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Analysis of Beetle Cuticular Hydrocarbons

Elizabeth A. Williams , '13

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Presented as a Senior Course Thesis in Chemistry

Elizabeth Williams
Swarthmore College
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Advisor: Alison Holliday

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Abstract

The insect cuticle, commonly known as the exoskeleton, consists of two layers: a bottom layer called the procuticule and a thin outer waxy coating called the epicuticle. The epicuticle contains hydrocarbons that prevent desiccation and are used for chemical communication both inter- and intra- specifically. For some insects, these compounds are important sociochemicals involved in recognizing nestmates and predators as well as identifying the sex of other insects. The established procedure to extract cuticular hydrocarbons is lethal, directly introducing the insects to toxic solvents. The goal of this study is to develop a non-lethal procedure to extract the cuticular hydrocarbons from forked fungus and carabid beetles, and to identify the extracted components. In the long term, an examination of the cuticular hydrocarbon profiles of beetles may have important biological implications including differences based on sex, fungus species they feed on, and colonies they inhabit.

Each beetle was sampled using the developed non-lethal procedure that uses C_{18} and the established lethal procedure that uses hexane. The results of the C_{18} procedure were compared to the results of the established solvent extraction to see if the developed procedure was as efficient as the established technique. The samples where analyzed using a Gas Chromatography-Mass Spectrometry (GCMS) instrument. Standard compounds were analyzed to identify some of the components of the cuticular profile.

Both procedures extracted the same compounds. Five components of the cuticular waxes were identified including four n-alkanes and one alcohol. The identified compounds are nonadecane, eicosane, heneicosane, docosane, and 1-eicosanol. *Chlaenius cordicollis* was found to contain all five components, while forked fungus beetles were found to contain nonadecane, eicosane and heneicosane. The differences in the components identified for the two different species of beetles suggests that there is variation in the components of cuticular waxes amongst different species as expected. Previous studies have found that n-alkanes, the majority of extracted components in this study, are primarily involved in the prevention of desiccation and not as sociochemicals. Unlike n-alkanes, alcohols have been shown to function as sociochemicals.

Chapter 1: Introduction to cuticular hydrocarbons, the species of beetles studied and the instrumentation used

Hydrocarbons

Hydrocarbons are nonpolar, hydrophobic, organic molecules composed entirely of carbon and hydrogen. They are naturally present in solid, liquid, and gas phases. There are several molecular types of hydrocarbons that differ in their arrangement of carbon and hydrogen atoms.[1] Examples include straight-chain hydrocarbons, branched hydrocarbons, and saturated rings.[2] There are two classes of hydrocarbons found in nature: petrogenic and biogenic. Petrogenic hydrocarbons can be found in natural gas, petroleum, coal and oil shale. Biogenic hydrocarbons can be found in trees, plants and in specific animals, mainly marine animals and insects. While biogenic hydrocarbons can easily be isolated from nature, only small amounts of hydrocarbons are found on animals.[1]

Insect Cuticular Hydrocarbons

The insect cuticle, commonly known as the exoskeleton, consists of two layers: the procuticle and the epicuticle. The procuticule is the bottom layer of the cuticule and the epicuticle is the waxy outer coating that contains hydrocarbons (Figure 1.1).[2] These hydrocarbons prevent desiccation by acting as a water barrier that keeps moisture in and water out. This protects insects from drowning and dehydration. In addition to serving as anti-desiccation agents, the cuticular hydrocarbons can be used for chemical communication both inter- and intra-specially.[3] These compounds can be important

sociochemicals involved in identifying gender, recognizing nestmates, predators, cheaters, and distinguishing between the roles the insects play in the colony.[2, 3]

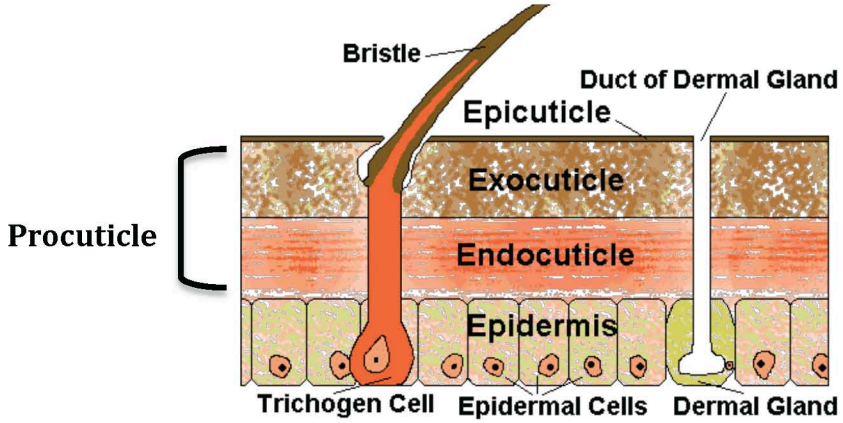


Figure 1.1 The layers that make up the exoskeleton of an insect. The two main components are the epicuticle, which is the outer layer and the procuticle which is the lower layer. The figure is adapted from Gordon.[4]

The hydrocarbon profile of the inset epicuticle can be complex and can include several hundred types of hydrocarbons.[2] Three classes of hydrocarbons commonly found on the epicuticle include n-alkanes, alkenes and methyl branched hydrocarbons.[3, 5, 6] It has been established that the main role of the n-alkanes is to control trans-cuticular water movement while the unsaturated compounds and methyl-branched hydrocarbons may be involved in communication.[3]

N-alkanes with chain lengths of 20 to 33 carbons are the most common hydrocarbon components found amongst insects.[2] In some cases they comprise less than one percent of the total hydrocarbons while in others they may comprise almost the entire profile. [5, 7, 8] Insect cuticular waxes have been found to contain anywhere from one n-alkane as the major component up to a series of n-alkanes.[7, 9, 10] Hydrocarbons with fewer than eighteen carbons are volatile making them unsuited to function as cuticular components. Instead, they are found as pheromones and defensive compounds.[2] There is

an array of unsaturated hydrocarbons[6, 11-14] and methyl-branched alkanes that have been identified on the epicuticle of insects.[5,15,16] Both are difficult to characterize due to the molecular complexities. Insects have been found to contain unsaturated hydrocarbons that have double bonds in an array of positions[9, 17, 18] Most alkenes found on the epicuticle of insects are straight-chain molecules[19, 20] and the double bonds are often not conjugated.[9, 17, 18] Identification of methyl-branched alkanes is often ambiguous because their mass spectra are so similar.[21]

In addition to hydrocarbons other chemical classes contribute to the insect cuticular waxes. Waxes generally contain a significant portion of hydrocarbons,[15] but alcohols and wax esters may be present as well.[14, 22] Alcohols ranging from C24 to C34 were found to constitute major lipid fraction of the cuticular waxes from the pupae of tobacco budworm, *Heliothis virescens*.[23] Wax esters were found to be the major compounds, constituting 86 percent of the cuticular waxes present in the cuticle of silverleaf whitefly nymphs, *Bemisia argentifolii*.[24]

Biosynthetic Pathway for Hydrocarbon Formation

Insects synthesize the majority of their own cuticular hydrocarbons with dietary lipid contributing very small amounts in certain insects.[14, 25] The pathway for the synthesis of cuticular hydrocarbons varies from insect to insect. An understanding of the basic pathway has been obtained through the study of insects including fruit flies (*Drosophila melanogaster*), cockroaches (*Periplaneta americana* and *Blatella germanica*), and termites (*Zootermopsis angusticollis*).[11, 14, 26,] In vitro studies with specific radio-labeled precursors, in vivo experiments with ^{13}C labeled precursors, and

analysis of metabolic products determined the biosynthetic pathways for the most common hydrocarbon components.[7, 27, 28]

It has been established that the major site of hydrocarbon biosynthesis occurs in the oenocytes, which are cells associated with the epidermal layer or peripheral fat body.[2] The anatomical location of oenocytes differs among insect species leading to different pathways for the synthesis and transfer of cuticular hydrocarbons.[29, 30] There is a specific synthesis pathway for each of the main classes of hydrocarbons, each producing compounds of varying lengths (Figure 1.2).[31] Cuticular hydrocarbons are synthesized in oenocytes from fatty acid precursors (Acetyl-CoA) by three related pathways.[11, 14] For the three main classes of hydrocarbons the fatty acids are lengthened by elongase enzymes. Decarboxylation follows the elongation of the fatty acids to form n-alkanes. Alkenes are produced by lengthening of fatty acids, followed by action of desaturase enzymes and then additional elongation and decarboxylation. Carbon skeletons of amino acids initiate the chain synthesis of methyl-branched hydrocarbons.[32, 33] The pathway consists of methylation of the amino acid carbon skeletons, followed by elongation and decarboxylation.[14, 31, 34]

