AN INVESTIGATION OF SAMPLING METHODS FOR THE ANALYSIS OF INSECT CUTICULAR HYDROCARBONS

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ABSTRACT

Several sampling methods were examined to determine their efficiency at recovering the cuticular hydrocarbons, including solvent washing, extraction, and dynamic headspace analysis. Hexane proved to be an acceptable solvent for obtaining a representative hydrocarbon sample, but is not a good solvent for quantitative recoveries, unless special measures are taken. This appears to be as a result of both limitations in solubility and kinetic problems. Dynamic headspace analysis using a pyroprobe for thermal desorption of the hydrocarbons proved to be a rapid and quantitative sampling method.

Key Words: Cuticular hydrocarbons, sampling, fire ants, *Solenopsis richteri.*

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INTRODUCTION

The cuticular hydrocarbons of insects have been receiving a great deal of attention in recent years, due to many reports of their being involved in chemical communication in many species. An important step in investigating these substances is removal of the cuticular hydrocarbons from the insect cuticle. This should be accomplished in a non-discriminatory fashion, so that the hydrocarbon sample recovered reflects the relative composition of hydrocarbons in the insect cuticle. This is especially important if the hydrocarbon profiles of different samples are to be compared. It is also important to avoid extracting any internal hydrocarbons in the haemolymph, particularly if they are different from the cuticular hydrocarbons.

The most widely used method for recovering insect cuticular hydrocarbons is hexane washing. The insects are usually immersed in the solvent for approximately 10 minutes, followed by several hexane rinses, which are then combined with the washing for further sample preparation (Lok et al. 1975; Nelson et al. 1980). It has been reported that hexane washings do not quantitatively remove the cuticular hydrocarbons. Jackson et al. (1974) have reported in studies on adult fleshflies that digestion of insects with a strong base prior to hexane extraction resulted in quantitative recovery of the hydrocarbons.

We have recently reported on another sampling method which avoids the use of a solvent and can be performed on a single insect or even part(s) of an insect (Brill and Bertsch 1985). This method is a dynamic headspace procedure, in which the hydrocarbons (or other volatiles) are thermally desorbed from the insect and transferred directly onto the column of a gas chromatograph for analysis. The hydrocarbons can be removed quantitatively from the sample. One of the major concerns of this procedure was whether or not hydrocarbons in the haemolymph of

the insects (imported fire ants in our studies) were being gas phase extracted to a significant degree as well. A series of experiments was performed to address this question. Additional studies were conducted to examine the extractability of cuticular hydrocarbons with non-polar organic solvents such as hexane. These studies are reported in this paper.

MATERIALS AND METHODS

Samples

For the experiments reported below, worker ants of *Solenopsis richteri* Forel (the black imported fire ant) were collected from a nest in Pickens County, Alabama. The ants were anesthetized with carbon dioxide and kept at−25°C.

The analyses were performed on a Hewlett-Packard 5830A gas chromatograph, fitted with a Perkin-Elmer 900 injection port and an inlet splitter. The split ratio was set to approx. 100:1. A 10 m × 0.25 mm glass capillary, coated with a 0.25 μm film of SE-30 was used. The column was temperature-programmed from 80° - 300°C at 8°C/min. The carrier gas was helium at a flow rate of approx. 1.0 ml/min.

Gas chromatography - mass spectrometry (GC/MS) was performed on a Hewlett-Packard 5985A instrument in the electron impact (EI) mode. A 15 mm × 0.35 mm fused silica column coated with a 0.10 μm film of DB-5 was used.

All solvents used were distilled in glass. For use as an internal standard mixture, a stock solution containing 250 ng/μl each of tricosane ($C_{23}H_{48}$) and dotriacontane ($C_{32}H_{66}$) in hexane was prepared.

Pyroprobe Dynamic Headspace Analysis (PDHA)

Single ants of similar size were examined individually by this sampling method, the details of which have been reported in a previous paper (Brill and Bertsch 1985). One μl of the internal standard mixture was added to the sample before analysis. Thermal desorption was performed at 300°C for a 5 second time interval.

