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Solid-phase microextraction for the detection of termite cuticular hydrocarbons

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Abstract

Solid-phase microextraction (SPME)–gas chromatography–mass spectrometry was used to identify the cuticular hydrocarbons of the subterranean termite *Coptotermes formosanus* Shiraki. Headspace SPME and direct contact SPME methods were evaluated and compared to the hexane extraction method. Variables, such as temperature, time, number of termites, condition of the termites, and the type of SPME fiber were evaluated. Methods were refined to increase the reproducibility as well as the sensitivity. Both SPME methods were successfully used for the identification of all the major termite cuticular hydrocarbons. Using the headspace SPME method, other compounds of interest could also be identified, such as fatty acids. Using the direct contact SPME method, termites could be repeatedly studied over time to monitor chemical changes. Published by Elsevier Science B.V.

Keywords: *Coptotermes formosanus*; Headspace analysis; Solid-phase microextraction; Hydrocarbons

1. Introduction

The chemicals produced by termites have various purposes, affecting behaviors such as foraging, caste regulation, nest-building, mating, and defense [1]. The cuticular hydrocarbons that are found in high concentrations on their outer surface are used by termites as protection from desiccation and for recognition of other species, and in some cases, other colonies of the same species [2,3]. Based on cuticular hydrocarbon profiles, several phenotypes of termite species have been identified [4–7].

The identification of termite cuticular hydrocarbons has traditionally been through a surface hexane extraction procedure. Several problems associated with solvent extraction of termites have been previously addressed [8]. There is no standard method and different results can be obtained by changing any variable such as the termite state (alive, dead, or dried), the method of killing or drying the termite, the choice of solvent, solvent volume, number of extraction repetitions, extraction duration, temperature, number of termites, and many more. Also, the main chemicals found from solvent extraction of whole termites frequently do not correspond to the

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chemicals that the termite actually uses for communication because unrelated chemicals are extracted with the ones being sought.

Solid-phase microextraction (SPME) is a solventless form of gas chromatography (GC) sample introduction that eliminates sample matrix problems. It has been used for the detection of insect cuticular hydrocarbons by sampling the headspace of heated pieces of cuticle [9] or by rubbing the cuticle membrane of an individual organism [10]. Pheromones of termites have recently been detected using SPME, by rubbing the fiber on the area of the gland producing the pheromone [11]. However, the parameters for use of this technique have not been thoroughly studied. This report investigates the parameters for the use of SPME as a method to detect and identify the cuticular hydrocarbons of termites. Also, new SPME methods for the detection of cuticular hydrocarbons are examined that do not interfere with the natural state of the termite.

2. Experimental

2.1. Insects

Coptotermes formosanus Shiraki were collected from field monitoring stations associated with live oak, cypress, and pine trees at the campuses of the University of New Orleans and US Department of Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA in January 1999–June 2000 and maintained on spruce blocks until needed. OmniSolv glass distilled hexane was acquired from EM Science (Gibbstown, NJ, USA).

2.2. GC–MS equipment

Gas chromatography–mass spectrometry (GC–MS) was performed on a Hewlett-Packard 6890 GC system equipped with a 7683 autosampler and a 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA, USA). Electron impact (EI) MS was obtained at 70 eV. A split/splitless injector was used in splitless mode with a purge flow to split at 2.0 min after injection. Chromatograms were run at a constant flow of 1 ml/min of He gas. The inlet temperature was set at 250°C. A HP-5MS (5% diphenyl–95% dimethylsiloxane) capillary column (30 m×250 μm, 0.25 μm nominal) was used with temperature programming from 60°C (1 min hold) to 300°C at 10°C/min with a final 10 min hold. Solvent samples (1 μl) were injected by an autosampler. SPME samples were manually injected by insertion of the fiber into the mass spectrometer inlet until after the purge flow to split occurred. Mass spectra were recorded from 40 to 750 m/z.