The chain length specificity of hydrocarbons is regulated by the elongation of fatty acids by the elongase enzymes.[9, 26, 35] The cuticular hydrocarbons are transported and distributed over the epicuticle, just after the larval molt, before adult emergence and permanently remain on the cuticle, but the specific mode of transportation of the cuticular lipid components to the surface of the insect is unclear.[36, 37] In addition, the specific arrangement of hydrocarbons on the epicuticle of the insect is unknown, but it is thought

that the components of most importance in chemical communication are on the outer surface of the cuticle.[38]

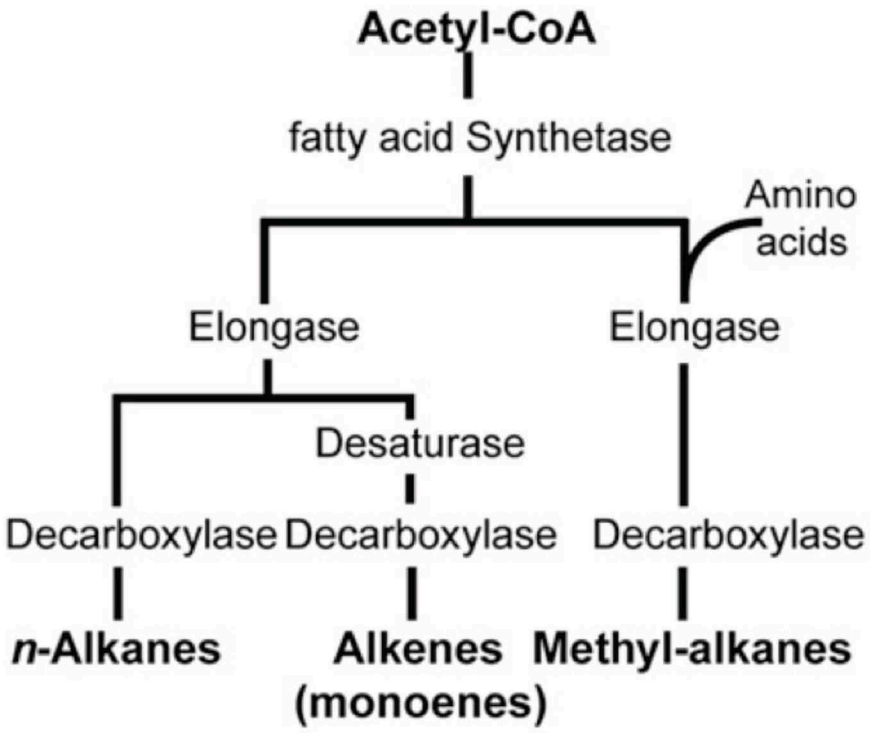


Figure 1.2 General synthesis pathway for cuticular hydrocarbons in insects. The pathway for the three main classes of hydrocarbons found in insects are depicted: n-Alaknes, alkenes and methyl-alkanes. Figure is adapted from Kent *et al.* (2008).[31]

Biological Implications of Cuticular Hydrocarbons

Cuticular hydrocarbon profiles have been found to differ between species and among insects intraspecifically.[2] Differences in the hydrocarbon profile include there being variations in abundance of specific hydrocarbons or different components. The chemical profiles can provide insight into the biological implications of cuticular hydrocarbons through their role as ecological, behavioral, and physiological signals. Cuticular profiles aid in species and gender recognition, nestmate recognition, task

specific cues, dominance and fertility cues, and chemical mimicry.[39] Chemical analyses and biological studies have been completed on cuticular hydrocarbon profiles in many different species of insects including ants, bees, flies, cockroaches, and beetles.[34, 38, 40, 41]

Chemical and biological studies conducted on longhorned beetles and ant colonies are two examples of relevant biological work and chemical analysis that have been completed on cuticular hydrocarbons. First, chemical analysis conducted on the longhorned (cerambycid) beetles of the species *Neoclytus acuminatus acuminatus*, in an experiment by Lacey *et al.* (2008) found differences in the cuticular profiles between males and females, including differences in the components and their abundance.[3] The cuticular lipids of females were found to contain the sex pheromone, 7-methylheptacosane along with 7-methylpentacosanol and 9-methylheptacosane. These compounds were found to comprise over 40% of the total hydrocarbons in the cuticular profile of the females and were not found to be contained by males.[3]

Biological studies of longhorned beetles confirmed that a mating response is elicited in males after they touch the cuticle of a female with their antennas.[3] Dose-response tests were completed to test the behavioral response of males to specific contact sex pheromones in the cuticular lipids of females. These studies also sought to confirm that males rely on a contact sex pheromone to recognize females and to determine the quantities of pheromones necessary to elicit responses from males. They tested the response of males to females that had their hydrocarbons extracted and to reconstituted females, which are beetles that have their hydrocarbons extracted and then replaced with a specific quantity of the extracted compounds. In addition, they tested the female-specific compounds individually, in pairs and all together including synthetic 7-

methylheptacosane, 7-methylpentacosanol and 9-methylheptacosane. Males fully responded to the reconstituted females, to all three components together and to 7-methylheptacosane alone or in combination with 9-methylheptacosane. There was no response elicited in the males to the females with their hydrocarbons extracted. These three contact pheromones of cerambycids appear to be more species-specific than the volatile sex pheromones they excrete.[3] This suggests that the contact pheromones may play a critical role on reproductive isolation of closely related, sympatric species (two different populations of closely related species inhabiting the same geographic area).[42]

Second, chemical analysis on ants conducted in an experiment by Greene and Gordon (2007) suggests that cuticular hydrocarbons play an important role in the social hierarchy of ants.[40, 43] Slightly different chemical profiles and their concentrations aid in distinguishing ants with different roles, such as workers from the queen.[40] Also, in many species of ants different concentrations of compounds help to distinguish the queen from other reproductive individuals.[43] In addition to chemical analyses, biological studies completed with the ant species, *Aphaenogaster cockerelli* provide direct evidence that reproductive cheaters (insects that synthesize cuticular waxes representative of the fertile queen) are identified by nestmates through changes in their cuticular compounds.[40, 43] Correlational evidence indicates that hydrocarbons contain sufficient information necessary to identify cheating egg-layers and their eggs. Studies have been completed where groups of non-reproductive workers from both queen and queenless colonies are experimentally separated and made to mimic reproductive cheaters by applying a synthetic compound typical of fertile individuals. Upon reuniting the workers with the manipulated cuticular profiles with their nestmates, aggression was immediately seen

towards the workers in the queen colonies but not in the queenless colonies. This observation indicates policing, in which the ants control for fertility. Studies in other ant species have shown that such aggression will lead to inhibition of any ovarian activity, resulting in only the queen having the ability to produce viable eggs.[40]

The study with *A. cockerelli* concluded that in queenless colonies, aggression was not observed towards the non-reproductive workers with manipulated hydrocarbon profiles.[40] This is most likely because reproductive workers are already present in the queenless group. It is known that when *A. cockerelli* workers are kept in queenless groups, some will activate their ovaries and eventually lay eggs. This change in ovarian activity is accompanied by a shift in the cuticular hydrocarbon profile, similar to a queen's profile. In the queen colonies the manipulated workers are still considered as nest-mates and the aggression received in these colonies is not a consequence of mistaking them as foreign workers.[40] Similar interactions have been identified in colonies of bees and wasps, among other insects.[41, 43]

An electoantennography study by Cortez *et al.* (2011) provides a clear connection between the biological studies and chemical analysis.[44] The study shows that the antennae of workers from the ant species, *Camponotus sericeiventris*, react to volatile compounds in secretions from the beetles, *Canthon cyanellus cyanellus* and *Canthon femoralis femoralis*. Specific compounds in the secretions of these beetles elicited coupled gas chromatography-electroantennogram (GC-EAD) activity in the antennae of the ants. This activity occurs when the eluent from a gas chomatography separation flows over an antennae wired to measure the electric response the antenna has to the compounds.[45] GC-EAD studies allow for the chemical relevancy and biological

response to be observed in tandem.[44, 45] The compounds that released an electroantennographic response were classified as eliciting a chemosensory response from the ant antennas. This shows how both biological and chemical analysis connected to provide insight into the communication amongst insects. In addition, biological studies showed that the ants respond when contacting the body of the beetles with their antennae. This suggests that chemicals on the cuticle of the beetles also elicit a response in the ants along with the defensive secretions.[44]

Insects

The nonsocial beetles focused on in this study are forked fungus beetles (*Bolithotherus cornutus*) from Virginia and various carabid beetles, from Manitoba, Canada and Poconos, Pennsylvania. A variety of carabid species that live within the same ecosystem were studied.[46] The carabid species of focus is *Chlaenius cordicollis* because this was the species most available. Additional carabid species studied include *Agonum extensicolle*, *Chlaenius tricolor*, *Chlaenius sericeus* and *Chlaenius impunctifrons*. The interactions observed amongst beetles within a given species, suggests that individuals could communicate using chemical signals such as hydrocarbons. The cuticular hydrocarbons of these species have not been previously classified. In addition, differences in the components of the defensive secretions have been identified between sexes for *Chlaenius cordicollis* [47] and between forked fungus beetles inhabiting two different species of fungus.[48]