Workers of approximately the same size were used in the experiments below. After washing or extraction, the ants were allowed to dry for at least an hour and then retained for PDHA/GC analysis.

Hexane Washings

The basic procedure followed in the solvent washing experiments is shown in Figure 1. The ant sample (between 200 and 400 workers) was immersed in 2 ml hexane for 10 min., then rinsed three times with fresh solvent. The combined washings were concentrated to approx. 0.2 ml under a stream of nitrogen gas. The concentrated sample was then applied to the top of a silicic acid column (500 mg in a Pasteur pipet). The hydrocarbons were eluted from the column with 2.0 ml hexane, and the solution was concentrated to 1 ant-equivalent per μl. The sample was then examined by regular injection into the gas chromatograph. In addition, a 1 μl aliquot of the sample plus 1 μl of the internal standard mixture were injected onto a small plug of silanized glass wool in the quartz sample tube of the pyroprobe. The sample was then thermally desorbed and analyzed as described previously.

In the first experiment, the ant sample was washed a second and a third time, using the same procedure. After each washing some of the ants were reserved for PDHA/GC analysis.

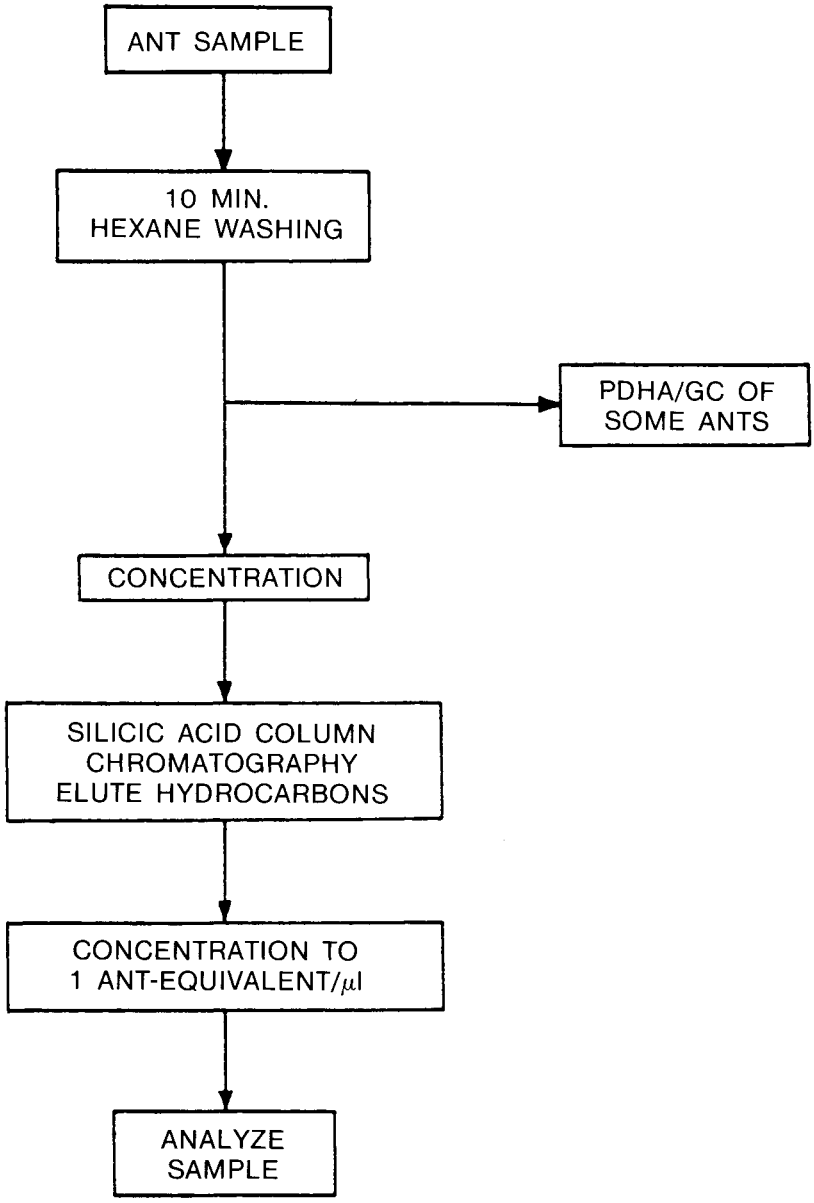

Fig. 1. Basic procedure used for hexane washings.

