

HYDROCARBONS OF *Nasutitermes acajutlae* AND COMPARISON OF METHODOLOGIES FOR SAMPLING CUTICULAR HYDROCARBONS OF CARIBBEAN TERMITES FOR TAXONOMIC AND ECOLOGICAL STUDIES

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Abstract—Using data from the arboreal nesting *Nasutitermes acajutlae* (Holmgren), we propose standard collection and extraction methodology for characterization of cuticular hydrocarbons of termites under field conditions in the tropics. Specifically, we evaluated: (1) the effect of the duration and the number of extractions; (2) the effect of drying termites before extraction; (3) the effect of sample size; (4) the effect of solvents (ethanol versus hexane) on cuticular hydrocarbon profiles. Olefins comprise ca. 70% of the cuticular hydrocarbons of *N. acajutlae*. Hydrocarbons consist of two distinct groups: early-eluting components, primarily *n*-alkanes and methyl-branched alkanes, and late-eluting compounds, which consist almost exclusively of unsaturated components with one to six double bonds. Soldiers have more early-eluting compounds than workers or alates. Nests from the same island had qualitatively similar, but quantitatively dissimilar hydrocarbon mixtures. Brief extractions of 300 live workers in 10 ml of hexane for only 20 sec produced a hydrocarbon mixture equivalent to a 10-min extraction. Long-term extraction of 300 workers in hexane for two years resulted in different mixtures of hydrocarbons. Drying workers tended to enhance extraction of the less abundant unsaturated compounds such as C_{41:4} and C_{41:5}. A single extraction of a

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minimum of 100 workers (live or dried), with hexane for 20 sec to 10 min is best; these extraction regimes resulted in mixtures of hydrocarbons that are quantitatively very similar. For quantitative comparisons, extracts from dried samples should not be compared to those from live samples. Storage in ethanol caused numerous unidentified, nonhydrocarbon compounds to be extracted either from the cuticle or from internal tissues.

Key Words—*Nasutitermes acajutlae*, chemotaxonomy, Isoptera, Termitidae, tropical termites, gas chromatography, cuticular hydrocarbons, olefins, mass spectrometry.

INTRODUCTION

Termites play an essential role in the ecological dynamics of many tropical ecosystems, recycling nutrients and aerating soils in forests, mangrove swamps, and grasslands. Some species of termites are also economically important as structural pests in urban, as well as rural, areas of the tropics. The termite fauna of tropical regions is known to be diverse, but species diagnosis remains equivocal in many groups. Cuticular hydrocarbons are useful for discriminating termite species in tropical (Haverty et al., 1990b, 1991a, 1992, 1997; Howard et al., 1988) and temperate (Haverty et al., 1988, 1991a; Howard et al., 1978, 1982a,b; Thorne and Haverty 1989; Thorne et al., 1993) regions. Species sorting and diagnosis based on such chemical separation may then be used to facilitate discovery of morphological criteria for discriminating species (Haverty et al., 1988; Thorne and Haverty, 1989) and delimiting geographic distributions (Thorne et al., 1993).

Comparative research for characterizing cuticular hydrocarbons for taxonomic or ecological studies will necessitate uniform protocols among investigators. Field circumstances must also be considered. In the tropics, termites are usually collected far from laboratory facilities. Hydrocarbon extractions must therefore be delayed until well after the insects have been collected. Standard preservation techniques, such as freezing, are usually impractical. Keeping subcolonies, groups, or samples of a specific size alive and healthy for more than a few hours is very difficult. Hot sun and predacious ants usually render field samples unusable, and live cultures are prone to problems with humidity, food stress, and pathogenic fungi. Collection of specimens directly into 70% or 85% ethanol may partially extract hydrocarbons or include some debris and potential contaminants. Long-term retention of specimens in ethanol or in a solvent such as hexane for extended periods, although potentially quite convenient, may also extract internal hydrocarbons and confound the characterization of cuticular hydrocarbons.

We have collected termites from the islands in the Caribbean and have also received specimens collected by colleagues, for characterization of cuticular

hydrocarbons. The method of collection has varied among these efforts. To interpret these data we must know if different collection/extraction regimes are equivalent or comparable. We also want to recommend the most appropriate technique for a given taxon to collaborating scientists.

In the earliest studies of termites that introduced the concept of species specificity of cuticular hydrocarbons, it was not specified how the insects were handled before extraction (Howard et al., 1978, 1982a). In later studies of chemical mimicry by termitophilous staphylinids, Howard et al. (1980, 1982b) separated the termitophiles from the termites and froze the beetles at -20°C before extraction. Haverty et al. (1988) froze *Zootermopsis* spp. individuals, then subsequently thawed them to room temperature before extraction. The termites were brought from the field to the laboratory alive (Haverty et al., 1988). However, after extracting a few live termites, one of us (L.J.N.) noticed that the termites convulsed and emptied their gut contents into the hexane during the process. Freezing the insects, followed by extraction of the specimens at ambient temperature, prevented this possible source of contamination.

Subsequent studies of cuticular hydrocarbons of termites have used live termites extracted in the field or in the laboratory [Howard et al., 1988 (for *Nasutitermes*); Haverty et al., 1990b (for *Nasutitermes*), 1991a, unpublished (for *Reticulitermes*)], or termites dried in the field and later extracted in the laboratory [Haverty et al., 1990a, 1996b (for *Coptotermes*), 1991a, 1992 (for *Reticulitermes* and *Coptotermes*)]. Our preliminary results comparing extraction of live versus dried *Nasutitermes acajutlae* (Holmgren) indicated that the resulting hydrocarbon mixtures were not equivalent.

Many of our colleagues find it difficult to dry termites while collecting termites in the tropics because of logistical problems. Ovens, heating lamps, and electricity are not always available. Since drying is often impractical, many researchers prefer to collect termites directly into alcohol or hexane. Collectors also do not want to divert their efforts away from maximizing the number of samples they can collect while in the field. The usual field technique is to place termites directly into 80% or 100% ethanol for later transmittal to the museum or laboratory. Detailed comparisons of cuticular hydrocarbon patterns derived from extractions of live or dried termites or termites stored for extended periods in ethanol or hexane are thus warranted and are one focus of this study.

Another factor affecting the assessment of the cuticular hydrocarbon pattern is the number of insects that are extracted or quantity of hydrocarbon extracted. In some studies a variable number of individuals (and mixture of castes) was included. Haverty et al. (1991a) used from 15 to 200 *Reticulitermes* spp. workers per sample in their preliminary study of this genus. In most of our studies an exact number of termites was extracted: individual *Zootermopsis* spp. pseudergates, nymphs, soldiers, or alates (Haverty et al., 1988); 100 *Coptotermes formosanus* Shiraki workers or soldiers (Haverty et al., 1990a); 200 *C. formo-*

sanus workers or 50 soldiers (Haverty et al., 1996b); and 100 *Nasutitermes costalis* (Holmgren) or *N. ephratae* (Holmgren) large workers (Haverty et al., 1990b).

Extraction of too few individuals can result in a diluted extract and require a deviation from a standard technique. Characterization of hydrocarbons from dilute extracts would likely underestimate or not detect the less abundant compounds and overestimate the abundant compounds. We know of no published studies to determine the minimum number of individuals necessary to characterize the cuticular hydrocarbons of any termite taxon.

One of the primary reasons we began studies of extraction methodology was to remedy a problem encountered during studies of the cuticular hydrocarbons of *N. corniger* (Motchulsky), *N. ephratae*, and *N. costalis* (Haverty et al., 1990b; Howard et al., 1988). Haverty et al. (1990b) experienced difficulty obtaining a sufficiently large hydrocarbon sample by extracting 100 large workers of *N. costalis* or *N. ephratae* in the field in Trinidad. The samples taken during that study were barely sufficient to allow quantification of the hydrocarbon mixtures. Thus, we designed a first set of experiments in 1989 to better understand the difficulties experienced with the characterization of cuticular hydrocarbons from *N. costalis* and *N. ephratae*.

Given that any taxonomic or ecological study entails processing a large number of samples, it is essential to keep the method of analysis as consistent as possible. We have developed chromatographic conditions and mass spectral acquisition parameters that give optimal results for the vast majority of insect samples. We therefore focused our efforts on comparing various collection and extraction regimes using our standard analytical technique.

In the present study, we attempted to define a standard methodology for collecting and extracting termites under field conditions. Our field work was based at our research site on Guana Island, British Virgin Islands (BVI) (Thorne et al., 1994; Haverty et al., 1997). This island has a diverse termite fauna consisting of nine species in three families. The most conspicuous, and apparently abundant, species is the arboreal nesting *N. acajutlae* (Thorne et al., 1994; Scheffrahn et al., 1994). This species, as well as a closely related species, *N. nigriceps* (Holmgren), is numerically and ecologically conspicuous on many of the Caribbean islands (Thorne et al., 1994). A better understanding of the appropriate procedure(s) for sampling and extracting cuticular hydrocarbons of *N. acajutlae* is important to our understanding of the taxonomy, ecology, and biogeography of this and other *Nasutitermes* species. The methodology described here is optimal for sampling and extracting cuticular hydrocarbons for this species and should be applicable to most termites in the tropics. Very small termites, such as *Parvitermes* spp., will likely require larger samples, whereas the larger termites, such as *Neotermes* spp. and *Incisitermes* spp., will require fewer individuals.

