded quantitatively and qualitatively similar data (Table 1, Figure 2 and Figure 3). The statistical analyses of each group of compounds revealed no significant difference in the relative proportions obtained by the two treatments (direct injection and solvent extract). The results for each group were – Alkanes: $F_{1,18} = 2.63$, $p = 0.122$; Alkenes: $F_{1,18} = 0.179$, $p = 0.677$; and Branched alkanes: $F_{1,18} = 1.722$, $p = 0.206$ (Fig. 1).

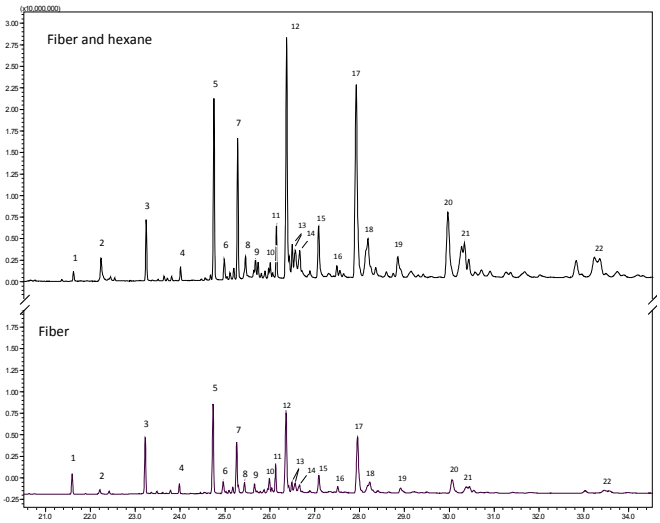

Figure 2 Chromatograms of cuticular hydrocarbons in a single *Dinoponera quadriceps* forager using two different techniques of GC-MS injection. Mainpeaks: 1) *n*-C_{23}2) *n*-C_{24} 3) *n*-C_{25} 4) *n*-C_{26} 5) *n*-C_{27} 6)13-, 11-,9-MeC_{27} 7)3-MeC_{27} 8) *n*-C_{28} 9) 13-,11-,9- MeC_{28}10) Z-?-C_{29} 11) n-C_{29} 12) 13-,11-,9-MeC_{29}13-,11-,9-MeC_{29} 13) 7-Me$C_{29}$and 5-MeC_{29} 14) 11,15- and 13,17-diMeC_{29}15) *n*-C_{30} 16) Z-?-C_{31} 17) *n*-C_{31} 18) 15-,13-,11-, 9-MeC_{31} 19) *n*-C_{32} 20) 17-,15-,13-,11-MeC_{33} 21) 15,19-, 11,15-, 13, 17-diMeC_{33} 22) 9,13-, 11,15-, 13,17-, 15,19-diMe C_{35}

In their study of *D. quadriceps*, Monnin et al. [27]showed that the cuticular profile of these ants varied little during repeated samples taken with SPME fibers (for ants of the same reproductive status), confirming the reliability of this extraction method. In general, the hydrocarbon profile of the *D. quadriceps*foragers analyzed in the present study was qualitatively similar to those recorded by Monninet al.[27] in sterile workers, although some compounds (branched alkanes of heptacosane) were found only in the previous study.

The SPME using commercial fibers presents a number of advantages over solvent extraction. As a non-destructive technique, it permits the study of individuals without sacrificing them. In addition, the use of fibers permits extraction from a specific part of the body, in contrast with solvent extraction involving dead insects, which may contain glandular compounds.[38]In *Solenopsis saevissima*, cuticular hydrocarbons and alkaloids from the venom gland were obtained by both hexane- and water-based extraction.[21] The collection of samples by SPME can be used to minimize or avoid the acquisition of glandular compounds altogether given that it can be directed to specific parts of the insect's body, in which these glands are absent. However, the disadvantage of the SPME procedure is that samples cannot be stored and must be analyzed immediately.[27]