Carabids belong to the *Carabidae* family, which is a large class of ground beetles consisting of more than 4,000 species world wide.[49] Carabids are common in North

America and are found in wetter regions, often on the shores of water sources.[46] These beetles are nocturnal, hiding under logs, rocks, or in soil crevices during the day and emerging in the evening. The adult beetles range from 3.2 to 6.4 mm long.[50, 51] While the beetles vary in shape, carabids are classified as being elongate, heavy bodied, and tapered near the head. They are often darker in color, with some metallic green or multi-colored.[46] They are fast-moving, with prominent, long legs and fairly threadlike antennae.[51] *Chlaenius* are sexually dimorphic differing in the shape of the legs.[50] Identifying characteristics of, *Chlaenius cordicollis* include: metallic green color, corset-shaped pronotum, (the first segment of the thorax) and fine hairs on the exterior of the cuticle (Figure 1.3, 1.4).[46, 52] The biological relevance of these fine hairs is unknown. In addition, these beetles aggregate, often being found in groups of two or more.[51]

Forked fungus beetles belong to the *Tenebrionidae* family, which consists of around 20,000 species and are found world wide.[53] Adult forked fungus beetles are sexually dimorphic. Males have two sets of horns used to compete for mates found on the clypeus, the lower region of the face, and on the thorax, specifically on the top of the head; females lack the presence of horns (Figure 1.5).[53, 54] Forked fungus beetles are found on the Northeastern part of the United Sates and the eastern part of Canada. They inhabit and feed off of brackets of many species of shelf fungus that grow primarily on rotting logs.[55] These beetles exist with a range of horn and body sizes.[56] The adult beetles are smaller than the carabid beetles, ranging from 8 to 12 mm long. The exoskeleton is rigid and rough.[55]

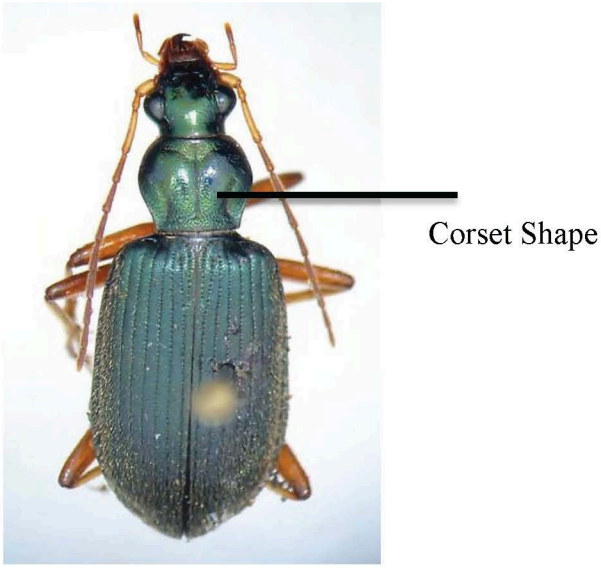


Figure 1.3 *Chlaneius cordicollis*, a species of the carabid family distinguished by their metallic green shell and corset shape beneath the head.[56]

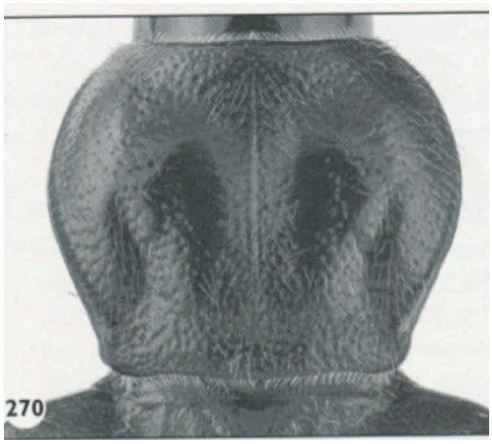


Figure 1.4 The pronotum, the first segment of the thorax of *Chlaneius cordicollis*, detailing the small hair on the cuticle. Figure is adapted from Bousquet (2010).[45]

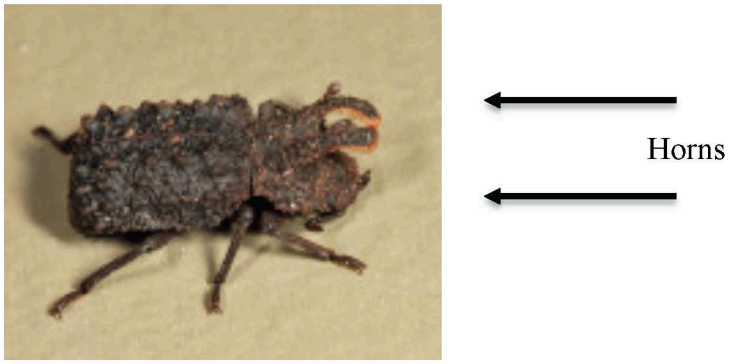


Figure 1.5 Male forked fungus beetle (*Bolithotherus cornutus*). The male can be distinguished by the two sets of horn, one on the clypeus and one on the thorax. These beetles have a rigid exoskeleton.[57]

Methods to Extract Cuticular Hydrocarbons

Different extraction methods have been developed to study the cuticular hydrocarbon profiles of insects. Existing procedures to extract cuticular hydrocarbons can be either lethal or nonlethal. The most established method to extract cuticular hydrocarbons is a lethal method in which the insects are directly introduced to toxic

solvents, mainly hexane.[58] This method is completed with freeze-killed insects and completely strips the insect of their cuticular hydrocarbons.[59] Additionally significant contaminates such as glandular products and internal products of the insect can be released when the insect is in direct contact with a solvent.[59, 60] The amount of hexane used to extract the hydrocarbons depends on the size of the insect, as it should be fully immersed in the solvent, and the amount of sample needed for the analytical technique. The amount of hexane is chosen to optimize the specific protocol.[61]

In some cases, composite sampling, in which multiple insects are used to complete one hexane sample, has been completed.[40] A composite sample provides a greater concentration of the individual components of the hydrocarbon profile so that they can be detected. This has been done for smaller insects, such as ants and bees that have less surface area and therefore contain a low abundance of hydrocarbons. Samples consisting of hydrocarbon extracts of thirty to sixty ants or five to twenty bees have been completed.[40, 41] This has been done for slightly larger insects as well when low signal intensities are obtained. Samples consisting of hydrocarbon extracts from two to ten larger insects have been completed.[38]

Another lethal procedure includes putting samples of glands or wings of an insect into micro-capillaries and directly injecting them into a gas chromatograph injection port using a holder for thermal desorption.[59] The holder for the samples is hard to come by and the samples do not give a full representation of the hydrocarbons the insect contains. The samples are only a small portion of the insect and may not contain many or all of the cuticular hydrocarbons.[58, 62]

Non-lethal extraction techniques allow for successive sampling and the completion of multiple samples over time.[3, 63] These extraction techniques are optimal for behavioral and ecology studies, where the insects are studied over time and need to be kept alive. There are a few established non-lethal extraction procedures. One established non-lethal procedure uses polydimethylsiloxane (PDMS) dual-layered Solid Phase Microextraction (SPME) fibers to trap non-polar high molecular weight compounds, volatile compounds and non-polar semi-volatiles.[38, 64] The SPME fibers are rubbed along the cuticle of the insect and directly thermally desorbed in the GC inlet.[38] While the results obtained using SPME fibers are similar to those using toxic solvent extractions, there are three important disadvantages to this method. First, the samples cannot be stored after analysis and therefore samples must be analyzed more rapidly than they would using alternative methods because the compounds will thermally desorb from the fiber over time. Second, the fibers can only be used a limited number of times as direct contact of the fibers with the cuticle damages the fibers. Finally, SPME fibers are very expensive.[60, 63, 65]

Some additional existing non-lethal procedures for extracting cuticular hydrocarbons from insects include using cotton wool or filter paper [63] and tepid water by which the water forms an emulsion with the hydrocarbons that can be extracted with solvent.[66] In addition, SPME fibers have been replaced with silicone tubing treated with bis trisfluoroacetamide.[67] The silicone tubing is washed with solvent instead of being directly heated, allowing for the fibers to be reused and decreasing the expense. These extraction procedures are not widely tested or commonly used.[2, 60]