SPME fibers [100 μm polydimethylsiloxane (PDMS), 70 μm Carbowax–divinylbenzene (CW–DVB), and 75 μm Carboxen–PDMS] were obtained from Supelco (Bellefonte, PA, USA).

2.3. Solid-phase microextraction

2.3.1. Headspace SPME analysis

Either one or 50 *C. formosanus* workers (either alive or killed by freezing at −80°C or being lyophilized) were placed in a 1 dram vial with septum. When a weighed amount of termites was used, 0.22 g was used as an equivalent to 50 termites. For experiments with one worker, specimens of equal mass (4.2 mg, 2.7% RSD) were used and the 1 dram vial (4.77 ml) was replaced by a tapered 100 μl microvial (catalog No. 78000-M; Scientific Resources, Lawrenceville, GA, USA) that had an actual volume of 456 μl. The β value (gas volume/solid volume) was increased from 31 for the 50 termite experiment to 120 for the one termite experiment. A SPME fiber was inserted into the vial (tip of fiber was 1 cm above top of termites) which was then inserted into a sand-filled heating block set to the desired temperature (30, 60, 90, or 120°C). After heating for 15, 30, 60, or 120 min, the fiber was removed.

2.3.2. Direct contact SPME analysis

2.3.2.1. Live termites

C. formosanus workers (50, 100, or 200, alive) were placed in a 1 dram vial with septum. The vial was laid on its side at a slight incline so the termites could walk up to the lip of the vial but not reach the septum. Termites were equilibrated for 0 min to 2 h prior to inserting SPME fiber. The SPME fiber was inserted so as to press against the glass under the

termites. After 30 min at 26°C, the fiber was removed.

2.3.2.2. Dead termites

C. formosanus workers (100, killed by freezing at −80°C, then brought to room temperature in a desiccator) were placed in a 1 dram vial with septum. The SPME fiber was inserted into the vial and the vial was rolled for 1 min, causing the termites to gently tumble over the fiber.

2.3.2.3. Cuticle rub

An anesthetized (cold or CO_2) *C. formosanus* worker was held by tweezers to expose the abdomen. The SPME fiber was rubbed across the abdomen cuticle several times.

2.4. Hexane extraction

C. formosanus workers (50, either alive or killed by freezing at −80°C) were placed in a 1 dram vial and 125 μl hexane was injected onto the termites. After 2 min of slight agitation, the hexane was removed via syringe. The process was repeated with another 70 μl hexane. Because of absorption of hexane by the termites, recovered hexane was less than the sum added. The combined hexane was diluted with hexane to a total volume of 140 μl.

3. Results

3.1. Headspace SPME

Several parameters were examined to find the optimum conditions. The type of SPME fiber was evaluated first. Three types of fibers were tested: 100 μm PDMS, an absorbent, nonpolar fiber; 70 μm CW–DVB, an adsorbent, polar fiber; and a 75 μm Carboxen–PDMS, an adsorbent, bipolar fiber. PDMS and CW–DVB gave similar profiles, while Carboxen–PDMS showed very little cuticular hydrocarbon adsorption, having mainly small molecule, early eluting peaks in the chromatogram (Fig. 1). The PDMS fiber was chosen over the CW–DVB for all other tests since it is more commonly used in experiments of this type.

The optimum condition of the termite used in the study was also determined. Live, dead by freezing, and lyophilized termites were tested for possible differences in the cuticular hydrocarbon profile. Live termites were killed by the experimental conditions within the first minute. Nearly identical profiles were obtained from the three initial conditions of the termites. To eliminate extra steps in the sampling process, we chose to use live termites in all subsequent tests.