For taxonomic studies we consider cuticular hydrocarbons useful primarily for separating species within a genus; obvious and consistent morphological characters usually suffice to separate taxa at higher levels (Haverty et al., 1991b). Hydrocarbons beneficial for taxonomic studies should be abundant, not minor components (at least 1.0%, but preferably 5.0% of the total hydrocarbon component). However, if a hydrocarbon is consistently detected, even in quantities below 1.0%, we would consider it a useful taxonomic character. Useful hydrocarbons should also be unique or present in only a few of the species, or conversely, they should be common in most of the species yet completely absent, rare, or of insignificant quantities in one or a few species. Furthermore, they should have a unique elution time so that they do not coelute with another hydrocarbon in the same species, nor should they elute at a time similar to that of a different hydrocarbon in a different species (Haverty et al., 1991b). From a set of hydrocarbons with these characteristics, dichotomous keys can be constructed. For ecological or biogeographic studies of the same species, examinations of the quantitative differences of the hydrocarbon mixture would be of paramount importance.

In this paper we report the results of studies conducted in 1989 and 1993 to compare and improve our sampling and extraction techniques for the characterization of cuticular hydrocarbons of tropical termites. In 1989 we examined influences of the number and duration of extractions. In 1993 we tried to design studies that would ultimately provide us with an optimal field method for characterizing both the composition and relative abundance of the cuticular hydrocarbons of *N. acajutlae*. We compare methodologies and suggest standard and alternative, acceptable methodologies for both chemotaxonomic and ecological studies of this termite. Specifically, we evaluate: (1) the effect of the duration and the number of extractions; (2) the effect of drying termite specimens before extraction; (3) the effect of sample size; and (4) the effect of solvents (ethanol versus hexane) on the reproducibility of cuticular hydrocarbon profiles.

METHODS AND MATERIALS

Collecting and Sorting Termites. We collected workers and soldiers of *N. acajutlae* during two separate trips (1989 and 1993) to Guana Island, BVI. During each trip different collection techniques were used. In 1989 two nests were sampled near White Sands Beach. Portions of nest material containing workers and soldiers were returned to the laboratory. Nest material was placed in pans and folded cardboard index cards were placed on top of this material. Workers and soldiers climbed onto the cards and then were tapped off into trays, where they were sorted, counted, and prepared for hydrocarbon analyses as described below.

In 1993 we selected 13 colonies from the same area, and we used a modification of a technique demonstrated to us by Dr. Jan Krecek. This is the best technique for collecting large numbers of workers and soliders with the least effect on nest structure. A tangential slice, 2–3 cm deep and 15–20 cm in diameter, was removed from the more fragile exterior portion of a nest and discarded. Over this breach we placed one or two 20-cm × 20-cm squares of moist corrugated cardboard. If we were able to cut the slice from the top of a nest, the corrugated cardboard squares were secured against the surface of the nest with a stone. If the slice was taken from the side of the nest (often we could not reach the top of a nest) the corrugated cardboard squares were secured to the nest with 7.5 cm, galvanized, finishing nails.

As soon as the slice of nest was removed and the corrugated cardboard squares put in place, soldiers swarmed out of the breach and covered the squares; workers immediately retreated into the nest. In less than 60 sec the squares were removed and a collection of nearly pure soldiers was tapped into a collection pan. To readily obtain a nearly pure sample of hundreds to thousands of soldiers, this process can be repeated several times. We then visually scanned all individuals in the collection pan and removed the few workers in the sample.

Workers were collected by leaving the moist squares of corrugated cardboard on the nest for up to 60 min. Once the alarm reaction of the soldiers began to dissipate, workers ventured to the underside of the squares and began to repair the breach. When the cardboard was gently removed, a dense sample of workers adhered to its surface. In contrast to the technique for collecting soldiers, the squares of corrugated cardboard containing the workers (and relatively few soldiers) were placed in a collection pan and returned to the field laboratory for sorting, counting, and preparation of workers for hydrocarbon analyses as described below.

Preparation of Termites for Extraction. Because soldiers squirt glue over the containers and themselves, they were difficult to count individually. For the purposes of cuticular hydrocarbon analyses, we measured ca. 5–8 ml of soldiers (about 500 individuals) in 20-ml scintillation vials (Wheaton scintillation vials, foil-lined caps) for extraction or drying. Workers were separated and individually counted into 20-ml scintillation vials for extraction or drying of the appropriate number of individuals. Alates were occasionally present; when collected, they were individually counted into vials and handled in exactly the same way as the workers.

Additional termites from each of the sampled colonies (with soldiers and alates, when available, as diagnostic castes) were placed in 80% ethanol (from Quantum Chemical Corp., 200-proof punctilious dehydrated alcohol) to serve as voucher specimens. These voucher specimens are kept at the Pacific Southwest Research Station, Albany, California, and the Department of Entomology, University of Maryland, College Park, Maryland.

Standard Extraction Process. Cuticular lipids were extracted by immersing termites in *n*-hexane (EM Science OmniSolv, suitable for HPLC, GC, and residue analysis). Our usual procedure has been a 10-min extraction of 100 termites in 10 ml of *n*-hexane. In this study, we used this procedure as the standard to evaluate the various extraction regimes described in the following sections. The lipid extracts resulting from each of the various methods were pipetted through 4 cm of activated BioSil-A (silica gel, BioRad Laboratories, 100–200 mesh) in Pasteur pipet minicolumns (5 mm ID) in order to isolate the hydrocarbon components. The resulting hydrocarbon extracts were evaporated to dryness under nitrogen and redissolved in 60 μ l of *n*-hexane for gas chromatography–mass spectrometry (GC-MS) analyses.

Duration and Number of Extractions. In 1989 we evaluated the following extraction regimes using *N. acajutlae* from Guana Island, BVI, to determine whether we could improve upon extracting 100 live workers in 10 ml of *n*-hexane for 10 minutes:

A. Extract 300 workers in 10 ml *n*-hexane for 10 min (standard technique with an increased sample size).

B. Extract 300 workers three times in 10 ml of *n*-hexane for 10 min, keeping each extract separate to determine if the standard technique left significant quantities of hydrocarbon on the sample.

C. Extract 300 workers in 10 ml of *n*-hexane for 20 sec followed by a 10-min extraction in 10 ml of *n*-hexane, keeping each extract separate to determine whether a brief extraction would produce a mixture of hydrocarbons comparable to the standard, A, extraction.

D. Extract 300 workers in 10 ml of *n*-hexane for 20 sec followed by extracting for 24 hr in 10 ml *n*-hexane, keeping each extract separate (same as extraction B with an extended second extraction) to determine whether an extended extraction would remove additional hydrocarbons from the cuticle or from other tissues.

E. Extract 300 workers in 10 ml of *n*-hexane for 24 hr to allow for a less stringent extraction schedule.

F. Long-term extraction of 300 workers in 10 ml of *n*-hexane for two years to allow field collection with subsequent laboratory storage for an extended period.

Effect of Drying. In 1993 six samples of 100 *N. acajutlae* workers from each of 13 nests were allocated to evaluate the effect of drying termites prior to extraction. The cuticular hydrocarbon profiles of the dried termites were compared to those generated from an extraction of live termites. Live termites were extracted at the Guana Island, BVI, field laboratory by placing each of three samples from each colony directly into separate 20-ml scintillation vials and extracting these samples in 10 ml of *n*-hexane for 10 min (standard procedure A). The hexane from each of 39 vials was then decanted into separate 20-ml

scintillation vials and subsequently returned to our laboratory in California for purification and analysis of cuticular hydrocarbons.

The other three samples of *N. acajutlae* workers from each of 13 colonies were dried by placing 20-ml scintillation vials, each containing 100 individuals, in a wire box over a single 75-W, reflecting, incandescent light. The amount of time required to completely dry termites varied slightly as a function of the position of the vials over the bulb. The position of the vials over the bulb was changed in an attempt to make the drying time similar for each sample. Once termites were completely dried, we attempted to keep them dry by tightly sealing the vials. The vials were returned to our laboratory in California for extraction, purification, and analysis of cuticular hydrocarbons. Until they were extracted, dried samples were kept in a freezer at -20°C in California to prevent possible fungal growth.

Effects of Sample Size. In 1993, samples of 25, 50, 100, or 200 *N. acajutlae* workers from five different colonies were extracted either live or dried as described above. Each combination (sample size $\times$ live versus dried $\times$ colony) was replicated three times.

Effects of Solvent. In 1993, for each of five colonies, three replicates of 200 workers were placed in 20 ml of 100% ethanol and left in the solvent for 60 days. The ethanol extract was decanted and evaporated to dryness under nitrogen. The extract was then redissolved in 10 ml of hexane and processed by the standard procedure. After the ethanol was drained from the termites, the insects were dried and extracted with hexane following the standard procedure.

Characterization of Cuticular Hydrocarbons. Each sample was analyzed by GC-MS in order that the presence of all compounds could be verified by mass spectral data. These data allowed us to obtain information about coeluting compounds in particular. GC-MS analyses were performed on a Hewlett-Packard (HP) 5890 gas chromatograph equipped with a HP 5970B Mass Selective Detector interfaced with HP Chemstation software. The GC-MS was equipped with an HP-1, fused silica capillary column (25 m $\times$ 0.2 mm ID) and operated in split mode (with a split ratio of 8:1). A 3- μl aliquot was injected into the GC-MS. Each mixture was analyzed using a temperature program from 200°C to 320°C at $3^{\circ}\text{C}/\text{min}$ with a final hold of 16 min. Electron impact (EI) mass spectra were obtained at 70 eV. *n*-Alkanes were identified by their mass spectra. Mass spectra of methylalkanes were interpreted as described by Blomquist et al. (1987) to identify methyl branch locations. Alkenes were identified by their retention times relative to *n*-alkanes and/or mass spectra; the latter only for olefins with 35 or more carbons. A typical alkene mass spectrum shows a molecular ion and a series of fragments at 14-mass unit intervals (e.g., 69, 83, 97), similar to those displayed by *n*-alkanes, less 2 mass units. Interpretation of the mass spectra of dienes and polyunsaturated hydrocarbons was extrapolated from this pattern, i.e., for each double bond, the molecular ion is decreased by 2 mass units.