Another non-lethal extraction procedure is a solid-phase extraction using styrene-divinylbenzene copolymers, Chromosorb® 101 which was originally used for chromatography in packed columns for binding non-polar compounds.[60] This is a porous divinylbenzene polymer and is very effective for the separation of hydrocarbons.[68] The advantages of using this resin include its insolubility in most solvents and its chemical resistance, which allows it to be reused. Also, when using a sorbent, an equal sampling of the hydrocarbons from the entire body of the insect is achieved unlike with SPME when sampling from one specific area.[60] Chromosorb® is made of diatomaceous earth, which is a naturally occurring, soft, siliceous sedimentary rock, functionalized with divinylbenzene. The supply of this functionalized diatomaceous earth is limited and therefore Chromosorb® is no longer commercially available.[69]

Ferreira-Caliman *et al.* (2012) developed the procedure to extract cuticular hydrocarbons with Chromosorb® from social insects, including species of bees and beetles: *Melipona marginata*, neotropical stingless bee in southeast Brazil, Africanized honey bees, *Apis mellifera* found in Brazil, and the solitary insect *Tenebrio molitor*,the Yellow Mealworm. They looked at the efficiency of recovery of the classes of hydrocarbon compounds in comparison to both the established lethal method for extraction of hydrocarbons using hexane and to existing non-lethal procedures using both PDMS/DVB and PDMS fibers. Gas chromatography- mass spectrometry was used to separate and analyze the components of the cuticular hydrocarbon mixture.[60]

To identify the compounds, the mass spectrum for each compound was compared with the NIST Library data and an alkane standard solution for compounds 21 to 40 carbon atoms.[60] They looked at the peak area from the total ion chromatograms to

estimate the relative abundance of each compound. Statistical analysis was also used to see the difference in the relative abundance of the classes of hydrocarbon compounds between the different methods used. Compounds were classified as linear and methyl alkanes, alkenes and alkadienes.[60] This method of identification is commonly used to identify the extracted hydrocarbons from insects.[2] The findings overall showed that each extraction method yielded qualitatively similar profiles of cuticular hydrocarbons and that the relative abundances of the different classes of hydrocarbons obtained after treatment with Chromosorb® are also similar to those found for the other extraction methods.[60] It is important to note that there is large qualitative variation in some individual compounds for all of the species studied. This is attributed to the fact that cuticular hydrocarbons play roles as individual chemical signatures.[60, 70] Beyond SPME and Chromosorb®, which are either expensive or unavailable respectively, none of these non-lethal procedures have been verified to be fully effective, therefore another method is needed.

Instrumentation

There are many techniques used to analyze cuticular hydrocarbons both as pure compounds and in a mixture. To identify the components in a mixture of cuticular hydrocarbons, these techniques exploit the physical properties, molecular mass, and chemical structures of cuticular hydrocarbons. Many of the techniques are used for both qualitative identification and quantitative measurements of the components, allowing for the determination of the composition of a cuticular hydrocarbon mixture.[1]

Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), is a very sensitive technique that has been used as a qualitative

technique to analyze cuticular hydrocarbons. This technique allows for the identification of high-molecular-weight hydrocarbons consisting of over 50 carbons, often containing highly unsaturated or cyclic components.[1, 71] The long chain hydrocarbons cannot be detected by other techniques due to their high boiling point.[2] This instrument was not available to use for the present study.

Near-Infrared Spectroscopy is another technique, used to analyze the cuticular hydrocarbons of intact insects.[1] The hydrocarbons of intact insects are rapidly scanned generating absorption spectra in the near-infrared without the need for extracting the hydrocarbons. Near IR provides pattern recognition based on C-H bonds. This technique examines the differences in cuticular hydrocarbons among insects but chemical specificity is not provided.[72, 73] Therefore, the components of the cuticular hydrocarbon profile cannot be determined.

GC-MS

Gas Chromatography-Mass Spectrometry (GC-MS) is the most common technique used to analyze cuticular hydrocarbons.[2] GC-MS is a selective and sensitive analytical technique for the separation, identification, and quantification of components of complex organic mixtures.[1] The components that are present in a complex mixture are separated by gas chromatography and then identified via their retention time and mass spectra. Chromatography produces nearly pure fractions of the chemical components in a mixture, while spectrometry produces selective information for identification of the components using standards or library spectra.[74] A two-dimensional identification is provided including a GC retention time and a mass spectrum for each component of the

mixture. The GC provides good separation and a clear mass spectra, normally with reliable structural assignments of the components.[1, 74]

Components of the Instrumentation

There are three components of the instrumentation: the gas chromatograph, the mass spectrometer and the data system.[1] The data system, namely the computer interface, has a dual role consisting of data acquisition and processing and instrument control. To determine quantitative results, the area of the chromatographic peak can be integrated using the data system. The area of the peak is proportional to the quantity of the component.[74]

Chromatography is a technique used on an analytical scale, which is used to separate and quantify components based on the elution characteristic of the mixture. This technique can therefore be used for both qualitative and quantitative analysis.[75] For gaseous or volatile mixtures of hydrocarbons, gas chromatography is the common technique used for analysis. This analytical method is limited to volatile compounds that will withstand thermal decomposition.[1]

Gas Chromatograph

Volatile liquids or gaseous samples can be directly injected into the GC through a rubber disk known as a septum into the heated injection port. The injection port is kept at a high temperature, well above the elution temperatures of all of the components of the sample. The sample quickly evaporates into the port and condenses onto the cool column, which is kept at a temperature well below the boiling point of the sample components. In

this way the entire sample condenses onto the column at once. As the temperature of the column rises, the entire component will elute at its elution temperature, which is based on the vapor pressure of the component. The carrier gas brings vapor into the column and the separated analytes flow through a detector that records their retention times. The detector is kept at a higher temperature than the column so the analytes remain gaseous upon detection.[74]

Chromatography phases

Chromatography consists of a mobile and stationary phase. In gas chromatography the mobile phase is a carrier gas, an inert gas such as helium. The mobile phase carries the sample mixture through the stationary phase in order for the mixture of compounds in the mobile phase to interact with the stationary phase. The stationary phase, which is contained in the column, is a chemical that can selectively attract components in the sample mixture. A common stationary phase in gas-chromatography columns is (alkyl) polysiloxanes that are chemically bonded to the column wall. After the polymer coats the column wall, it is often cross-linked to increase the thermal stability of the stationary phase and prevent it from gradually bleeding out of the column. This allows for higher temperatures of up to 400°C to be used.[74] At very high temperatures the stationary phase can decompose which leads to bleeding of the column. This is a decomposition of the products from the column. In addition, other components of the GC including the septum and o-ring in inlet can bleed at high temperatures. These products cause elevated background signal in the detector, which can contaminate

the detector. An advantage to the presence of peaks in the spectra due to bleeding is that they provide a continual check on the mass spectrometer calibration.[1, 74]

The stationary phase used in this research is relatively non-polar as are the hydrocarbons to be separated. The compounds elute based on equilibrium between the gas and liquid phase. The elution of the compounds is determined by the order of increasing vapor pressure, almost in the order of increasing boiling point, and affinity for the stationary phase.[74] The vapor pressure is the pressure that a vapor exerts in theromodynamic equilibrium with the liquid or solid phase of the compound at a given temperature in a closed system. The equilibrium vapor pressure indicates the evaporation rate of a liquid.[76] The affinity of the nonpolar compounds for the nonpolar stationary phase is based on dispersion forces. A compound with a high vapor pressure will have low dispersion forces and thus will have less affinity for the stationary phase. Compounds whose equilibria favors the gas phase more elute first. Generally elution occurs before the boiling point is reached because molecules have substantial vapor pressures below their boiling points. The temperature at which there are more molecules in the gas phase than the liquid phase should be slightly below the boiling point for the compound, as the entire compound does not need to fully be in vapor form to elute. The characteristics of the mobile and stationary phase can be changed so that different mixtures of chemicals can be separated. The temperature of the stationary phase and the pressure of the mobile phase can be adjusted to better refine the separation.[74]

A temperature program is used to slowly elute the components of a mixture. The equilibrium constant changes with the temperature, so each component will elute at a

different temperature.[1] Once the vapor pressure is great enough, there are more molecules of the component in the gas phase than the liquid phase, so the gas phase is favored and the compound will elute. By raising the temperature of the column the components separate and elute at different times. Raising the temperature too quickly will cause more than one component to elute at a time or result in overlapping peaks on the chromatogram. In addition, raising the temperature too slowly can result in the entire component not eluting at once, resulting in broad peaks.[74]

GC columns

Capillary columns are commonly used in gas chromatography as they provide high separation efficiency and a short analysis time. These columns are a thin fused-silica (SiO_2) capillary, which is a narrow open tubular column with the stationary phase coated on the inner surface. The inner diameter of the column is typically between 0.10 and 0.53 mm with lengths between 15 and 100 m. A column length of 30m is typical.[74]