The effects of temperature and extraction time were also examined. Fifty termites were extracted by headspace SPME at four temperatures (30, 60, 90, or 120°C) and four extraction times (15, 30, 60, or 120 min). As seen in Fig. 2, the total hydrocarbon peak

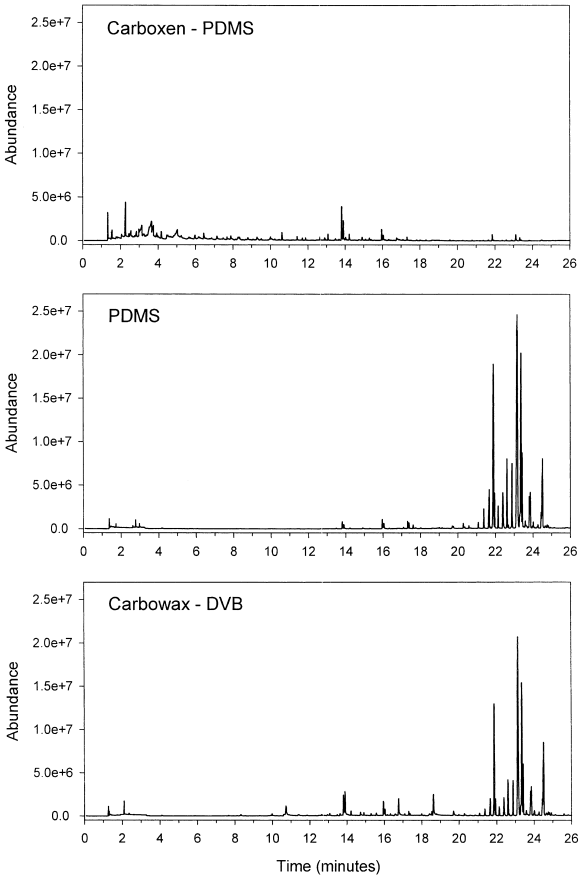

Fig. 1. Comparison of SPME fiber types. Total ion chromatograms (TICs) of GC–MS analyses of headspace SPME injections of 50 *Coptotermes formosanus* (termite) workers heated at 120°C for 60 min in a 1 dram vial. Fiber types indicated on chromatograms. Cuticular hydrocarbons elute between 21 and 25 min.

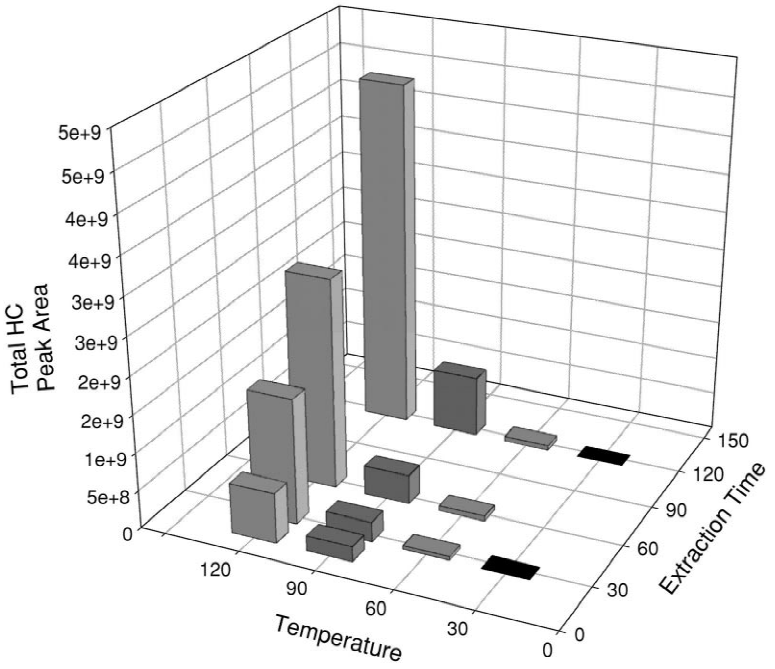

Fig. 2. Headspace SPME analysis parameter test. Changes of total hydrocarbon peak area from headspace SPME injections of 50 *Coptotermes formosanus* (termite) workers in relation to sample temperature and absorption time. Temperature in °C; extraction time in min.