In the text and tables, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the location of methyl groups (X-Me), the total number of carbons (subscript XX) in the hydrocarbon component excluding the methyl branch(es), and the number of double bonds following a colon (subscript Y). Thus, pentacosane becomes $n\text{-C}_{25}$; 11-methylpentacosane becomes 11-MeC₂₅; and pentacosene becomes C_{25:1}. Hydrocarbons are presented in the tables in the order of elution on our GC-MS system.

Integration of the total ion chromatogram was performed by the data analysis software (HP59974J Rev. 3.1.2) in the HP Chemstation. GC-MS peak areas were converted to percentages of the total hydrocarbon fraction. These percentages for each hydrocarbon peak were the response variables used to make statistical comparisons among preparation and extraction techniques.

Statistical Analyses. The effect of drying termites before extraction was assessed by a series of t tests of the differences between the mean percentage of a given hydrocarbon (three samples from each colony were averaged, which resulted in 13 replicates, one for each colony) from the two conditions (Steel and Torrie, 1960). The significance of the calculated t value was tested at $\alpha = 0.00227$ [0.05/22, the number of consistently abundant (0.3% or greater of the total) hydrocarbon peaks for workers]. Our null hypothesis was that the 10-min extraction of 100 dried termites was not significantly different from the standard 10-min extraction of 100 live termites.

The effect of group size was tested with an analysis of variance for each hydrocarbon (Steel and Torrie, 1960). Each treatment combination (sample size $\times$ colony) was replicated three times. The four sample sizes were compared separately for termites extracted live or dried. Our null hypothesis was that all sample sizes provide extracts with the same percentages of each hydrocarbon. The significance of the F statistic was also tested with $\alpha = 0.00227$. Significant differences among mean percentages for each hydrocarbon were determined by Tukey's honestly significant difference (HSD) procedure. We were looking for the smallest sample size that results in a hydrocarbon profile equivalent in percentage of hydrocarbons to that of the next greatest sample size. A sample size was considered inadequate if it yielded a hydrocarbon mixture that had significantly different percentages from that of the next larger sample size.

RESULTS AND DISCUSSION

Cuticular Hydrocarbons of N. acajutlae

We identified 33 hydrocarbons from workers, 45 from soldiers, and 43 from alates of *N. acajutlae*; a total of 60 different hydrocarbons or isomeric mixtures in this species (Table 1; Figure 1). The hydrocarbons found in all three

TABLE 1. RELATIVE ABUNDANCE (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS OF WORKERS, SOLDIERS, AND ALATES OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI^a

Hydrocarbon ^b	Workers		Soldiers		Alates	
	Mean	SD	Mean	SD	Mean	SD
C _{23:1}	0.32	0.32	0.41	0.17	0.00	0.00
C ₂₃	0.97	0.33	4.04	0.91	0.08	0.04
11-; 9-MeC ₂₃ ^c	0.00	0.00	0.51	0.15	0.00	0.00
3-MeC ₂₃ + C _{24:1} ^d	0.00	0.00	0.18	0.15	0.00	0.00
C ₂₄	0.22	0.22	0.66	0.19	0.00	0.00
11-MeC ₂₄	0.00	0.00	0.37	0.32	0.00	0.00
C _{25:1}	0.68	0.27	2.96	0.69	0.00	0.00
C ₂₅	3.05	1.14	9.16	1.28	0.55	0.14
13-; 11-MeC ₂₅ ^c	0.16	0.17	1.98	0.43	0.12	0.03
5-MeC ₂₅	0.00	0.00	0.00	0.00	0.07	0.01
2-MeC ₂₅	0.00	0.00	0.00	0.00	0.20	0.03
3-MeC ₂₅ + C _{26:1} ^d	0.00	0.00	0.57	0.13	0.00	0.00
5,17-DimeC ₂₅	0.00	0.00	0.00	0.00	0.08	0.01
C ₂₆	0.26	0.27	0.59	0.16	0.14	0.02
13-; 12-; 11-MeC ₂₆ ^c	0.00	0.00	0.42	0.35	0.00	0.00
2-MeC ₂₆	0.00	0.00	0.00	0.00	1.42	0.16
C _{27:1}	0.86	0.42	3.88	0.63	0.00	0.00
C ₂₇	1.90	1.60	3.67	0.92	1.44	0.28
13-; 11-MeC ₂₇ ^c	0.07	0.11	1.14	0.21	0.00	0.00
5-MeC ₂₇	0.00	0.00	0.00	0.00	0.08	0.04
2-MeC ₂₇	0.15	0.13	0.14	0.09	1.67	0.29
3-MeC ₂₇	0.00	0.00	0.00	0.00	0.72	0.13
C ₂₈	0.15	0.19	0.13	0.12	0.30	0.08
2-MeC ₂₈ + C _{29:1} ^d	0.00	0.00	0.19	0.10	0.53	0.11
C _{29:1} ^e						
C ₂₉	0.73	0.56	0.56	0.25	1.08	0.25
5-MeC ₂₉	0.00	0.00	0.00	0.00	0.07	0.05
2-MeC ₂₉	0.00	0.00	0.00	0.00	0.03	0.03
3-MeC ₂₉	0.00	0.00	0.00	0.00	0.04	0.05
C ₃₀	0.00	0.00	0.00	0.00	0.05	0.04
C ₃₁	0.06	0.15	0.02	0.04	0.58	0.08
C ₃₃	0.00	0.00	0.01	0.03	0.12	0.03
C _{35:1}	0.00	0.00	0.00	0.00	0.11	0.06
13-MeC ₃₅	0.00	0.00	0.00	0.00	0.12	0.06
C _{37:1}	0.40	0.17	0.65	0.17	0.40	0.14
C _{38:1}	0.53	0.20	0.58	0.13	0.47	0.07
C _{39:5} ^f	0.36	0.33	0.28	0.25		
C _{39:4} ^f	2.00	0.73	1.33	0.61	3.89	0.78
C _{39:2}	0.43	0.48	1.29	0.56	1.14	0.11
C _{39:1}	20.01	2.20	15.77	2.79	14.40	1.26
15-MeC ₃₉	0.04	0.12	0.36	0.27	0.00	0.00

TABLE 1. Continued

Hydrocarbon ^b	Workers		Soldiers		Alates	
	Mean	SD	Mean	SD	Mean	SD
C _{40:5}	0.00	0.00	0.00	0.00	0.90	0.35
C _{40:1}	2.51	0.42	2.89	0.62	0.00	0.00
C _{41:4} + C _{41:5} ^g	18.77	3.81	11.01	3.92	21.72	2.41
C _{41:2}	1.21	0.63	1.50	0.49	3.31	0.20
C _{41:1}	24.43	2.74	17.42	3.52	20.24	1.18
15-MeC ₄₁	0.13	0.23	0.46	0.24	0.76	0.15
C _{42:1}	1.60	0.65	1.99	1.03	0.00	0.00
C _{43:6} + C _{43:5} ^h	4.35	1.37	2.41	1.09	6.24	1.11
C _{43:2}	0.83	0.52	1.14	0.43	2.63	0.27
C _{43:1}	10.40	1.61	7.66	1.48	10.28	1.09
15-MeC ₄₃	0.00	0.00	0.00	0.00	0.43	0.11
C _{45:5} ^e						
C _{45:2}	0.00	0.00	0.00	0.00	0.32	0.11
C _{45:1}	2.41	0.82	1.69	0.64	3.26	0.87

^aThree subsamples of 100 workers from each of 13 colonies. Four subsamples of ca. 4 ml of soldiers from each of 13 colonies. Two subsamples of 25–31 alates from four colonies.

^bThis shorthand (X-MeC_{XX} and C_{XX:Y}) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

^cAn isomeric mixture. These monomethylalkanes coelute.

^dThis monomethylalkane and the olefin coelute.

^eThese hydrocarbons were not detected in samples from Guana Island, but were found in samples from Tortola, BVI. C_{29:1} was found in trace amounts in soldiers only; C_{45:5} was found in all three castes, but in amounts averaging <1.0%.

^fThese two isomers occasionally did not resolve in alates. Therefore, the areas for the two isomers were summed for alates only.

^gThese two isomers occasionally did not resolve. Therefore, the areas for the two isomers were summed.