Injection of Samples into the GC

To inject liquids into the GC a sandwich injection technique can be used. This involves sandwiching the sample between air by taking up a certain volume of air, then sample, then air, so that the sample is surround by air.[77] The purpose of the sandwiching technique is to ensure that there is no sample in the needle, so the sample does not immediately evaporate when the needle is introduced to the hot injection port through the septum. If there is sample in the needle, the most volatile components would evaporate before the whole sample was injected into the column. The air plug behind the sample

expels the sample from the needle. The common amount of sample injected is about 0.1 to 2 μl of liquid sample.[74]

Split vs. splitless mode of injection

A split injection is best for high concentrations of analyte for gas analysis because it injects only a fraction of the sample into the column, about 0.2 to 2% of the sample. When the analyte constitutes >0.1% of the sample the split mode of injection is preferable, especially when using a narrow column. High resolutions are achieved with a very small amount of sample, the smallest amount that can be detected. This is about ≤1 μl of liquid sample, containing about≤1 ng of each component of the sample.[74] The split ratio is the proportion of sample that does not reach the column. The excess sample that does not reach the column is released into a waste vent, which is controlled by the pressure regulator leading to a needle valve.[74, 77] One disadvantage of the split ratio mode of injection is that it is not reproducible from run to run and therefore quantitative analyses are often inaccurate.[74]

Splitless mode is an alternative mode of injection used for very dilute samples.[77] This mode of injection is for samples that contain less than 0.01% of the analyte of interest. About 80% of the sample is injected onto the column. Since there is a larger volume of sample being injected it spends more time in the injection port. The splitless injection is slower than that of the split mode of injection because in the splitless mode the sample spends more time in the injection port, and spending a long period of time in the high temperature of the injection port might decompose the sample. This mode of injection uses solvent focusing. This is when the column temperature is set at least 10 to

20 °C below the boiling temperature of the solvent. The solvent will fully condense at the front of the capillary column before traveling down the column. This is to concentrate the components and to prevent each species from vaporizing at different times. After the sample is condensed, the column temperature is slowly raised to vaporize the solvent that is trapped at the start of the column. All of the molecules of the component travel down the column together, resulting in sharp peaks on the chromatograph, which otherwise would be broader. If the component does not evaporate at once, broad or multiple peaks can occur for the species.[74]

Mass Spectrometer

Mass spectrometry (MS) is the most powerful analytical technique used to characterize hydrocarbon mixtures at the molecular level. Mass spectrometry is used in tandem with gas chromatography to separate and analyze the cuticular hydrocarbons.[1] Directly introducing the capillary column into the ion source of the mass spectrometer is the common method used for interfacing the two techniques. Once the components in the hydrocarbon mixture are separated, their structures and molecular masses can be determined by MS. In this technique, the molecules are ionized and the ionic species are separated by an ion trap. Electron impact ionization (EI) with an electron beam at 70ev is the most common ionization technique used in conjunction for gas chromatography. The EI spectrum produces a fragmentation pattern from which the structure of the molecule can be found.[74]

The fragmentation patterns produced by the mass spectrometer can be used to identify the compounds of the chromatographic peaks. To identify compounds from the

fragmentation patterns, a spectra database and standards can be used as a comparison for identification. A specific mass to charge ratio (m/z) can be selected for. By selecting for a specific ion, only the chromatographic peaks that contain the specific ion will appear at their respective retention times. This allows for compounds to be more easily selected for in a complex chromatogram.[1] Fragmentation patterns can be similar for isomers, such as branched and un-branched compounds. There can be extensive fragmentation, which makes it difficult to identify the compounds.[74]

Experimental Goal

The goal of this thesis research is to develop a non-lethal procedure using a sorbent to efficiently extract the cuticular hydrocarbons from the cuticles of forked fungus and carabid beetles. A non-lethal procedure will allow long-term and continued ecological observation of the beetles. Intra-specific differences in defensive spray components have been found for carabid [47] and forked fungus beetles.[48] In addition, the majority of studies on insect cuticular waxes are on social insects. Since these are nonsocial species with unknown cuticular profiles, I am investigating to see if there are differences in these beetles' cuticular hydrocarbons aiding them in chemical communication.

Another goal of the study is to identify the major components in the cuticular profiles of the beetles and to compare the components of the profile and their abundances between beetles. Classifying the components of the profile is an important step in learning what compounds are essential in intraspecific communication and if these differ between species. Cuticular hydrocarbon samples will be analyzed using gas

chromatography-mass spectrometery. The focus is on classifying the cuticular hydrocarbons with shorter chains, consisting of 21 to 40 carbons, due to instrumentation limits using the GC-MS. I do not have access to other instruments, such as MALDI, that would allow of the identification of longer hydrocarbon chains.

Chapter 2: Experimental Procedures

The cuticular hydrocarbons were extracted from forked fungus beetles from Virginia and carabid beetles, including *Chlaenius cordicollis*, *Agonum extensicolle*, *Chlaenius tricolor*, *Chlaenius sericeus* and *Chlaenius impunctifrons* from Manitoba, Canada and Poconos, Pennsylvania. Extractions were completed at room temperature (25°C), first using the optimized novel and established non-lethal procedures, extraction procedures for cuticular hydrocarbons using C_{18} and SPME respectively, and then using the established lethal procedure. Both the novel non-lethal and lethal procedures were completed for each sample to compare the compounds extracted and the intensities between the two. The same novel non-lethal and established lethal procedures were completed for the fungus inhabited by the forked fungus beetles. This served to compare the hydrocarbon profiles of both the beetles and their food source, upon which they live. No uninhabited fungus was located to sample.

Non-lethal extraction procedure for cuticular hydrocarbons using Porapak P•

A novel non-lethal procedure using SUPELCO Porapak P• with particles between 180 and 300 μm was initially developed. The procedure was first tested with freeze-killed beetles. After these tests, this procedure was determined not to work and was no longer used. This protocol was modified from a non-lethal procedure from Ferreira-Caliman *et al.* (2012) using Chromosorb• 101 [SUPELCO] with particles between 250 and 180 μm, 0.3 g/cc.[60] Chromosorb• was initially used for chromatography in packed

columns for the analysis of free fatty acids, glycols, alcohols, esters, ketones, hydrocarbons, and ethers. The advantages of this resin include its insolubility in most solvents and its chemical resistance, allowing it to be reused.[60] Chromosorb® is made of divinyl benzene diatomaceous earth; although diatomaceous earth is a naturally occurring, soft, siliceous sedimentary rock, the supply of the derivatized sorbent is limited.[69] Therefore, Chromosorb® is no longer commercially available and an alternative was needed. Porapak P® was used instead because it is similar to Chromosorb® and is often used in its place in industry. Both are porous divinylbenzene polymers, and are very effective for the separation of hydrocarbons.[68]

Before use, Porapak P® was washed with hexane and then with chloroform, both in a 1:10 ratio of Porapak P® to solvent. Chloroform dissolved the Porapak P®, therefore hexane was used as the only solvent from then on. Porapak P® was dried in an oven for 30 minutes and was then allowed to cool to room temperature for 30 minutes before being used. A vial was filled with 0.1 g Porapak P®. The beetle was placed in the vial on its back and gently agitated for 10 minutes, rotating the beetle along the edge of the vial, so that the beetle came into contact with all of the beads. The beetle was removed from the vial and the Porapak P® was washed in 2 mL hexane to recover the extracted compounds for analysis. The vial was securely capped and repeatedly inverted for one minute to transfer the hydrocarbons to the hexane. The hexane was removed using a VWR disposable Borosilicate glass Pasteur pipette 9″ and placed in a 2mL, 9mm diameter screw top GC vial.

Final non-lethal extraction procedure for cuticular hydrocarbons using C_{18}

A non-lethal procedure was developed using porous octadecyl carbon chain - bonded silica 50 µm particles (C_{18}) from Agela Technologies, which is a sorbent used for Reverse Phase Fast Protein Liquid Chromatography (RP-FPLC).[78] This protocol was further adapted from the procedure completed with Porapak®. The procedure was originally developed for freeze-killed carabid beetles and was used for both live *Chlaenius* and forked fungus beetles once the procedure was established to work.

C_{18} was washed with hexane in a 1:10 ratio of C_{18} to hexane, dried in an oven for 30 minutes and allowed to cool to room temperature for 30 minutes. The beetle was gently removed from the fungus with tweezers around the posterior, taking care to avoid the legs and placed in a vial filled with 0.1 g C_{18}, on its back. The beetle was gently agitated in the C_{18} powder for five minutes, by rotating the beetle along the edge of the vial, so that the beetle came into contact with all of the powder. The beetle was removed from the vial with tweezers and placed onto a scoopula. While on the scoopula, the remaining C_{18} was gently rinsed off of both sides of the beetle with purified water. A minimal amount of water to remove all of the C_{18} from the beetle was used. The end of the scoopula was held over the vial to ensure that neither water nor beetle was lost. The beetle was moved between pools of water on the scoopula to transfer all of the C_{18} from the beetle to the water and the water was poured from the scoopula into the vial. After rinsing the beetle, it was placed back in its environment in the laboratory and assessed for longevity over a week to see if the procedure had an affect on the mortality of the beetle.