In the second experiment, the procedure was modified in the following way. After hexane washing, the concentrated crude washing was divided into two equal portions. The first portion was analyzed before the silicic acid column cleanup step, while the second portion was treated in the normal manner (see Fig. 1).

In the third experiment, the ants were immersed in hexane for 24 hr. The rest of the sample workup was as described in Fig. 1.

Hexane Micro-Soxhlet Extractions

One hundred workers of the same size were placed in a micro-Soxhlet extractor and extracted continuously with 15 ml hexane for 2 hr. The extract was concentrated to approx. 0.2 ml and then worked up in the same manner as described above. The final solution was concentrated to 1 ant-equivalent per μl. The ants were then extracted a second time with fresh hexane for 2 hr. The second extract was worked up as described before. The extracts were examined by GC and PDHA/GC.

The micro-Soxhlet extractions were then repeated, using toluene as the solvent.

Hexane Homogenates

One hundred workers were homogenized in a 0.25 ml borosilicate glass Potter Elvehjem tissue grinder (Fisher Scientific, Norcross, GA) with approx. 0.2 ml hexane. After the ants had been completely ground up, the supernatant was decanted off and the residue rinsed several times with fresh aliquots of hexane. The rinses were added to the extract and the solution concentrated under N_2 gas to 200 μl. The concentrated extract was then divided into two equal portions. The first sample was set aside for analysis, while the second was placed on a silicic acid column and the hydrocarbons eluted, as described above. The final solution was concentrated to 1 ant-equivalent per μl. The samples were examined by GC and PDHA/GC, as described above.

Each of the samples prepared above was also examined by gas chromatography - mass spectrometry for quantititative and qualitative analysis.

Five replicates were examined from each of the samples prepared. The experiment was also repeated with four different colonies.

RESULTS AND DISCUSSION

When the same sample was examined by regular gas chromatographic injection as well as pyroprobe dynamic headspace analysis (PDHA), no significant differences were observed in the resulting profiles. GC/MS analysis also resulted in almost identical profiles. Thus, the PDHA/GC procedure generates a representative sample and shows no significant discrimination. In the PDHA/GC method hydrocarbons are removed almost completely, since, when the same ant is reexamined, a negligible amount of hydrocarbons can be recovered. The temperature at which the pyroprobe is operated (300°C for 5 seconds) is too low to pyrolyze the sample. This has been confirmed by studies to determine the optimum thermal desorption temperature (Brill and Bertsch 1985; Brill 1985). Pyrolysis products only begin to appear when the operating temperature is raised to 500°C or higher.

The hydrocarbon profiles from each of the different sampling procedures are very similar (see Figs. 2 and 3). The only differences are in the absolute amounts of hydrocarbons recovered. When the liquid column chromatography cleanup step was omitted, other components [mainly piperidine venom alkaloids (Brand et al. 1972)] appeared in the sample. However, these substances show no significant interference, since they elute prior to the diagnostic (hydrocarbon) region of the chromatogram (compare Fig. 3A and B).