^hC_{43:6} was identified only in alates. There were two isomers of C_{45:3} that occasionally did not resolve. Therefore, the areas for the two isomers were summed.

castes segregate into two distinct groups. The early-eluting components are primarily *n*-alkanes, methyl-branched alkanes, and a few normal alkenes. The late-eluting compounds consist almost exclusively of unsaturated hydrocarbons with chain lengths of 37–45 carbons and one to six double bonds and a few monomethyl alkanes in trace amounts (especially in alates). Soldiers have considerably greater proportions of the early-eluting compounds (23–29 carbons) than do workers or alates (Table 1; Figure 1). Whereas workers and alates have at least 88% of the cuticular hydrocarbons with 31 or more carbons, soldiers have only about 69% of these late-eluting compounds. The predominant class

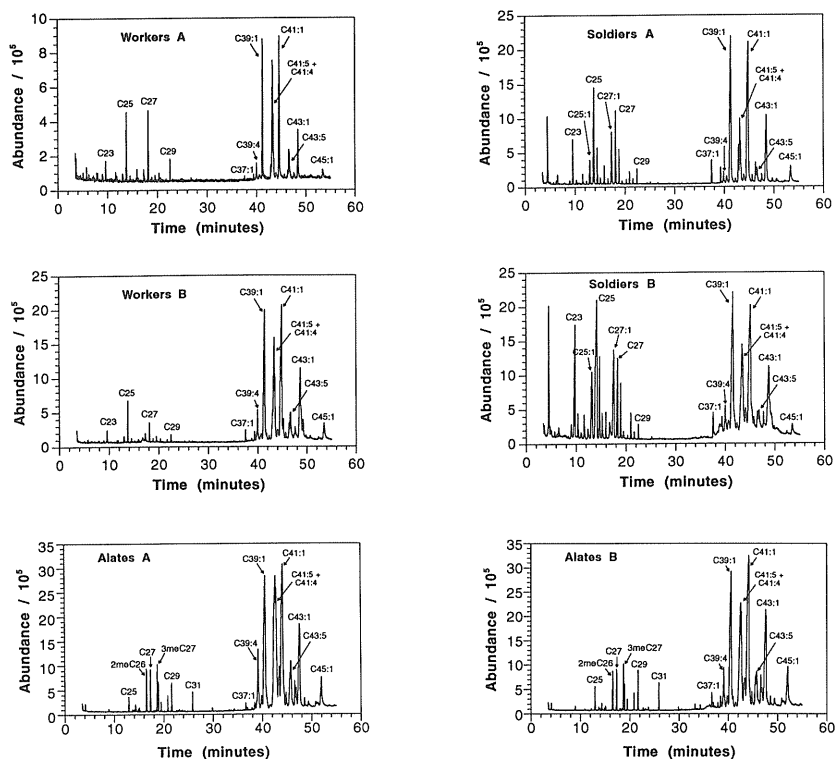


FIG. 1. Total ion chromatograms of the cuticular hydrocarbons of workers, soldiers, and alates of *Nasutitermes acajutlae* (Holmgren) from two colonies (A and B) from Guana Island, BVI. The cuticular hydrocarbon extracts were derived from dried samples of 100 workers, ca. 8 ml of soldiers, and 25 alates collected in October 1993.

of hydrocarbons in all three castes is the olefin fraction (Table 1). No dimethyl- or trimethylkanes were found in workers or soldiers.

n-Alkanes. Normal alkanes ranged from $n\text{-C}_{23}$ to $n\text{-C}_{33}$; $n\text{-C}_{25}$ and $n\text{-C}_{27}$ were usually the most abundant. The n -alkanes were least abundant in alates, moderately abundant in workers, and most abundant in soldiers (Tables 1; Figure 1).

Internally Branched Monomethylalkanes. This class of hydrocarbon was nearly always encountered, in trivial amounts in workers and in significant amounts in soldiers (5.2% of the total hydrocarbon), and accounted for only 1.4% of the total hydrocarbon in alates. For soldiers most of this class of hydrocarbon eluted early, while those found in alates were in the late-eluting fraction (Table 1).

Terminally Branched Monomethylalkanes. The 2- and 3-methylalkanes occurred in trivial amounts in workers and soldiers. Alates usually had a significant component of 2-methylalkanes. These hydrocarbons were always found in the early-eluting constituents (Table 1; Figure 1).

Dimethyl- and Trimethylalkanes. Only one dimethylalkane was identified: 5,17-dimeC₂₅. It was found only in trivial amounts in alates (Table 1).

Olefins. The unsaturated component is the paramount class of hydrocarbons in the cuticular lipids of *N. acajutlae*. Olefins comprise over 90% of the total hydrocarbons in workers and alates. Because soldiers contain a larger proportion of early-eluting *n*-alkanes, the olefin component amounts to an average of 75.6% of the total hydrocarbons in this caste. For all castes, C_{39:1}, C_{41:5}, C_{41:4}, C_{41:1}, and C_{43:1} combined account for >70% of the total olefin fraction (Table 1).

We are confident in our identification of the late-eluting monounsaturated alkenes. The spectra of C_{39:1} and C_{41:1} display the parent ion (546 and 574, respectively) as well as the characteristic fragmentation pattern with the predominant peaks of fragments (83 and 97) being 2 mass units less than would be expected from *n*-alkanes and exceeding fragment 67 in abundance (Figure 2). The polyunsaturated components, for example, C_{41:4} and C_{41:5}, are identified by the presence of parent ions (568 and 566, respectively) which are 8 and 10 mass units less than the normal alkane with the same number of carbons (Figure 3).

Polyunsaturated alkenes have not been commonly reported from the cuticular hydrocarbons of termites. However, Moore (1969) was the first to describe the cuticular lipids from a termite, *Nasutitermes exitiosis* (Hill), from Australia. He found a complex mixture of unsaturated components with the predominant component identified as a quadrupally unsaturated, unbranched hydrocarbon, C_{39:4}. Similarly, we have identified high-molecular-weight dienes, trienes, and tetraenes from the cuticular lipids of *Cryptotermes brevis* (Walker), *Procrystotermes corniceps* (Snyder), *Incisitermes* spp., *Parvitermes wolcotti* (Snyder), and *N. acajutlae*, as well as other species of *Nasutitermes* from the Caribbean Basin (Haverty et al., 1992, 1997).

Effect of Duration and Number of Extractions

Early in our research on the chemotaxonomy of termites we extracted live or recently frozen individuals. The standard 10-min extraction of 300 live workers of *N. acajutlae* (Figure 4A) allowed us to resolve and characterize most of the components identified for this species (Tables 1 and 2). Additional components were identified by drying termites first (see Table 1 and *Effects of Drying* below). We also discovered that different nests of this species from the same island produced qualitatively similar (not identical), but quantitatively dissimilar, hydrocarbon mixtures (Figure 1; and unpublished observations). Further-

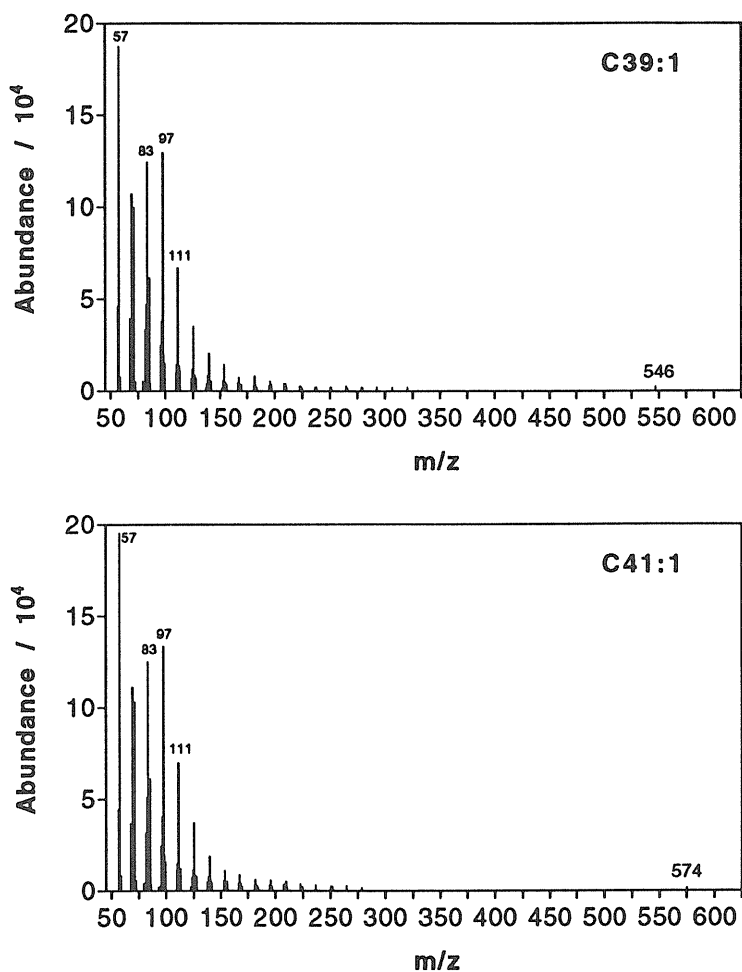


Fig. 2. Mass spectra of nonatriacontene (C_{39:1}) and hentetracontene (C_{41:1}) from cuticular hydrocarbons from workers of *Nasutitermes acajutlae* from Guana Island, BVI.

more, these colony-specific hydrocarbon analyses are repeatable; when a second group of 300 live workers was extracted using the standard procedure, the hydrocarbon mixtures from each colony were qualitatively identical and quantitatively quite similar (Figure 4A and B1; Table 2). A second 10-min extraction of the same 300 workers resulted in a hydrocarbon mixture that was quite similar to the mixture from the first extraction (Figure 4B2), although the ion abundances of the peaks were about half those in the first extract. A third, 10-min extraction resulted in an extract that did not resemble those from either of the