Two milliliters of hexane was added to the C_{18} and water to complete a liquid-liquid extraction to recover the extracted compounds from the C_{18} for analysis. The vial was securely capped and repeatedly inverted for one minute to transfer the hydrocarbons to the organic layer. The organic layer (hexane) was removed using a VWR disposable Borosilicate glass Pasteur pipette 9″ and placed in a 2mL, 9mm screw GC vial.

Non-lethal extraction procedure for cuticular hydrocarbons using SPME

The non-lethal protocol to extract cuticular hydrocarbons from beetles using SPME fibers was adapted from Ginzel *et a*l. (2003).[38] The cuticular hydrocarbons were extracted using a two cm-long Solid Phase Microextraction (SPME) fiber, coated with polydimethylsiloxane (PDMS, 100 μm) (SUPELCO). The fiber was rubbed gently along the cuticle, from beneath the head to the end of the beetle for 20 seconds, repeatedly turning it for the entire fiber to absorb the chemical compounds. The extracted compounds were analyzed using a GC-MS. The SPME fiber was directly introduced into the GC-MS injection port for five minutes to desorb the compounds.

Lethal extraction procedure for cuticular hydrocarbons using hexane

For each beetle, after completing one or both of the non-lethal procedures, the established lethal procedure was completed using the toxic solvent hexane. This protocol was modified from Bagneres and Morgan (1990) to optimize the parameters: the time of the extraction and the volume of the solvent.[58] This was completed in order to establish the efficiency of recovery of cuticular hydrocarbons of the novel non-lethal procedure by comparing the chromatograms, the compounds extracted and their intensities, from the

novel non-lethal procedure and the established lethal procedure. The beetle was freeze-killed at -15°C and the hydrocarbons were extracted by placing the insect in 2 mL hexane and gently swirling for one minute. The amount of hexane was chosen so that the entire beetle was immersed in the solvent and for the fact that the larger the volume of solvent, the shorter the extraction time.[1] The beetle was removed and allowed to air dry before storing. Beetles were stored in a vial in the freezer

Additional procedural steps

These additional procedural steps were only completed with a subset of the trials. These steps were completed to try to obtain greater concentrations of the individual components and to eliminate contamination in the samples. For some trials, the hexane extracts of the C_{18} and those from the lethal procedure were concentrated to achieve greater intensities of the extracted compounds. The samples were evaporated under an air stream and re-suspended in 30 μL hexane. Some of the hexane extracts of the C_{18} were filtered to eliminate the C_{18} powder and other contaminants. Disposable 3 mL BD Luer-Lok Tip Syringes were used with a 0.45 μm PTFE filter. 50 μL of the hexane extract was filtered and placed into a GC vial.

Controls

Positive controls were completed to ensure that the sampling method and GC-MS program were efficient. Fifty micro-liters of C7-C40 saturated alkane standard (1000 μg/mL in hexane) was placed on wax paper to represent the cuticle of the beetle or

directly onto the C_{18}, allowed to dry, and treated in the same manner as the beetle samples.

A negative control consisting of pure hexane was run. In addition, hexane and hexane with water, with each sorbent material used (Porapak® and C_{18}), were treated in the same manner as the beetle samples. Both sorbent materials were also left in hexane and water for one week to check for decomposition of the compounds of the sorbent.

Instrumental Analysis

The samples for each procedure were stored in vials at room temperature until analysis was completed, excluding the samples, which were absorbed by SPME fibers. The analyses of the extracted compounds and controls were conducted within a week after sampling. Analysis was completed with a Varian Saturn 2100T gas chromatograph-mass spectrometer (GC-MS). Separation was achieved on a FactorFour VF-5 ms column (30 m, 0.25 mm i.d., 0.25-μm) with a 1.2 mL/min helium mobile phase. The samples were introduced both by manual injections and injections completed by the autosampler. A sandwich injection method was used, consisting of 1 μL hexane extract of each sample sandwiched between 1 μL of air on both sides. Different temperature programs were used to optimize the parameters to achieve the best separation and resolution of the compounds (**Table**). The parameters varied include the ramping speed and injection mode.

Table 2.1 Parameters for GC-MS temperature programs. The injection split ratio was 20 and the injection port temperature was 280 °C for each run. These temperature programs were used to optimize the parameters of ramping speed to achieve the greatest separation and resolution of the compounds. Only the final program was used to evaluate the samples.

	Initial oven temperature (°C)	Initial oven temperature duration (minute)	Increment temperature increases by (°C min^{-1})	Oven temperature at end of initial ramp	Increment temperature increases by (°C min^{-1})	Final oven temperature (°C)	Final oven temperature duration (minutes)	Total Program Duration (minutes)
Program 1	40	1	3			300	5	92.67
Program 2	40	1	10	150	3	300	5	67.00
Program 3	40	1	5			300	5	58.00
Program 4	40	1	5	150	3	300	5	78.00
Final Program	40	1	10			300	5	32.00

Final optimized temperature programs

The temperature program used for both manual injections and autosampler injections began with an initial oven temperature of 40°C and after one minute was increased by 10°C min^{-1} to 300°C and held for five minutes. The total run time was 32 minutes. The injection port temperature was 280°C. The autosampler injections were performed in the splitless mode with a split ratio of 100 at 1.01 minutes and a split ratio of 20 at 2.00 minutes.

The temperature program for the SPME injections was the same as the manual and autosampler injection programs. The temperature program was performed in the splitless mode with a split ratio of 20 starting at 1 minute. The injection port temperature of 260°C was lower than it was for the other injection methods so not to damage the fiber. The SPME fiber was cleaned between analyses at 270°C for 10 minutes.

Parameters for the mass spectrometer

The mass spectra were obtained after70 eV electron impact ionization. Ionization started at 5.00 minutes, with analysis *m/z* range from a low mass of *m/z* 42 and a high mass of m/z 650 with ionization mode EI Auto.

Identifying compounds

The chemical compounds were initially identified based on their mass spectra by comparison with the NIST Library data. To identify the hydrocarbons extracted, a C7-C40 saturated alkane standard (1000 μg/mL in hexane) was analyzed using the optimized temperature programs for the GC-MS. The alkane standard was analyzed at a concentration of 1.50 ppm. To obtain this concentration, alkane standard was allowed to dry for two minutes to evaporate the hexane it was originally in before adding the hexane used to dilute the sample.

Standards for eicosane, 1-eicosanol, hexatriacontane, heneicosane, n-docasane and nonadecane were analyzed at a concentration of 1.50 ppm. Concentrations of the standards were made using hexane as a solvent. The eicosane and hexatriacontane standards were cross-referenced with the alkane standard chromatogram to confirm the correct peaks for these standards and to calibrate the alkane standard. These standards along with the 1-eicosanol standard were then used to identify the corresponding peaks on the chromatograms of the beetle samples. All standards used are from Sigma Aldrich.

Data Analysis

The chemical composition of the cuticular hydrocarbon profiles was quantified by integrating peak areas of the chromatograms (Table 2). The chromatogram with all mass-to-charge ratios was evaluated to look at the overall signal intensities of the peaks. Only the identified peaks using standards are included in the analysis to determine the percent that each component is of the identified cuticular profile. The relative peak areas of each component identified in the profile were reported as percentages of the total summed cuticular hydrocarbon peak area. The percentage of each component is dependent on the absolute value of the other peaks. The absolute values for peak area for each identified component was reported as well.

In addition, ion *m/z* 57 (mass-to-charge ratio) was selected for because the majority of hydrocarbons contain this ion fragment. The mass extracted chromatogram for *m/z* 57 shows the times that all of the ion 57 fragments come out of the GC column. This is comparing the selected signal intensity for ion 57 to the total signal intensity for each peak. The mass extracted chromatogram was compared to the total chromatogram to ensure that the peaks identified are from hydrocarbons and that other components are not contributing to the peak.

Table 2.2 Parameters for integrating peak areas for the GC chromatograms.

Base Parameters	**Setting**
Threshold	700
Minimal Abundance	30
Max Pre-Search Hits	6000
Max Final Search Hits	100
Integration Parameters	
Peak Width (seconds)	4.0
Slope Sensitivity (SN)	3
Tangent %	10
Peak Size Reject	2000

Chapter 3: Results and Discussion

Overview

To allow for continued ecological studies, the cuticular waxes of carabid and forked fungus beetles were extracted with a novel nonlethal procedure using C_{18}. The samples were analyzed using GC-MS with a temperature program designed specifically for these samples. To establish the efficiency of the nonlethal method, it was compared to the established lethal method using hexane. The main components in the profiles for the cuticular waxes of the different species of beetles were identified and compared. The identified components were specifically compared between the two extraction methods. The resulting signal intensities for the extracted components were low, making them difficult to identify. Different techniques were used to increase the signal intensities. In addition, contamination was observed in the chromatograms about halfway through this study, preventing many of the results from being used. Much of the time was spent identifying the source of contamination so the study could be continued.