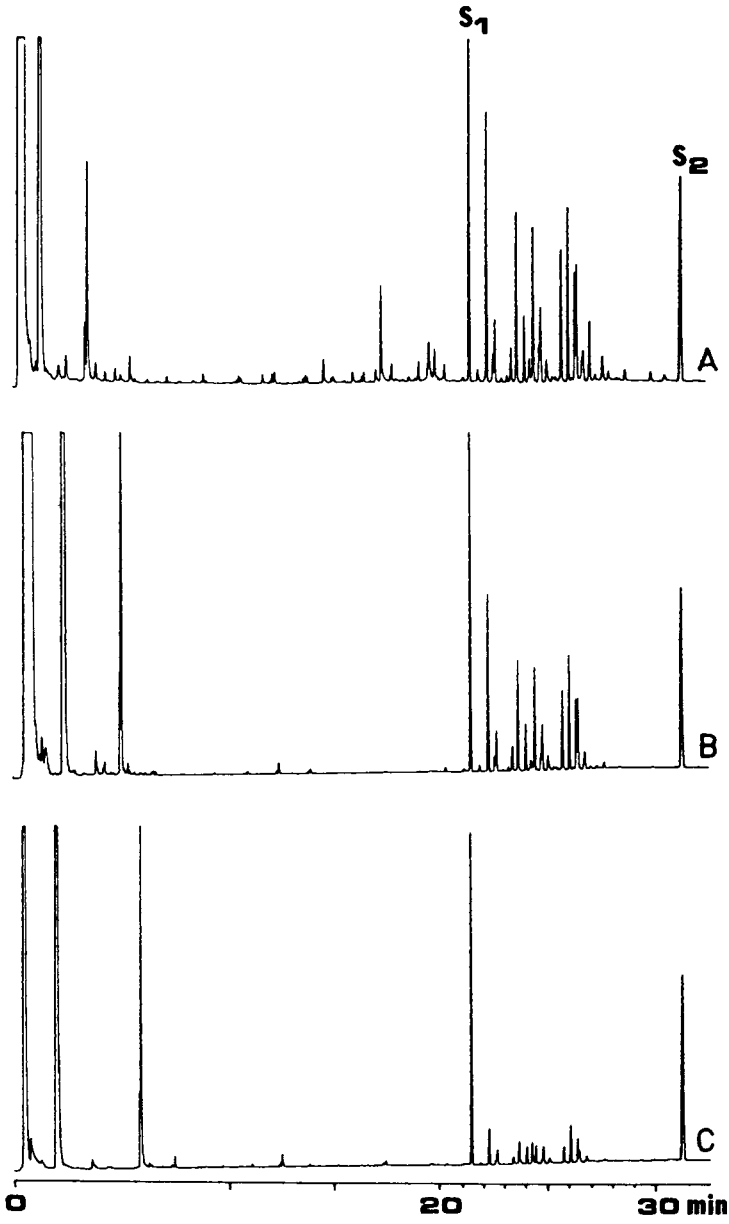

Fig. 2. Gas chromatographic profiles[1] obtained from the different sampling procedures. A, hexane micro-Soxhlet extraction (2 hr); B, hexane washing (24 hr); C, hexane washing (10 min). S_1, internal standard (tricosane); S_2, internal standard (dotriacontane).

[1] All chromatograms are 1 ant-equivalent.