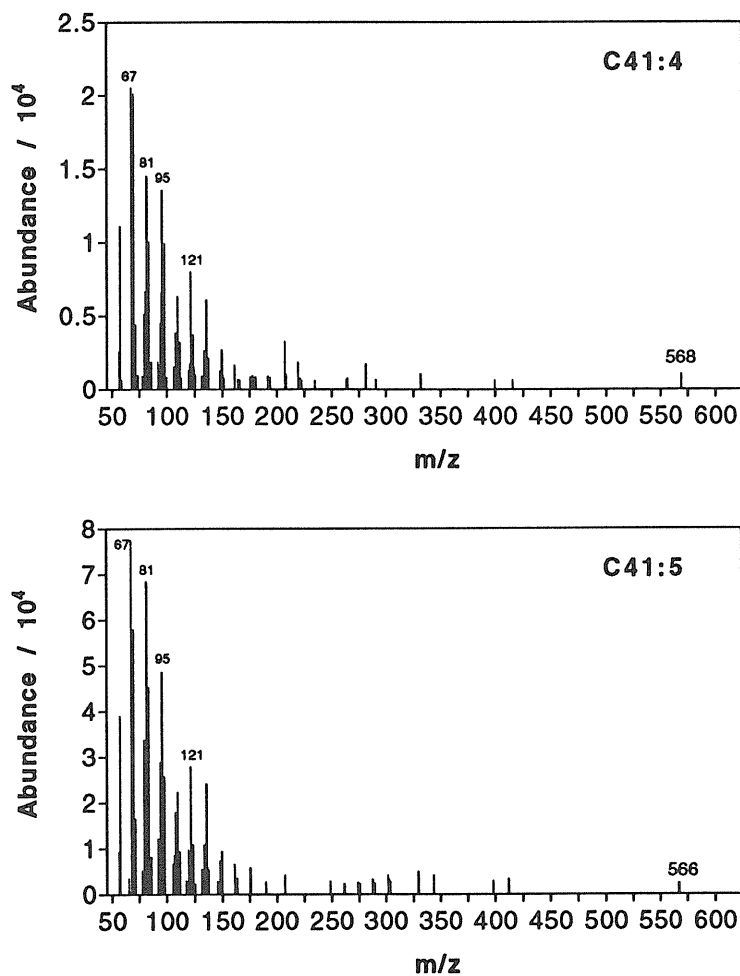


FIG. 3. Mass spectra of hentetracontatetraene ($C_{41:4}$) and hentetracontapentaene ($C_{41:5}$) from cuticular hydrocarbons from workers of *Nasutitermes acajutlae* from Guana Island, BVI.

first two extractions; only the predominant peaks (C_{25} , C_{27} , $C_{39:1}$, and $C_{41:1}$) were detected (Table 2; Figure 4B3).

An extraction of 300 live workers in 10 ml of hexane for only 20 sec appeared to be equivalent to a 10-min extraction (Table 2; Figure 4A, C1, and D1). Furthermore, the 20-sec extraction was repeatable. A subsequent 10-min extraction of the same workers produced a hydrocarbon mixture similar to that of a 20-sec extraction, although the ion abundances of the peaks were about

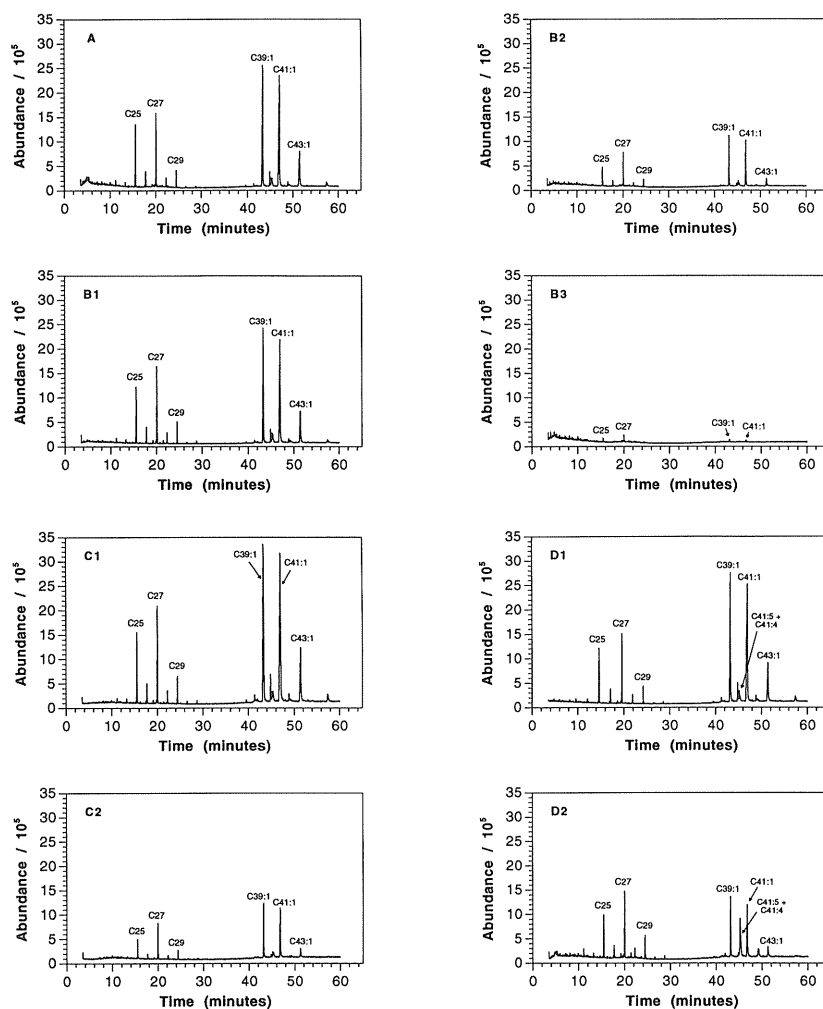


FIG. 4. Chromatograms of cuticular hydrocarbons extracted from 300 live workers from one nest of *Nasutitermes acajutlae* from Guana Island, BVI. A = extraction with 10 ml hexane for 10 min (standard); B1 = first extraction with 10 ml hexane for 10 min (equivalent to the standard); B2 = second extraction of the same termites as B1 with 10 ml hexane for 10 min; B3 = third extraction of the same termites as B1 and B2 with 10 ml hexane for 10 min; C1 = first extraction with 10 ml hexane for 20 sec; C2 = second extraction of the same termites as C1 with 10 ml hexane for 10 min; D1 = first extraction with 10 ml hexane for 20 sec (equivalent to C1); D2 = second extraction of the same termites as D1 with 10 ml hexane for 24 hr; E = extraction with 10 ml hexane for 24 hr; F = extraction with 10 ml hexane for two years. Samples A–F were each derived from independent groups of 300 workers.

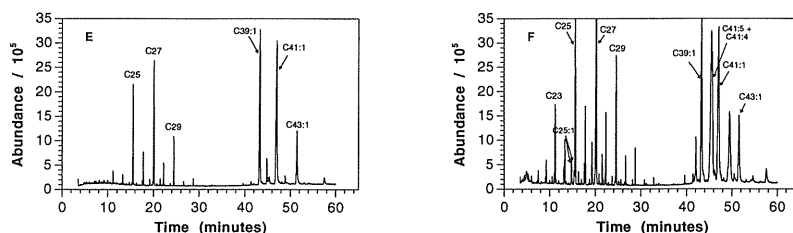


FIG. 4. Continued.

TABLE 2. RELATIVE ABUNDANCE (MEAN PERCENT FROM TWO COLONIES) OF CUTICULAR HYDROCARBONS OF WORKERS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, RESULTING FROM DIFFERENT EXTRACTION REGIMES^a

Hydrocarbon ^b	A	B1	B2	B3	C1	C2	D1	D2	E
C ₂₃	0.3	0.4	0.0	0.0	0.2	0.0	0.3	1.5	0.6
C ₂₄	0.3	0.3	0.0	0.0	0.2	0.0	0.2	0.5	0.3
C _{25:1}	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C ₂₅	4.7	4.7	5.3	18.4	3.5	4.9	3.9	7.5	5.6
C ₂₆	0.9	1.0	0.9	0.0	0.7	0.9	0.7	1.4	1.1
2-MeC ₂₆ + C _{27:1}	0.4	0.3	0.0	0.0	0.1	0.0	0.2	1.2	0.5
C ₂₇	5.1	6.1	7.5	20.1	4.2	7.5	4.3	9.5	6.7
C ₂₈	0.4	0.6	0.8	0.0	0.4	0.8	0.4	1.3	0.7
C ₂₉	1.0	1.3	1.8	0.0	0.9	1.8	0.8	3.0	1.7
C _{37:1}	0.2	0.2	0.2	0.0	0.3	0.0	0.3	0.0	0.3
C _{38:1}	0.4	0.6	0.7	0.0	0.8	0.0	0.6	0.2	0.4
C _{39:4}	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
C _{39:1}	32.0	30.0	31.7	34.3	31.3	32.6	31.4	23.0	28.4
C _{40:1}	2.7	2.9	2.4	0.0	2.9	1.6	3.0	1.8	2.5
C _{41:5}	0.7	0.7	1.6	0.0	0.7	1.2	0.7	3.9	0.7
C _{41:4}	0.9	0.9	2.4	0.0	0.9	2.3	1.5	8.0	0.7
C _{41:1}	37.1	35.4	35.9	27.2	37.7	38.2	37.2	25.9	35.3
C _{42:1}	0.9	1.1	0.4	0.0	1.2	0.0	1.2	0.0	1.0
C _{43:5}	0.0	0.1	0.0	0.0	0.0	0.0	0.4	2.0	0.0
C _{43:1}	11.2	11.2	8.0	0.0	11.8	8.3	11.3	6.3	10.9
C _{45:1}	0.6	1.5	0.4	0.0	1.6	0.0	1.7	0.1	1.4

^aExtraction regimes A-F are outlined in the Methods and Materials and in the legend of Figure 4. Extraction procedure F was not included because of numerous unique, unknown, undetermined, or different peaks.

^bThis shorthand (X-MeC_{XX} and C_{XX:Y}) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

half those in the first extract (Table 2; Figure 4C1 and C2). Extraction of 300 workers for 24 hr after a 20-sec extraction resulted in an extract that was different from the standard extraction (10 ml for 10 min) or the 20-sec extraction. Proportional relationships changed dramatically: C_{25} and C_{27} were much more prominent as were $C_{41:4}$ and $C_{41:5}$, in extracts from the second extraction (Table 2; Figure 4A, D1, and D2).