Development of a GC temperature program

Different parameters were tested in developing a temperature program to analyze the samples including time and temperature. The parameters were altered to achieve a short duration of the program and clear peak resolution with good separation and sharp peaks. Five different temperature programs were tested, each with an initial injection port temperature of 280 °C. All programs began with an initial oven temperature of 40 °C, increasing to 300 °C after one minute and remaining at this temperature for five minutes. The injection port temperature of 280 °C was chosen to elute all of the molecules from

the injection port to the start of the column. The initial oven temperature of 40 °C is low, well below the boiling temperature of all components of interest, to ensure that all components condense at the start of the column before eluting and begin the separation at the same time. The final oven temperature of 300 °C is held for five minutes because this is above the boiling temperature of all of the expected components and will elute any component left on the column.

Different temperature increments were tested on an alkane standard to see which resulted in the best peak resolution. Three microliters of a C7-C40 saturated alkane standard (1000 μl/mL in hexane) was air dried for three minutes to evaporate the hexane it was stored in, and then 2000 μl hexane was added. This sample was used for each temperature program because the resolution of the peaks could be clearly observed. All of the expected n-alkane peaks were observed for each trial. Program 1 resulted in good peak resolution but is a long time period per sample, 92.67 minutes. Programs 2, 3 and 4 have slightly broad peaks (Table 3.1). The final optimized temperature program has the shortest duration and has clear peak resolution (Figure 3.1).

All of the samples were run using the optimized temperature program for both manual and autosampler injections. The initial temperature of the injection port was 280 °C. The oven temperature began at 40 °C for one minute and was increased by 10 °C min^{-1} to 300 °C and held for five minutes. The duration of this temperature program is 32 minutes. A splitless injection with a slow injection speed is used for three reasons. First, it prevents the gas products of solvent flash vaporization from forming too quickly and creating backflow.[77] Second, it ensures that the injection port does not overflow with liquid, which would prevent the entire sample from being injected into the

column and could contaminate upstream parts of the system. Finally, the analyzed components are found in low concentrations, which would limit the results of a split injection. With a split different amounts of each component may be put onto the column, as something more easily vaporized will not condense onto the column and will go into waste. This is avoided with a splitless injection.[74]

The solvent for this procedure is hexane, which is commonly used to extract cuticular hydrocarbons and has been shown to be the most successful solvent in extracting the cuticular waxes from insects.[2, 39] Pure hexane was analyzed using the GC-MS to find the retention time for hexane. The retention time for hexane is 2.57 minutes (Figure 3.2). Therefore the mass spectrometer was set to start collecting data at three minutes so that the solvent peak was not included in the data set. During the three-minute delay, compounds coming off the GC column are not ionized. Since the mass spectrometer cannot detect neutral species, data is not collected.[1] None of the cuticular wax components are expected to elute before three minutes.

Table 3.1 Parameters for the different GC-MS temperature programs tested. Each program increases in temperature by different amounts and time intervals. The injection split ratio was 20 and the injection port temperature was 280 °C for each run. The different parameters were tested to achieve the greatest separation and resolution of the compounds. This table is also shown in Chapter 2.

	Initial oven temperature (°C)	Initial oven temperature duration (minute)	Increment temperature increases by (°C min^{-1})	Oven temperature at end of initial ramp	Increment temperature increases by (°C min^{-1})	Final oven temperature (°C)	Final oven temperature duration (minutes)	Total Program Duration (minutes)
Program 1	40	1	3			300	5	92.67
Program 2	40	1	10	150	3	300	5	67.00
Program 3	40	1	5			300	5	58.00
Program 4	40	1	5	150	3	300	5	78.00
Final Program	40	1	10			300	5	32.00

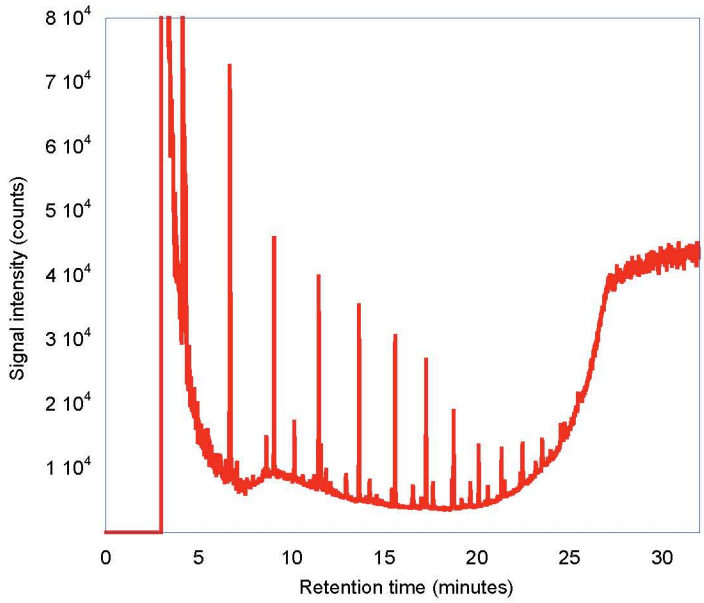


Figure 3.1 Final optimized GC temperature program to analyze cuticular waxes extracted from forked fungus and carabid beetles. This program has a total duration of 32.00 minutes. The temperature program is detailed in table 3.1.

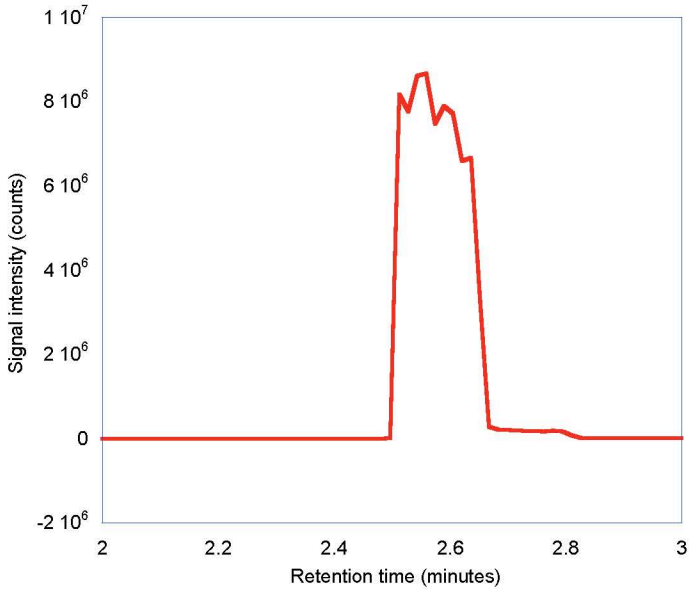


Figure 3.2 Chromatogram for pure hexane with a retention time of 2.57 minutes.

Peak Identification

Identifying components of the cuticular waxes for *Chlaenius cordicollis* and forked fungus beetles

The components of the cuticular waxes for both *Chlaenius cordicollis* and forked fungus beetles were identified using standards. Each standard was diluted using hexane to concentrations of 3333 ppm and 1.50 ppm. Both concentrations of each standard were analyzed with the GC-MS with the final optimized method and used to clearly visualize the peak for the standard. In addition, the chromatograms for the standards were compared to the C7-C40 saturated alkane standard to correctly identify the increments in the alkane standard and to confirm that the peaks observed for the standards were correct. The retention time was found for each standard. The peaks in the beetle samples were identified by retention time. In addition the lower concentration samples for each standard, which were closer in intensity to the beetle sample peaks, were directly compared to the beetle samples to confirm that the peak was correctly identified.

In choosing standards to analyze, the NIST mass spectra library was used. The compounds that were commonly identified by the NIST library were chosen to analyze. Though compounds identified using the NIST library did not correctly correlate to the specific peak, the compound was often a component of the cuticular profile. Straight-chain alkanes are a common class of hydrocarbons found on the cuticle of insects.[3, 5, 6] In addition, different fatty alcohols have been found to be a component in the cuticle profile of different insects.[12, 24]

Standards for four straight-chain alkanes including docosane, eicosane, heneicosane and nonadecane and one straight-chain fatty alcohol, 1-eicosanol, were analyzed and identified as components of the cuticular waxes for the beetles (Figure 3.3) A standard for hexatriacontane was purchased but this was not soluble in either hexane or dichloromethane. Therefore it is unlikely that it would have been a component extracted in the procedures. The peaks elute in the order of increasing boiling point as expected (Table 3.2). The compounds elute in the order of increasing vapor pressure, which is based on increasing boiling point.