area increased exponentially with increasing temperature. Both 90°C and 120°C gave peak heights with good signal-to-noise ratios, however, peaks at 120°C were five times larger than the 90°C peaks. Also, at 90°C, the earlier eluting cuticular hydrocarbon peaks were preferentially absorbed. This may be a result of incomplete volatilization of the higher boiling, later eluting, hydrocarbons. Peak area also increased as the extraction time increased. For the 90°C temperature, the slope of the increase between points continued to rise at longer extraction times, while at 120°C, the amount of increase became smaller at the longer extraction times. We chose the 120°C, 60 min extraction as our standard because of good signal to noise and an extraction times that would allow several experiments per day.

Sample reproducibility was evaluated for the headspace SPME analysis of multiple samples of 50 termites, using a 120°C absorption temperature and 60 min absorption time (Table 1). Total sample peak area varied by 13% with individual peaks varying 9–29% (18% average). If the samples were normalized to the average total peak area, the average RSD of the individual peaks was reduced to 10%. This is within the range of precision (<1–12% RSD) reported for most SPME applications [12].

The use of tetracosane (C_{24}) as an internal standard was studied as a method to reduce the deviation between samples, but the RSD for the pure standard was 16%, probably due to partial evaporation during the removal of the organic solvent used to dilute the C_{24}. When C_{24} was used as the internal standard in vials of termites, the amount of C_{24} recovered was reduced to one fifth the amount obtained from the standard alone, and the RSD was increased to 67%. This may be due to competition of the absorption of the C_{24} by the termite cuticle.

It was also found that cuticular hydrocarbon peak reproducibility was improved by a change in protocol, where instead of using 50 termites, an equivalent mass of 50 termites was used. Samples of 50 termites had varying masses associated with them. By choosing a standard mass, the variability of the termite's surface area is reduced. Using equivalent masses of termites, the average RSD of the individual peaks was 9%, similar to the normalized

Table 1
Headspace SPME peak area reproducibility for 50 termites, 120°C absorption temperature, 60 min absorption time

Peak	t_R (min)	Sample					Average	SD	RSD (%)	Normalized RSD (%)
		A	B	C	D	E				
1	21.39	3.6	5.2	2.8	4.0	3.1	3.8	0.9	24	13
2	21.67	9.7	11.4	8.9	9.7	8.5	9.6	1.0	11	5
3	21.90	50.8	49.8	50.6	54.7	42.3	49.6	4.5	9	9
4	21.96	5.8	6.7	5.3	5.7	4.9	5.7	0.6	10	5
5	22.15	4.1	5.6	2.9	4.6	3.2	4.1	1.0	24	13
6	22.40	9.8	11.5	8.7	10.7	7.8	9.6	1.4	15	3
7	22.62	14.8	18.8	12.9	17.1	11.6	15.0	2.9	20	7
8	22.89	12.9	17.1	10.6	15.6	10.2	13.3	3.0	23	10
9	23.17	98.8	111.0	91.0	107.4	83.5	98.3	11.3	12	2
10	23.37	54.5	63.4	48.5	63.5	45.9	55.1	8.2	15	3
11	23.44	14.0	16.8	9.7	16.2	11.3	13.6	3.0	22	12
12	23.60	2.8	3.0	2.9	3.9	2.7	3.1	0.5	16	12
13	23.86	15.4	20.3	10.0	14.4	10.8	14.2	4.1	29	18
14	24.27	1.1	1.3	0.6	1.1	1.3	1.1	0.3	28	29
15	24.52	25.8	28.5	23.7	27.7	23.0	25.8	2.4	9	4
							Average RSD (%)		18	10
	Sum	324.0	370.2	289.4	356.0	270.0	321.9	42.6	13	0

Peak numbers as shown in Fig. 4, t_R=peak retention time, peak area is $\times 10^7$, SD=standard deviation, normalized RSD are data normalized to average sample sum.

data reported above. Therefore, an internal standard is not necessarily needed to obtain reproducible results if an equal mass of termites is used instead of an equal number.