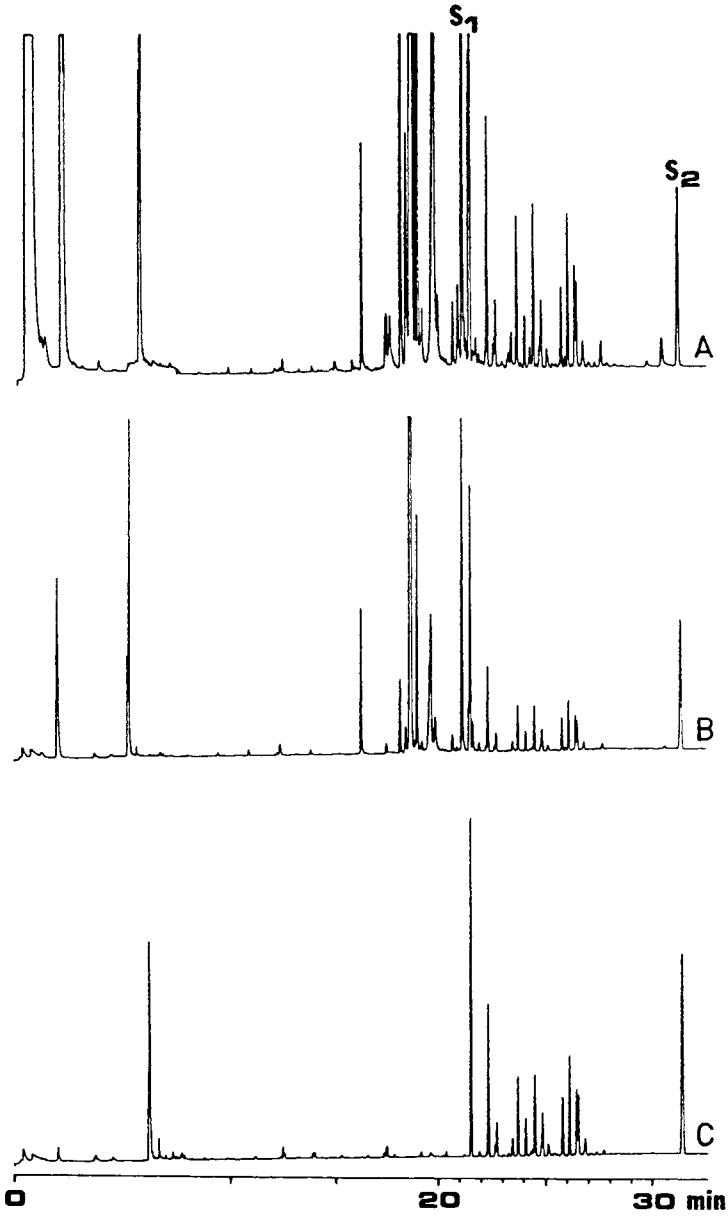

Fig. 3. Gas chromatographic profiles[2] obtained from the different sampling procedures. A, pyroprobe dynamic headspace analysis of a single worker; B, hexane homogenation (no liquid chromatography cleanup); C, hexane homogenation. S_1, internal standard (tricosane); S_2, internal standard (dotriacontane).

[2] All chromatograms are 1 ant-equivalent.

The total amount of hydrocarbons recovered was different for the various sampling procedures examined. The 10 min. hexane washing had the lowest recovery (compare Figs. 2 and 3). When the ants were washed a second time, recovery was approximately the same amount. Some of the washed ants were also examined by PDHA/GC. The amount of hydrocarbons recovered was approx. 5 times that of the first hexane washing. After washing the ants a third time, significant amounts of hydrocarbons were still being recovered (approx. half to two-thirds of the amount recovered in the first washing).

The 24-hour hexane washing recovered approx. 3 times as much hydrocarbons as the 10 min. washing (compare A and B in Fig. 2). Some of the washed ants from this sample were examined by PDHA/GC. The amount of hydrocarbons recovered was approx. the same as in the washing.

The 2-hour hexane micro-Soxhlet extraction recovered approx. 5 - 10 times the amount of hydrocarbons as the 10 min. washing (compare A and C in Fig. 2). The cycle time of the micro-Soxhlet was about 2 min., giving approx. 60 washes in the 2-hour period of extraction. The ants were extracted for a second 2-hour period and the amount of hydrocarbons recovered was about one fifth that of the first extraction. Analysis of some of the extracted ants by PDHA/GC showed very small amounts of hydrocarbons remaining.

Homogenation and extraction of ants followed by liquid chromatography yielded the same hydrocarbon profile as the washing and solvent extraction methods (see Fig. 3A). The amount of hydrocarbons recovered was approx. the same as the 24 hour washing. When a crude homogenate (i.e. no liquid chromatography cleanup step) was examined, the hydrocarbon profile remained unchanged. The more polar components (mainly piperidine alkaloids) now appeared in the chromatogram, eluting before the hydrocarbons (see Fig. 3B).

Identical results were obtained when the replicates from each of the samples were examined. In addition, the overall results obtained using different colonies were similar to those discussed above.

It can be concluded that hexane washing does not give a quantitative extraction of the hydrocarbons. However, this method does yield a representative sample, which is useful in qualitative studies. The results of these experiments also point to two problems with hexane as a solvent for extraction of the hydrocarbons. The first is a solubility problem. Hydrocarbons of high boiling point (such as cosane, triacontane, etc.) are not very soluble in hexane. This can be seen by comparing chromatograms B and C in Fig. 2. A larger amount of hydrocarbons can be recovered in the 2-hour Soxhlet extraction than in the 24-hour washing. In the latter the solvent was at room temperature (21°C), whereas the solvent temperature in the Soxhlet extraction was well above room temperature but below the boiling point of hexane. The elevated temperature enhanced the solubility of the hydrocarbons in the hexane. This was further shown by switching the solvent to toluene. A 30 min. extraction with toluene yielded approx. the same quantity of hydrocarbons as a 2-hour extraction in the hexane micro-Soxhlet. In addition, the 2-hour hexane micro-Soxhlet extraction removed more hydrocarbons than a hexane extraction of an ant homogenate (in which the solvent was at room temperature). The solubility effect was further exemplified when a sample of ants were first extracted in a micro-Soxhlet with hexane, followed by a second micro-Soxhlet extraction for an equal time interval using toluene as the solvent. The second (toluene) extraction recovered approx. twice the amount of hydrocarbons as the first (hexane) extraction.