Extraction of 300 workers for 24 hr resulted in a mixture of hydrocarbons that appeared similar to the standard 10-min extraction or to a 20-sec extraction (Table 2; Figure 4A, C1, D1, and E). Holding a sample of 300 workers in hexane for a period of two years provided a radically different mixture of hydrocarbons than the standard 10-min extraction (Figure 4F). Many hydrocarbons that we rarely see in *N. acajutlae* workers (such as C_{22} , $C_{23:1}$, $C_{24:1}$, two isomers of $C_{25:1}$, $C_{26:1}$, 11- and 13-Me C_{25} , $C_{27:2}$, 2-Me C_{26} , $C_{29:2}$, 5-Me C_{29} , $C_{31:1}$, and $C_{43:2}$) were present in quantities exceeding trace ($\bar{X} < 0.3\%$) amounts. One isomer of $C_{25:1}$ was identified in extractions of workers of *N. acajutlae* in 1993. However, the additional isomer (same mass spectrum with a different retention time) was not seen in the 1993 extractions. Furthermore, some compounds (C_{25} , C_{27} , $C_{41:5}$, and $C_{41:4}$) were present in much greater proportions. As a result of these qualitative and quantitative differences, we feel that a characteristic mixture of cuticular hydrocarbons can be obtained with extractions lasting from 20 sec to 24 hr. We do not consider the hydrocarbon mixtures from two-year extractions to be comparable to the standard 10-min extraction.

Effect of Drying

Mixtures of cuticular hydrocarbons extracted from live or dried workers were quantitatively different from one another. Of the 32 hydrocarbon peaks, the percentages of 20 of them were significantly different (Table 3). The most striking differences were exhibited in the late-eluting alkenes (Table 3; Figure 5). Drying the workers before extraction resulted in highly significant differences in the relative amounts of $C_{41:4}$ and $C_{41:5}$. Related to the apparent increased efficiency of the extraction of these compounds was the apparent decrease in the relative amounts of the most abundant compounds, $C_{39:1}$, $C_{41:1}$, and $C_{43:1}$.

In general, drying workers prior to extraction tended to enhance extraction of the less abundant unsaturated compounds. Extraction of 100 dried workers did not result in equivalent mixtures of hydrocarbons when compared to extraction of 100 live workers; these extraction regimes are not comparable for taxonomic or ecological purposes. Using either one of these extraction regimes exclusively would suffice for characterization of cuticular hydrocarbons for ecological studies, because the primary consideration would be for comparing the relative or absolute quantities of hydrocarbons. However, for taxonomic studies, the goal is to identify hydrocarbons that are consistently present even in quan-

TABLE 3. RELATIVE ABUNDANCES (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS FROM SAMPLES OF 100 WORKERS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, EXTRACTED ALIVE OR AFTER DRYING

Hydrocarbon ^a	Extracted live ^b		Extracted dry ^b		<i>t</i> value ^c
	Mean	SD	Mean	SD	
C ₂₃	0.80	0.29	0.97	0.33	-3.029
C ₂₄	0.45	0.27	0.22	0.22	3.348
C _{25:1}	0.36	0.35	0.68	0.27	-4.445
C ₂₅	2.64	0.95	3.05	1.14	-2.618
C ₂₆	0.53	0.32	0.26	0.27	4.453
C _{27:1}	0.34	0.33	0.86	0.42	-6.610
C ₂₇	1.40	0.95	1.90	1.60	-2.706
C ₂₈	0.37	0.22	0.15	0.19	3.251
C ₂₉	0.53	0.34	0.73	0.56	-2.438
C _{37:1}	0.45	0.22	0.40	0.17	1.724
C _{38:1}	0.63	0.25	0.53	0.20	3.537
C _{39:4}	0.54	0.40	2.00	0.73	-7.526
C _{39:2}	0.08	0.36	0.43	0.48	-3.272
C _{39:1}	28.12	2.49	20.01	2.20	17.314
C _{40:1}	2.84	0.27	2.51	0.42	3.928
C _{41:4} + C _{41:5}	6.23	3.61	18.77	3.81	-16.929
C _{41:1}	33.77	2.73	24.43	2.74	15.720
C _{42:1}	1.46	0.50	1.60	0.65	-1.650
C _{43:5}	1.01	1.13	4.35	1.37	-9.746
C _{43:2}	0.04	0.12	0.83	0.52	-5.876
C _{43:1}	13.15	1.41	10.40	1.61	11.519
C _{45:1}	3.44	0.66	2.41	0.82	6.233

^aThis shorthand (X-MeC_{XX} and C_{XX:Y}) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

^bThree subsamples of 100 workers from 13 colonies were either placed in a scintillation vial alive, then extracted for 10 min with 10 ml of hexane or placed in a vial, dried over an incandescent light, then extracted for 10 min with 10 ml of hexane.

^cThe critical *t* for $\alpha = 0.00227$ with 12 *df* is 3.859 for each comparison for each hydrocarbon. If $|t| > t_c$ then the difference is significant.

ties as low as 0.3% of the total hydrocarbon. Drying termites prior to the extraction greatly increases the chance of consistently detecting hydrocarbons of low abundance, especially the olefin fraction; therefore we feel dried samples should be used for taxonomy.

There is the possibility that these differences are due to the extraction of hydrocarbons from internal tissues. By definition, these hydrocarbons are not

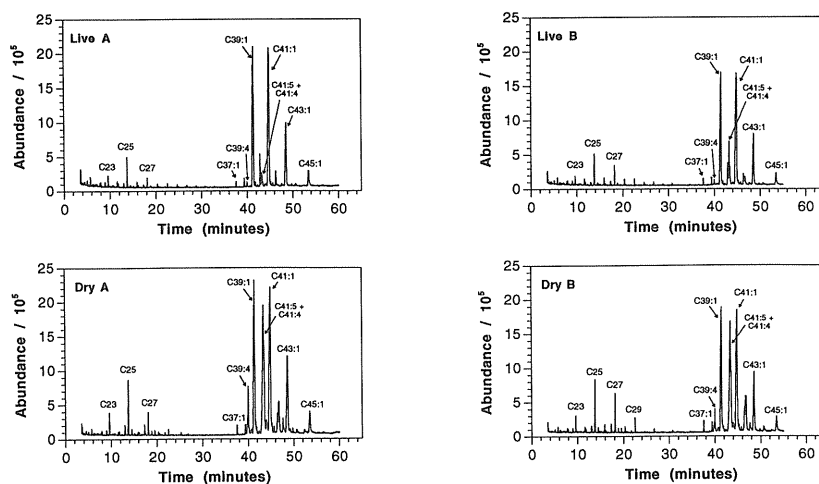


FIG. 5. Chromatograms of cuticular hydrocarbons extracted from 100 workers from two nests (A and B) of *Nasutitermes acajutlae* from Guana Island, BVI. Workers were extracted with 10 ml of hexane for 10 min either alive or after drying.

cuticular lipids, although the components may be the same (de Renobales et al., 1991). Dried termites are more fragile and often lose legs, antennae, or even heads during shipment, and also, the cuticle can become cracked. These conditions could allow the hexane to penetrate and extract lipids from the inner layers of cuticle and internal tissues. It is conceivable that some of the hydrocarbons extracted from our dried samples are not surface hydrocarbons, but those deposited on the external surface of the cuticle of the next instar (Howard et al., 1995). We found no evidence that any abundant hydrocarbons were unique to dried samples. However, the minor components $C_{41:2}$ and $C_{43:2}$ may have been unique to dried samples ($\bar{X} < 0.1\%$ in live samples; $\bar{X} > 0.8\%$ in dried samples for each component).

After examining hundreds of samples of termites extracted alive or after drying, it appears to us that the chromatograms from dried individuals are sharper and have a flatter baseline than those from live, field-extracted individuals. Three possible reasons are: (1) live termites void gut contents when placed in hexane and thus introduce contaminants, (2) live insects have a higher water content in the cuticle and less hydrocarbon is extracted because hexane is hydrophobic, or (3) hexane extracts in vials solubilize contaminants from the vial lids during transit from the field to the laboratory. The vials used were lined with foil, not Teflon. When we stored clean hexane in vials that were upside down, the resulting chromatogram had an uneven baseline similar to that resulting from extraction of live termites; however, no hydrocarbon peaks were seen. Further study of this phenomenon is warranted.