In addition to headspace SPME analyses of 50 termites (or their equivalent mass), the headspace SPME absorption from one termite was also tested. Termite workers of equal mass were used for this comparison. Smaller vials were used so the headspace to solid volume ratio would not be considerably different. As seen from Fig. 3, changes in temperature and absorption time showed the total hydrocarbon peak area to vary similar to that seen for 50 termites, although peak areas were eight times smaller.

Peak areas for one termite were very consistent between multiple samples taken with the 120°C, 2 h absorption (5% RSD for total area and 10% average RSD for the individual peaks). This was uncorrected for variations of termite mass (2.7% RSD). However, for the shorter absorption times variability became much greater (18% RSD for total area and 27% for the average of the individual peaks in the 30 min experiment; 9 and 20%, respectively, when normalized to the total peak area).

Comparison of the cuticular hydrocarbon profile from the headspace SPME method (120°C for 1 h with 50 termites) with the standard hexane extraction method shows good correlation between the two methods (Fig. 4). All peaks present in the hexane extract are present in the headspace SPME absorption (peak identifications shown in Table 2), although relative peak heights may not be the same. The peak areas from the 50 workers by headspace SPME were equal to seven worker equivalents of the hexane extract injection. The peak areas from the one worker experiment (2 h, 120°C) were equal to 0.8 worker equivalents of the hexane extract. In the headspace SPME absorption, earlier eluting peaks, not found in the hexane extraction were sometimes present. Some were identified as oleic, linoleic, and palmitic fatty acids (Fig. 5). To verify these were not artifacts of the procedure used for headspace SPME, a sample of evaporated hexane extract was tested by the headspace SPME procedure. The headspace SPME chromatogram of the hexane extract was identical to the hexane extraction chromatogram except the headspace SPME-produced peaks were three times smaller for an equal number of worker equivalents.

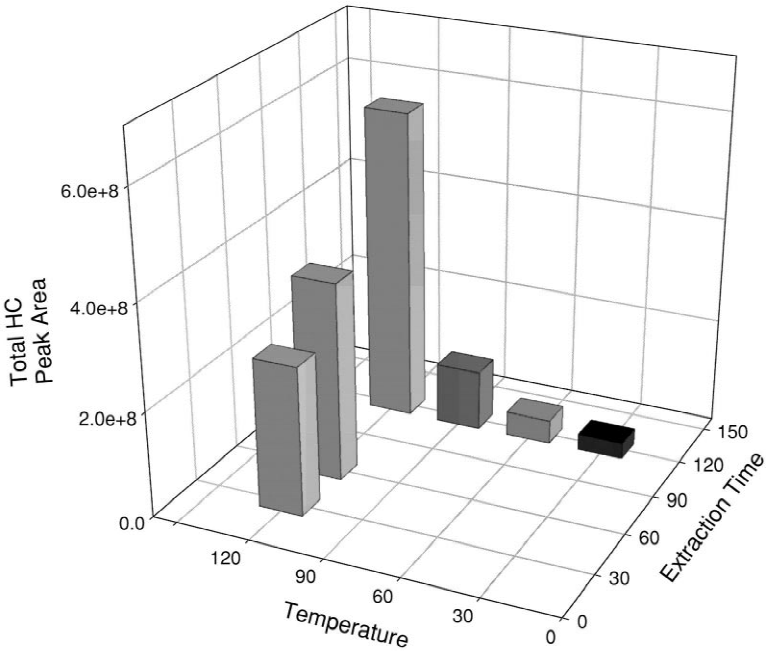

Fig. 3. Headspace SPME analysis parameter test. Changes of total hydrocarbon peak area from headspace SPME injections of one *Coptotermes formosanus* (termite) worker in relation to sample temperature and absorption time. Temperature in °C; extraction time in min.