A second problem associated with hexane extraction of hydrocarbons from insect material is apparently a kinetic phenomenon, linked to diffusion. The cuticular hydrocarbons are located in the epicuticular matrix, which contains wax and sterol esters, alcohols and free fatty acids as well as the hydrocarbons (Jackson and Baker 1970). Therefore, in order to extract the hydrocarbons, they must be moved out of the matrix and into the bulk of the solvent. For this to occur, solvent molecules must penetrate the lipid matrix, or the matrix itself must be broken down. The longer the extraction the more hydrocarbons are recovered, since with time the solvent molecules are better able to penetrate the matrix and leach out the hydrocarbon solutes. The recovery of significant amounts of hydrocarbons upon second and third solvent washings also points to a diffusion problem. By raising the temperature, the hydrocarbon solutes become more soluble in the solvent. This can be seen by switching from room temperature extraction to a micro-Soxhlet extraction in which the temperature of the solvent is near its boiling point (compare Fig. 2B and C).

Toluene seems to be a better solvent to use than hexane, since higher molecular weight hydrocarbons are more soluble in it. However, there are some disadvantages to its use. Firstly, toluene has a boiling point of approx. 110°C. This means that the evaporation/concentration steps must be performed at elevated temperature. Toluene extractions at room temperature are not as efficient as hexane extractions. Toluene is more dense and viscous than hexane and appears to remain trapped in the epicuticular matrix, yielding poor recoveries. At elevated temperatures, such as in a Soxhlet extraction, the problem seems to diminish.

Hexane, therefore, is not a good solvent when the cuticular hydrocarbons are to be extracted quantitatively. However, it does give a representative sample and can be used in qualitative extractions. The dynamic headspace analysis method quantitatively removes the hydrocarbons. This method is also rapid, requiring little or no sample preparation. Although other substances are desorbed together with the hydrocarbons, their interference with the diagnostic region is minimal. A selective detector such as a mass spectrometer can also be used to eliminate interferences from non-hydrocarbon material.

It has been reported that the post-pharyngeal glands of red imported fire ants contain hydrocarbons which are identical to the major cuticular hydrocarbons of these ants (Thompson et al. 1981). It was suggested in the report that the post-pharyngeal gland hydrocarbons might play a role in certain social functions, such as species and/or caste reognition, etc. The authors are not aware of any similar studies on black imported fire ants. However, an examination of the hydrocarbons from different body regions (antennae, head, thorax, legs and abdomen) of black imported fire ants yielded large recoveries from the head, although the profiles for the different body regions were very similar (Brill 1985). This suggests that the hydrocarbons from the post-pharyngeal glands of these ants are the same as the major cuticular hydrocarbons. The hydrocarbon profiles (i.e. relative peak ratios) appear to be the same, irrespective of the sampling method used. This suggests further that the relative component ratios of the post-pharyngeal gland hydrocarbons are the same as those for the cuticular hydrocarbons. Thus, although the PDHA method may desorb the hydrocarbons from the post-pharyngeal glands, the profiles of these substances are identical to the profiles of the cuticular hydrocarbons. Therefore, when the hydrocarbon profiles of different samples (colonies, etc.) are compared, the PDHA/GC method is a suitable and rapid procedure for collecting

large data sets for statistical analysis. This procedure is also useful in profiling the volatiles from different types of samples, such as species, subspecies, etc., for chemotaxonomic purposes (Brill 1985). The detector, such as a mass spectrometer, would enhance such a procedure.

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