Effects of Sample Size

Statistically significant differences ($\alpha = 0.00227$) in the percentage of hydrocarbon components among sample sizes were found for 16 hydrocarbon peaks from workers extracted alive (Table 4). The most abundant components, $C_{39:1}$ and $C_{41:1}$, were not significantly different among sample sizes. Samples of 25 or 50 workers were found to be significantly different in only three cases: C_{26} , C_{28} , and C_{29} . Samples of 25 workers yielded extracts with hydrocarbon proportions significantly different from samples of 100 workers in 13 cases and from samples of 200 workers in 16 cases (Table 4). Samples of 50 workers yielded extracts with hydrocarbon proportions significantly different from sam-

TABLE 4. RELATIVE ABUNDANCE (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS FROM FOUR SAMPLE SIZES OF WORKERS FROM FIVE NESTS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, EXTRACTED ALIVE^a

Hydrocarbon ^b	25 workers		50 workers		100 workers		200 workers		<i>F</i>
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
C_{23}	1.33a	0.50	1.26ab	0.40	0.85bc	0.20	0.74c	0.14	10.82
C_{24}	1.18a	0.59	0.99a	0.25	0.41b	0.18	0.41b	0.09	20.52
$C_{25:1}$	0.00a	0.00	0.01a	0.05	0.33b	0.30	0.25b	0.13	15.31
C_{25}	4.73a	1.31	4.05ab	1.15	2.84b	0.48	2.78b	0.44	15.66
C_{26}	1.49a	0.53	1.01b	0.24	0.49c	0.18	0.45c	0.13	39.79
$C_{27:1}$	0.14a	0.44	0.11a	0.21	0.42a	0.31	0.36a	0.15	4.03
C_{27}	2.48a	1.23	1.97a	0.73	1.59a	0.65	1.44a	0.66	4.44
C_{28}	1.17a	0.41	0.75b	0.19	0.32c	0.21	0.25c	0.10	41.75
C_{29}	1.18a	0.39	0.73b	0.15	0.46bc	0.26	0.35c	0.10	32.58
$C_{37:1}$	0.07a	0.18	0.14a	0.22	0.50b	0.18	0.48b	0.15	22.52
$C_{38:1}$	0.21a	0.38	0.33ab	0.27	0.68bc	0.23	0.80c	0.16	15.89
$C_{39:4}$	0.10a	0.22	0.15a	0.27	0.73b	0.34	0.81b	0.38	21.89
$C_{39:2}$	0.00a	0.00	0.00a	0.00	0.00a	0.00	0.08a	0.16	3.70
$C_{39:1}$	29.2a	2.47	28.7a	1.62	27.9a	2.70	26.4a	1.32	5.04
$C_{40:1}$	2.01a	0.85	2.47ab	0.40	2.87b	0.13	3.23c	0.52	14.10
$C_{41:4} + C_{41:5}$	5.47a	2.51	6.43a	2.44	7.92a	4.12	9.27a	2.52	4.70
$C_{41:1}$	35.5a	2.79	32.2a	8.88	32.4a	2.90	30.4a	1.98	2.79
$C_{42:1}$	0.99a	0.78	1.50ab	0.77	1.54ab	0.58	2.12b	0.66	6.50
$C_{43:5}$	0.23a	0.50	0.94ab	0.97	1.49ab	1.44	2.20b	0.71	11.14
$C_{43:2}$	0.00a	0.00	0.02a	0.08	0.03a	0.11	0.33b	0.11	49.69
$C_{43:1}$	10.7a	1.26	11.5ab	0.87	12.5b	1.48	12.4b	1.11	7.10
$C_{45:1}$	1.29a	1.10	1.96ab	0.84	3.21c	0.59	2.70bc	0.98	13.18

^aMeans are from three samples from each of five separate nests ($N = 15$). Means for a given hydrocarbon followed by the same letter are not significantly different at the $\alpha = 0.00227$ level.

^bThis shorthand (X-MeC_{XX} and C_{XX:Y}) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

ples of 100 workers in seven cases and from samples of 200 workers in 11 cases. Samples of 100 and 200 workers yielded extracts with hydrocarbon proportions significantly different in only two cases. If workers were extracted while alive, it appears that the cuticular hydrocarbon mixtures change the least in samples of 100 or more.

Statistically significant differences ($\alpha = 0.00227$) in the percentage of hydrocarbon components among sample sizes were found for 13 hydrocarbon peaks when workers were extracted after drying (Table 5). Contrary to the results from live workers, the most abundant hydrocarbons $C_{39:1}$ and $C_{41:1}$, did display statistically significant differences among sample sizes. Samples of 25 workers yielded no statistically significant differences from samples of 50 workers for any hydrocarbons. Samples of 25 workers yielded extracts with hydrocarbon proportions significantly different from samples of 100 in 12 cases and from 200 workers in only five cases. Samples of 50 workers yielded extracts with hydrocarbon proportions significantly different from samples of 100 or 200 workers in only one case each (Table 5). Samples of 100 and 200 workers were not found to be significantly different for any of the 22 hydrocarbons analyzed. Similar to the result of extracting live workers, extracting workers after drying changed the least in sample sizes of 100 or more; however, extracts of samples of 50 workers were significantly different only for $C_{39:2}$, which had a relative abundance of 0.0% in samples of 25 or 50 workers and ca. 0.7% in samples of 100 or 200 workers.

The less abundant or trace compounds from extractions of the standard group size (100) are either missing or infrequently recorded (resulting in a lower mean value) in samples of 25 or 50 workers (Tables 4 and 5; Figures 6 and 7). The most abundant compounds, such as $C_{39:1}$ and $C_{41:1}$, have a lower mean value in samples of 100 or 200 workers than in groups of 25 workers (Tables 4 and 5; Figures 6 and 7). This undoubtedly results from the greater contribution of the minor compounds to the total hydrocarbon mixtures in the larger sample sizes; many of these trace compounds are not recorded in the samples of 25 or 50 workers, and they do not add to the total hydrocarbons (Figures 6 and 7). Most of the less abundant components fall below the line of equality (dashed line).

Thus, for workers of *N. acajutlae*, 100 appears to be the optimum sample size for adequately characterizing the cuticular hydrocarbons for quantitative comparisons using our operating parameters, regardless of whether the workers are extracted while alive or after drying. If the goal is to characterize all hydrocarbon components, even though they might be present in trivial amounts, then a larger sample (200–300 workers) should be taken, the extracts should be concentrated beyond our standard 60 μ l, or the split ratio of the gas chromatograph should be altered. Collecting fewer insects in the field would save time and add to the convenience of field workers, but altering the chromatographic

TABLE 5. RELATIVE ABUNDANCE (MEAN PERCENT AND STANDARD DEVIATION) OF CUTICULAR HYDROCARBONS FROM FOUR SAMPLE SIZES OF WORKERS FROM FIVE NESTS OF *Nasutitermes acajutlae* (HOLMGREN) FROM GUANA ISLAND, BVI, EXTRACTED AFTER DRYING^a

Hydrocarbon ^b	25 workers		50 workers		100 workers		200 workers		<i>F</i>
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
C ₂₃	1.20a	0.38	1.08a	0.28	0.93a	0.20	1.12a	0.26	2.36
C ₂₄	0.34a	0.34	0.39a	0.28	0.24a	0.23	0.36a	0.20	0.89
C _{25:1}	0.24a	0.41	0.40ab	0.27	0.64b	0.21	0.59ab	0.19	6.29
C ₂₅	5.25a	2.44	3.83ab	1.09	3.04b	0.77	3.69ab	0.81	6.20
C ₂₆	0.41a	0.35	0.41a	0.31	0.35a	0.29	0.41a	0.26	0.14
C _{27:1}	0.33a	0.43	0.58ab	0.22	0.89b	0.56	0.74ab	0.25	5.71
C ₂₇	2.75a	1.62	2.49a	1.47	2.13a	1.44	2.65a	1.46	0.49
C ₂₈ ^c	0.05a	0.13	0.25a	0.20	0.26a	0.22	0.29a	0.17	5.55
C ₂₉	0.62a	0.50	0.69a	0.42	0.84a	0.52	0.85a	0.50	0.81
C _{37:1}	0.12a	0.22	0.22ab	0.15	0.47b	0.07	0.43ab	0.15	16.98
C _{38:1}	0.26a	0.35	0.45ab	0.16	0.57b	0.10	0.63b	0.21	8.03
C _{39:4}	0.97a	0.75	1.30ab	0.43	2.00b	0.68	1.91ab	0.92	7.07
C _{39:2}	0.00a	0.00	0.00a	0.00	0.67b	0.46	0.69b	0.28	31.89
C _{39:1}	22.6a	1.99	21.3ab	1.57	19.6b	1.97	19.6b	1.79	9.23
C _{40:1}	1.90a	0.29	2.05ab	0.33	2.41b	0.19	2.27ab	0.55	5.85
C _{41:4} + C _{41:5}	18.6a	4.60	18.9a	3.05	19.4a	3.39	17.4a	2.43	0.87
C _{41:1}	28.7a	2.20	26.3ab	1.85	23.1b	1.77	23.4b	3.16	19.09
C _{42:1}	1.33a	1.02	1.75a	0.80	1.72a	0.52	1.44a	0.49	1.19
C _{43:5}	3.10a	1.58	4.17a	0.98	4.25a	1.92	4.40a	1.09	2.54
C _{43:2}	0.12a	0.47	0.48ab	0.32	0.91b	0.63	0.94b	0.37	10.62
C _{43:1}	9.58a	1.01	9.84a	0.76	10.0a	1.77	10.3a	1.30	0.78
C _{45:1}	1.35a	0.72	1.89ab	0.54	2.47b	0.55	2.26ab	0.83	7.99

^aMeans are from three subsamples from each of five separate nests ($N = 15$). Means for a given hydrocarbon followed by the same letter are not significantly different at the $\alpha = 0.00227$ level.

^bThis shorthand (X-MeC_{XX} and C_{XX:Y}) uses descriptors for the location of the methyl group (X-Me), the total number of carbons (XX), excluding the methyl branch(es), and the number of double bonds (Y). Locations of double bonds are undetermined.

^cThe F statistic has a $P < 0.00227$, yet the Tukey's procedure did not separate any of the means for this hydrocarbon.

parameters would increase the complexity and repeatability of laboratory operations. If a sample of 100 workers is not attainable, then concentration of the extract or altering the split ratio must be done or the sample will not be usable.