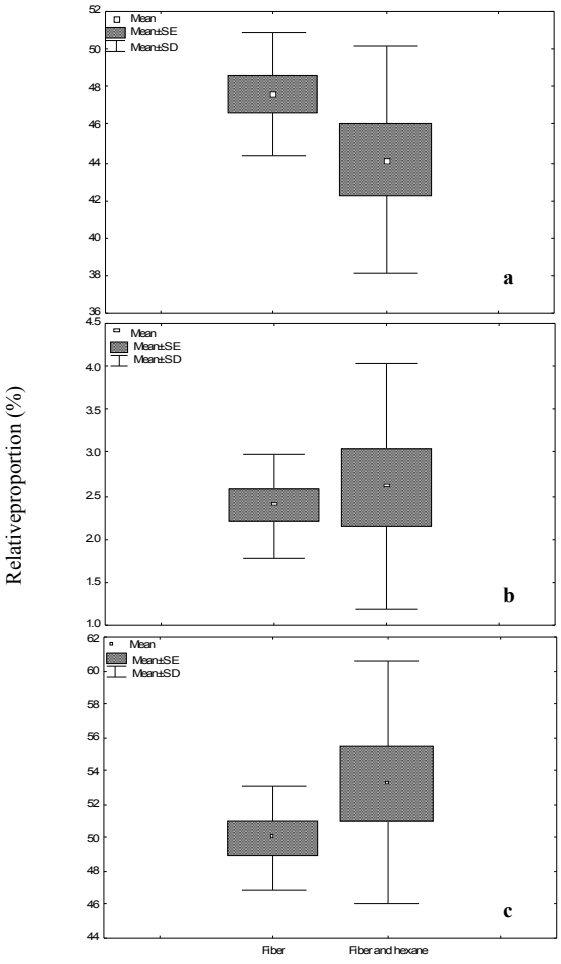

Figure 3 Comparison of the amounts (%, mean ± SD) of **a** n-alkanes, **b** alkenes and **c** branched alkanes between different treatments (Fiber vs. Fiber and Hexane) in *Dinoponera quadriceps* foragers (N=10).

The SPME approach is suited for situations in which the euthanization of the animals is not possible or undesirable, such as studies of the linear dominance hierarchy found in a number of different social hymenoptera species, including some wasps,[39,40] ants[41-43, 32] and bees.[44,45] In these species, all females have the potential to mate and lay fertilized eggs, but only dominant individuals reproduce.[46]Monnin and Peeters[33,34] emphasized the importance of chemical analyses for the understanding of the dominance interactions that regulate the linear dominance hierarchy in *D. quadriceps*. The authors identified differentcuticular hydrocarbon signatures in dominant and sterile workers, and concluded that this approach provides reliable information on the reproductive status of the ants. The acquisition

of multiple samples from a single female may thus provide valuable insights into the reproductive status of the individual and in particular the processes underlying the replacement of the dominant female in a nest. Furthermore, it may be possible to use this new technique to analyze nest materials[47-50] in orientation experiments in ants[17] or analyses of bee comb waxes.[47, 49]

4. Conclusions

This study describes a novel technique for the storage of cuticular hydrocarbon samples in solvent following SPME extraction using polydimethylsiloxane fibers. The study indicates that this technique can be used to collect and store samples of cuticular hydrocarbons from live insects under field conditions. Following collection by SPME, the cuticular compounds of the *Dinoponera quadriceps* foragers were eluted from the fibers with hexane, and the samples were stored prior to analysis. This hexane extract facilitates the collection of multiple samples from different phases of an individual's life, providing an important analytical tool to a variety of studies in chemical and behavioral ecology.

Overall, the present study has shown that desorption of SPME fiber in hexane is an effective non-lethal method for the extraction of cuticular hydrocarbons. This technique was developed specifically for the long-term monitoring of individuals, as well as for situations in which gas chromatography equipment is unavailable at the sampling site.

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