3.2. Direct contact SPME

The optimum number of termites needed to give reproducible results was evaluated first. Using a 30 min absorption period in a 1 dram vial, 50, 100, and 200 termites were tested. Cuticular hydrocarbon peak areas increased threefold from 50 to 200 termites. However, it was also found that as the number of termites reached 200, the destruction of the SPME fibers increased, as did the variability of the peak areas. Increase of the absorption period above 30 min also resulted in a similar premature degradation of the SPME fiber. Therefore, the conditions chosen for this experiment were 100 termites and a 30 min absorption time.

The condition of the termite, whether distressed or calm, was also considered. To reduce stress, an equilibration time was used prior to the SPME absorption and they were left in the same vial for subsequent tests. Non-equilibrated termites were produced by rotation of the vial, which limited the number of times a sample could be measured, or by placing termites in a fresh vial for each test. The variability of non-equilibrated termites (same termites transferred to new vials for each test) was equal to that obtained in the equilibrated experiments (see Table 3). It was noticed however, that the variability was closely related to the variability in the overall mass deposited (or absorbed onto the fiber) as measured by the sum of the peak areas. If the samples were normalized to the total peak area, the variability of both the equilibrated and non-equilibrated tests was reduced to 4–11% average RSD for the individual peaks.

Comparison of direct contact SPME with the hexane extraction method showed the two to be very similar (see Fig. 4). The same peaks were found in both methods with only a slight variation in the relative peak heights. No extra peaks were observed from either method. As with the headspace SPME method, there was a difference in the peak height relative to the number of termites used in the experiment. The direct contact SPME method using 100 termites gave peak heights equivalent to those

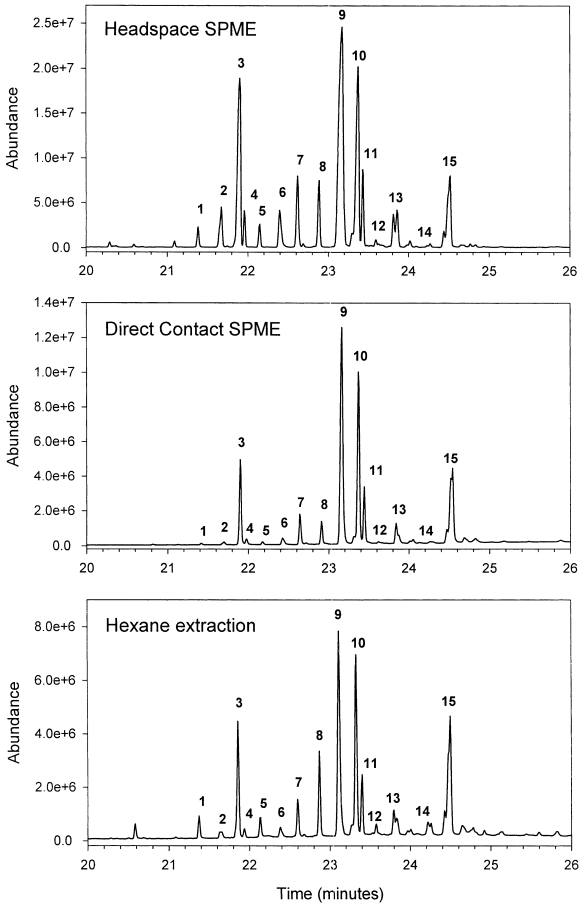

Fig. 4. Comparison of methods. Total ion chromatograms (TICs) of GC–MS analyses of cuticular hydrocarbons from 50 *Coptotermes formosanus* (termite) workers by headspace SPME, direct contact SPME, and hexane extraction methods, using optimized conditions. Peaks are identified as (1) *n*-C_{25}; (2) 9-, 11-, 13-MeC_{25}; (3) 2-MeC_{25}; (4) 3-MeC_{25}; (5) *n*-C_{26}; (6) 11-, 12-, 13-MeC_{26}; (7) 2-MeC_{26}; (8) *n*-C_{27}; (9) 11-, 13-MeC_{27}; (10) 9,13-diMeC_{27}+2-MeC_{27}; (11) 3-MeC_{27}; (12) *n*-C_{28}; (13) 11-, 13-, 15-MeC_{28}; (14) *n*-C_{29}; (15) 13-, 15-MeC_{29}+13,15-diMeC_{29} [hydrocarbons are designated using a descriptor for the location of the methyl group (X-Me) and the total number of carbons (C_{XX}) in the hydrocarbon component, excluding methyl branches].

obtained from a 1.2 termite equivalent hexane extract injection.