It is likely that our earlier problem with quantifying hydrocarbons from *N. costalis* and *N. ephratae* (Haverty et al., 1990b) resulted from two confounding problems. First, the workers were extracted live, in the field, which resulted in a less efficient extraction of the hydrocarbons, especially the less abundant components. Second, while 100 workers were used in the extractions, due to the

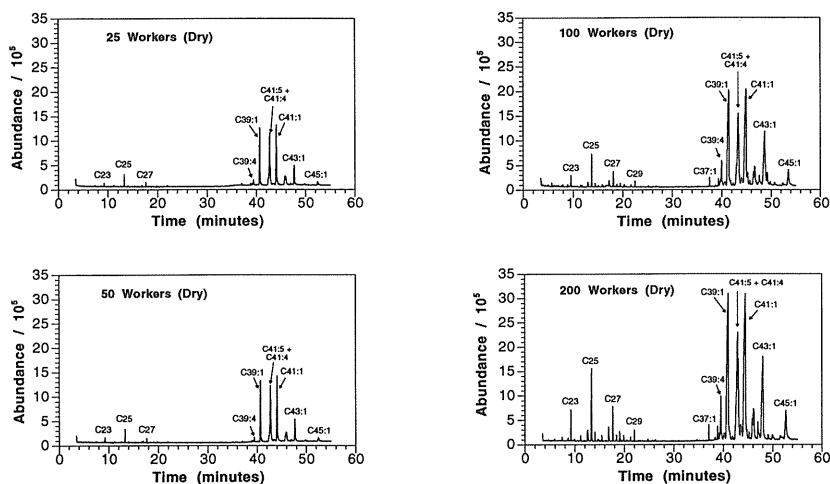


FIG. 6. Chromatograms of cuticular hydrocarbons extracted with 10 ml hexane for 10 min from four sample sizes (25, 50, 100, and 200) of dried workers from one colony of *Nasutitermes acajutlae* from Guana Island, BVI.

size of *N. ephratae*, these collections could very well have been below an acceptable size. Large workers of *N. ephratae*, (dry weight = 0.6 mg) are considerably smaller than large workers of *N. acajutlae* (dry weight = 1.3 mg) (Thorne, 1985); 100 large workers of the former species may not have had sufficient surface lipids to provide an adequate chromatogram.

Effects of Solvent

For field entomologists working in the tropics it would be convenient if storage of termites in 100% ethanol (EtOH) allowed for equivalent extraction of hydrocarbons. Preliminary observations of S. J. Seybold and L. J. Nelson (personal communication) indicated that EtOH will extract cuticular hydrocarbons of adult *Ips pini* (Say) (Coleoptera: Scolytidae). Furthermore, the longer beetles were in EtOH, the greater the extraction efficiency. Their work showed: (1) that the EtOH-extracted insects can be further extracted with hexane, (2) that the EtOH extract can be dried and the lipids redissolved in hexane, and (3) that the hexane solutions of the EtOH extract and the EtOH-extracted-insect extract can be recombined to provide a reconstituted extract for characterization. They have not shown whether the resulting hydrocarbon mixture is comparable to an extract gathered by a standard 10-min extraction with hexane of live or dead insects.

Unfortunately, the resulting extracts for both workers and soldiers of *N.*

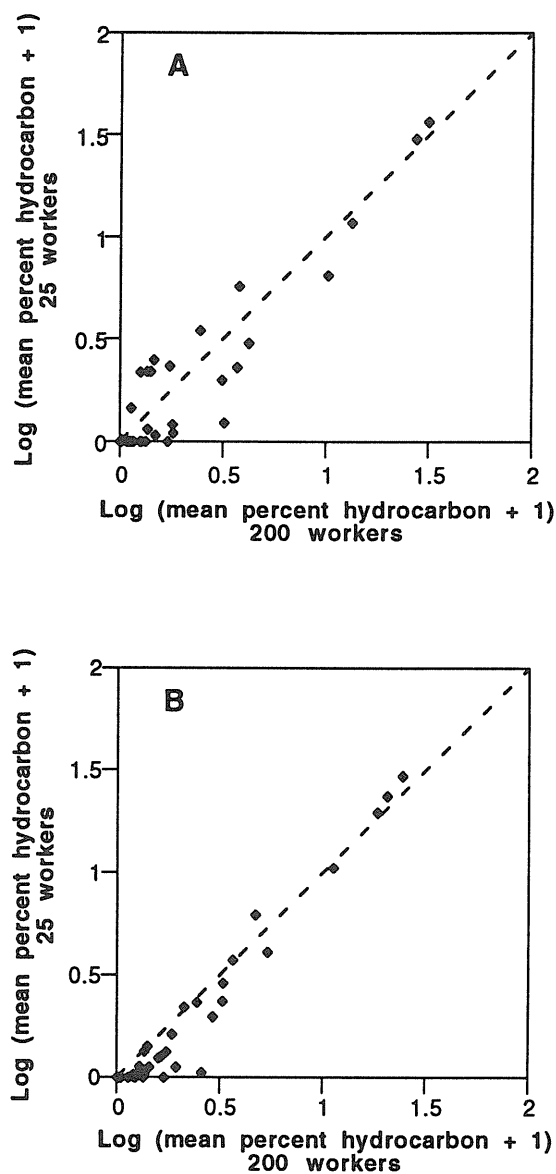


FIG. 7. Log (mean percent + 1) of 34 cuticular hydrocarbon components for groups of 25 and 200 workers extracted either alive (A) or after drying (B). Note: transformation was not done for statistical purposes, but to spread out the points for the less abundant components.

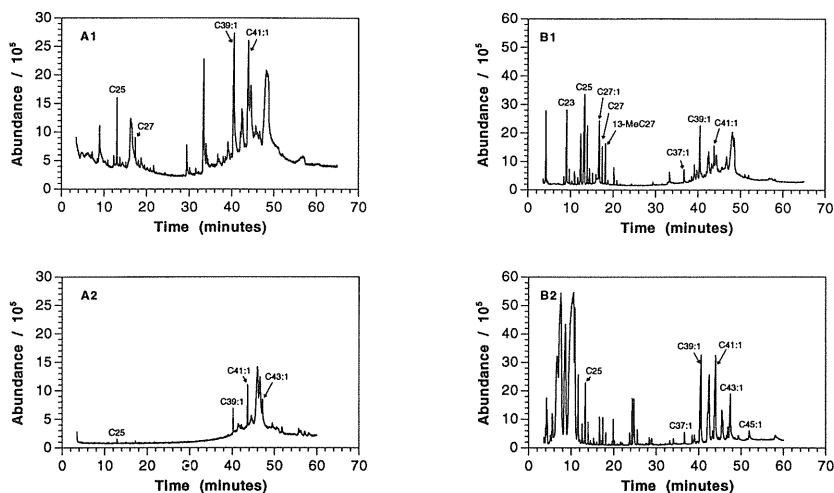


FIG. 8. Chromatograms of cuticular hydrocarbons (and other compounds) from 200 workers (A) or 200 soldiers (B) collected into 100% ethanol. For each caste the first chromatogram (A1 or B1) represents the pattern of compounds that were extracted by the ethanol. The second chromatogram for each caste (A2 or B2) represents the pattern of compounds extracted from the termites after they were stored in ethanol for 60 days, subsequently dried, then extracted with 10 ml of hexane for 10 min.

acajutlae stored in ethanol were not comparable to those where the standard technique was utilized. Storage in ethanol caused numerous, hexane-soluble, unidentified, nonhydrocarbon compounds to be extracted or these nonhydrocarbon peaks were in the ethanol as denaturing components (Figure 8). These compounds were not removed after evaporating the ethanol under nitrogen, redissolving in hexane, and pipetting through activated BioSil-A. Furthermore, enormous amounts of nitrogen and time were required to dry the ethanol samples. As a result of these problems, we did not characterize all 15 samples, because we presumed such a course would only waste resources and could affect our GC-MS. Therefore, unless a different cleanup procedure is developed, storage in ethanol is unacceptable for characterizing the hydrocarbons from *Nasutitermes*.

CONCLUSIONS

Different colonies of *N. acajutlae* produce qualitatively similar, but quantitatively dissimilar hydrocarbon mixtures. These colony-specific profiles appear to be reproducible; multiple extracts from separate samples of each colony are qualitatively identical and quantitatively quite similar. Only one extraction of a

sample of workers is necessary. A very brief extraction (in 10 ml of hexane for only 20 sec) of live workers produces a mixture of hydrocarbons equivalent to a 10-min or a 24-hr extraction. Holding a sample of workers (or soldiers) in hexane for a period of two years results in a radically different extract than the standard 10-min extraction and is not recommended.

Drying workers of *N. acajutlae* before extraction results in highly significant increases in the relative amounts of $C_{41:4}$ and $C_{41:5}$ and an apparent decrease in the relative amounts of the most abundant compounds, $C_{39:1}$, $C_{41:1}$, and $C_{43:1}$. In general, drying workers prior to extraction tends to enhance extraction of the less abundant unsaturated compounds and does not result in equivalent mixtures of hydrocarbons when compared to extraction of live workers. Extracting a minimum of 100 workers (live or dried) with hexane for 20 sec to 10 min seems to be the best method for characterizing cuticular hydrocarbons of *N. acajutlae*. For smaller species, samples of at least 200 would probably guarantee a satisfactory chromatogram.

For quantitative comparisons, the extraction technique should ideally be the same for all samples, i.e., hydrocarbon mixtures extracted from dried samples should not be compared to those extracted from live samples. Extraction of either live or dried termites would suffice for characterization of cuticular hydrocarbons for ecological studies but may not be comparable for taxonomic purposes. For quality of chromatograms and for several logistical reasons, we obtained the best results by drying at least 100 termites and then extracting them by the standard technique (10 min in 10 ml hexane) in the laboratory. Extraction of live termites in the field requires twice the number of vials and transportation of flammable liquids. Even with the potential logistical difficulties involved with drying termites in the tropics, we recommend this as the optimal technique to use.

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