The direct contact SPME analysis of dead termites was also studied. The absorption of cuticular hydrocarbons was also obtained by rubbing the SPME fiber on the cuticle of an anesthetized termite. Both were found to give comparable chromatograms to those obtained from live termites.

4. Discussion

The type of samples normally used with SPME are either an aqueous sample where the SPME fiber is inserted in the liquid, or a solid or aqueous sample where the SPME fiber is positioned above the sample and absorbs the chemicals in the headspace. SPME is well suited for the headspace detection of volatile compounds emitted by insects. However, cuticular hydrocarbons, with chain lengths of 25–29 carbons, are not volatile. Therefore, either the termite must be heated to a temperature to volatilize the hydrocarbons or the SPME fiber must come in direct contact with the cuticle to absorb the hydrocarbons.

In the headspace SPME method, it was demonstrated that 120°C was about the minimum temperature that was able to efficiently volatilize the termite cuticular hydrocarbons. Reproducibility was improved if equal masses of termites were used instead an equal number. Cuticular hydrocarbon profiles could be obtained from a single termite also, making this method more convenient than hexane extraction for the determination of individual differences. Headspace SPME was also able to detect other compounds of interest that were not seen from the hexane extraction method (i.e., fatty acids). A previous report of the use of headspace SPME for the detection of insect fatty acids gave mixed result [13]. The fatty acids are clearly not specific components of the cuticle since they are not observed by direct contact SPME, and may be fat degradation products or from an internal source.

A second sampling method studied was direct contact SPME. This method absorbs cuticular hydrocarbons directly from the termite's outer surface. The cuticular hydrocarbons or any chemical absorbed on the SPME fiber would be equivalent to what would be available to another termite for species, colony, caste, or mate recognition. Although the use of dead or anesthetized termites may alter the chemical signals of the termite, the use of live termites walking on and around the SPME fiber has great potential for the study of the chemicals associated

Table 2
EI-MS identification of cuticular hydrocarbon peaks and their percent abundance

Peak No.	Identity	% CH	EI-MS diagnostic ions
1	*n*-C_{25}	0.5	352
2	9-, 11-, 13-MeC_{25}	0.6	140, 252, 168, 224, 196
3	2-MeC_{25}	11.6	323
4	3-MeC_{25}	1.0	337
5	*n*-C_{26}	0.9	366
6	11-, 12-, 13-MeC_{26}	1.2	168, 238, 182, 224, 196, 210
7	2-MeC_{26}	3.8	337
8	*n*-C_{27}	3.6	380
9	11-, 13-MeC_{27}	29.3	168, 252, 196, 224
10	2-, 4-, 6-MeC_{27}+9,13-diMeC_{27}	21.1	379, 351, 323; 211, 295
11	3-MeC_{27}	7.0	365
12	*n*-C_{28}	1.0	394
13	11-, 13-, 15-MeC_{28}	4.1	168, 196, 238, 210, 225
14	*n*-C_{29}	0.7	408
15	13-, 15-MeC_{29}+13,15-diMeC_{29}	13.5	196, 252, 224; 196, 239, 267

Peak Nos. as shown in Fig. 4, identity nomenclature described in Fig. 4, % CH=peak area as percent of the total hydrocarbon peak area.

with termite or insect behavior. It can alleviate many of the potential problems associated with chemical degradation or reactivity confronted with most sampling methods, including headspace SPME, where the chemical being studied is sampled from a non-living insect specimen or one that is not in its natural habitat.

The major problem foreseen with this method was the reproducibility of the results would be dependent on the disposition of the termites. When the termites are removed from their nest and placed in a new environment, such as a glass vial, their behavior is altered. It has been reported that there is a calm-down period of about 40 min as determined by the amount of carbon dioxide released by *C. formosanus* and *Reticulitermes flavipes* [14]. Attempts were made to lessen this alteration in behavior by allowing a time period for equilibration before the test began or doing sequential tests from the same vial. How-

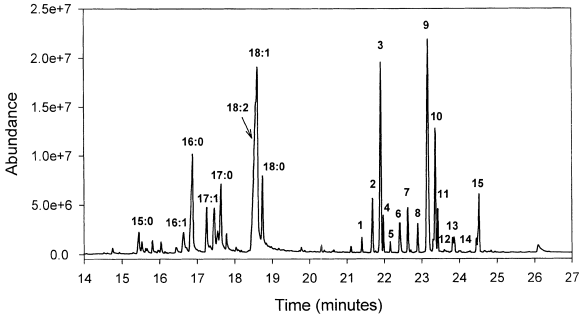

Fig. 5. Total ion chromatogram (TIC) of GC–MS analysis of 50 *Coptotermes formosanus* (termite) workers showing fatty acid peaks observed by headspace SPME. Fatty acid peaks are labeled with their total carbon number and number of unsaturation sites (e.g., oleic acid=18:1). Cuticular hydrocarbons are labeled as shown in Fig. 4.

Table 3
Direct contact SPME peak area reproducibility

Experiment	RSD (%)			
			Sample normalized[a]	
	Peak[c]	Sample[d]	Peak[c]	Sample[d]
Equilibrated[b]				
50 termites (n=7)	42	42	8	0
100 termites (n=9)	23	22	7	0
100 termites (n=7)	9	9	4	0
200 termites (n=9)	44	43	4	0
Non-equilibrated[e]				
100 termites (n=6)	29	25	11	0
100 termites (n=6)	33	32	4	0
100 termites (n=9)	36	35	7	0
100 termites (n=9)	45	44	6	0

[a] Peak areas were normalized so the sample's total peak area equaled the average of all samples in the experiment.
[b] Sequential samples in experiment were from the same vial.
[c] Average RSD of largest eight peaks in sample.
[d] The RSD of sample's total peak area (eight peaks).
[e] Sequential samples in experiment were placed in a new vial.

ever, the variability was found to be similar for equilibrated and non-equilibrated termites.

A problem not foreseen was how the live termites treated the fiber. As with the vial and any other object they came into contact, this involved gnawing and depositing a sticky substance and using any piece of debris or dirt they may be carrying with them to build a new carton to enclose themselves. With large numbers of termites or long periods of time, this led to the fiber being coated with various materials and thus causing it to be replaced by a new fiber after a small number of experiments. If the parameters chosen for the study (100 termites, 30 min absorption) are used, fiber degradation was not significant.

In conclusion, the use of SPME was shown to be a viable alternative method to solvent extraction for the determination of termite cuticular hydrocarbons. Direct contact SPME produced a chromatogram that was very clean with no extraneous peaks. Both methods are complementary to solvent extraction because of the additional information discovered by these methods. The headspace SPME method is beneficial for the observation of compounds other than cuticular hydrocarbons, such as the fatty acids that were occasionally detected. One of the major benefits of the direct contact SPME method was the ability to observe changes over time because the study could be performed on the same (live) termites.

Both methods were found to be reproducible. The reproducibility of the headspace SPME method was found to be comparable to the solvent extraction method when an equivalent mass of termites was used instead of an equal number. This was observed even for tests of single termites. The direct contact SPME method reproducibility was maximized when samples were normalized to an equal total peak area, due to the natural variation in cuticular hydrocarbon amounts.

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