Both species of beetle were found to contain a different variation of the compounds. *Chlaenius cordicollis* was found to contain all five components: docosane, eicosane, heneicosane, nonadecane, and 1-eicosanol (Figure 3.4 A). Forked fungus beetles were found to contain only three of the components: eicosane, heneicosane and nonadecane (Figure 3.4 B). This difference can be attributed to the fact that the beetles synthesize the cuticular waxes through different mechanisms.[14, 23] Therefore, each species is not expected to synthesize the same compounds. In addition the cuticular profiles are species specific; not all species will contain the same compounds.[2, 29]

When sampling live beetles with the non-lethal extraction procedure, they often released defensive secretions. The secretions for both *Chlaenius cordicollis* and forked fungus beetles have a pungent smell noticable when sampling. This was taken into account when identifying components of the cuticular waxes. The most abundant defensive secretions are phenols for *Chlaenius cordicollis* [47] and quinones and phenols for forked fungus beetles.[48] The defensive secretion for *Chlaenius*

cordicollis is predominantly 3-methylphenol[47] and the common components in the defensive secretions of forked fungus beetles include alkylated benzoquinones, methyl-*p*-benzoquinones and ethyl-*p*-benzoquinones.[48] The chromatograms for the samples were compared with the NIST library to identify peaks for the defensive secretions and to ensure that the peaks identified were components of the cuticular waxes and not of the defensive secretions.

Identifying components of other carabid beetles

Other species of carabid beetles were sampled including *Agonum extensicolle*, *Chlaenius tricolor*, *Chlaenius sericeus* and *Chlaenius impunctifrons*. These beetles were found when collecting *Chalenius cordicollis*, as these species of beetles often live in the same niches. Therefore, these beetles may interact with one another. The cuticular waxes were analyzed for these beetles to see if they contained similar components to *Chlaenius cordicollis*.

Each species of beetle was sampled with both the lethal hexane extraction procedure and the novel non-lethal C_{18} extraction procedure. Only freeze-killed beetles were available and were used for both extraction methods. The peaks were identified using standards in the same manner as with *Chlaenius cordicollis* and forked fungus beetles. For consistency, the GC chromatograms shown are for the hexane extraction. The signal intensities are low and many of the peaks are not clearly resolved, making them difficult to identify. Of the five compounds identified in *Chlaenius cordicollis*, only two components could be clearly identified for each species. Heneicosane and 1-Eicosanol were identified for *Agonum extensicolle*,

Chlaenius tricolor and *Chlaenius sericeus* (Figures 3.5, 3.6, 3.7). Eicosane and 1-Eicosanol were identified for *Chlaenius impunctifrons* (Figure 3.8). 1-Eicosanol was identified for all five species suggesting that it is a component of the cuticular waxes for beetles in the carabid family and may play an important role as a sociochemical. The peaks were hard to identify, therefore these species may contain the other three components even though they were not seen in the resulting chromatograms for the beetles sampled. Further trials would need to be completed to confirm these results.

Confirming the identification of the components

To confirm that the hydrocarbons identified are straight-chains, Kovat's retention index was used. Kovat's retention index can be used to estimate the expected retention time of a compound for gas chromatography. For a straight-chain alkane the retention index is 100 times the number carbon atoms the compound contains. The retention time of a compound is compared to its adjacent n-alkanes. The Kovat's index is given by the following equation, where I = Kovat's retention index, n = the number of carbon atoms in the smaller n-alkane, N = the number of carbon atoms in the larger n-alkane, $t_{r(n)}$=the retention time for the of the smaller alkane and $t_{r(N)}$=the retention time for the of the larger alkane.[74]

$$I = 100x\left[n + (N-n)\frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}}\right]$$

Branched alkanes were compared to the NIST index of Kovat's retention values and were found to have a different retention index from the corresponding n-alkanes. For example,

3-methyleicosane ($C_{21}H_{44}$) has a retention index of 2045. This means that this compound would be expected to have a retention time about half way in between those for eicosane ($C_{20}H_{42}$) and heneicosane ($C_{21}H_{44}$). Therefore, the branched alkanes would not have the same retention time.

Alkanes have inter-molecular van der Waals forces; the stronger the forces, the greater the boiling point. The van der Waals forces are stronger for molecules with a greater surface area and for molecules with a greater number of electrons surrounding the molecule, which increases with molecular weight. Straight-chain alkanes have higher boiling points than branched alkanes. Straight-chain alkanes have a greater surface area and pack together more tightly, and therefore have greater van der Waals forces between adjacent molecules.[74] Therefore, the branched alkane is expected to have a lower boiling point than the alkane with the same molecular formula, as shown for 3-methyleicosane ($C_{21}H_{44}$) and heneicosane ($C_{21}H_{44}$).

The mass spectra were also used to ensure that the compounds identified are straight-chain alkanes. The mass spectra of branched alkanes have slightly different fragmentation patterns from the straight-chain alkanes. In general the branched alkanes have a greater abundance of ions with a higher mass to charge ratio than the n-alkanes. Similar ions between the straight-chained and branched alkanes also have slightly different mass to charge ratios. While the mass spectra did not clearly match a specific compound for the identified peaks, they had mass spectra resembling the n-alkanes. Some of the mass spectra for the unidentified peaks in the cuticular profiles did contain fragmentation patterns resembling branched hydrocarbons.

Challenges with peak identification

The original method to identify the component in the cuticular profile for the beetles was by the mass spectra. Extensive fragmentation in the mass spectra for the components made it difficult to identify the compounds by their spectra. In the case of this experiment, the structural assignments provided by the mass spectra are not clear, causing complications in identifying the components in the cuticular profile. Therefore, standards were used in identifying the compounds.

The fragmentation patterns for the n-alkanes are similar, making it hard to differentiate between the compounds based on their spectra. As shown in figure 3.9, the mass spectra for two of the identified n-alkanes, eicosane and nonadecane, only have slight differences in their fragmentation patterns. The extensive fragmentation for the components in the beetle sample also made it difficult to identify the compounds based on the spectra. Therefore, the assignment of compounds could only be confidently completed using standards.

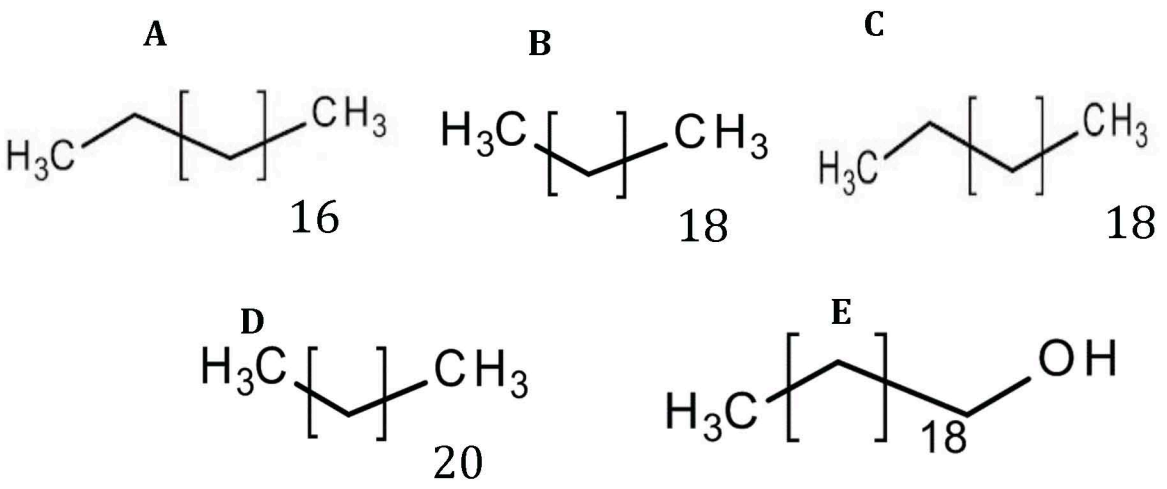


Figure 3.3 Structures of the components identified in the cuticular waxes of *Chlaenius cordicollis* and forked fungus beetles. **A)** Nonadecane ($C_{19}H_{40}$), **B)** Eicosane ($C_{20}H_{42}$), **C)** Heneicosane ($C_{21}H_{44}$), **D)** Docosane ($C_{22}H_{46}$) and **E)** 1-Eicosanol ($C_{20}H_{42}O$).

Table 3.2 Components identified in the cuticular waxes of *Chlaenius cordicollis* and forked fungus beetles. The retention times were found from standards run on the GC-MS using the final optimized temperature program. The numbers correspond to the peaks in figure 3.4 A and B.

Cuticular Wax Components	Number corresponding to Figure 3.4	Retention Time (minutes)	Boiling Temperature (°C)
Nonadecane	1	18.82	330.0
Eicosane	2	19.81	342.7
Heneicosane	3	20.75	356.5
Docosane	4	21.66	369
1-Eicosanol	5	22.29	372

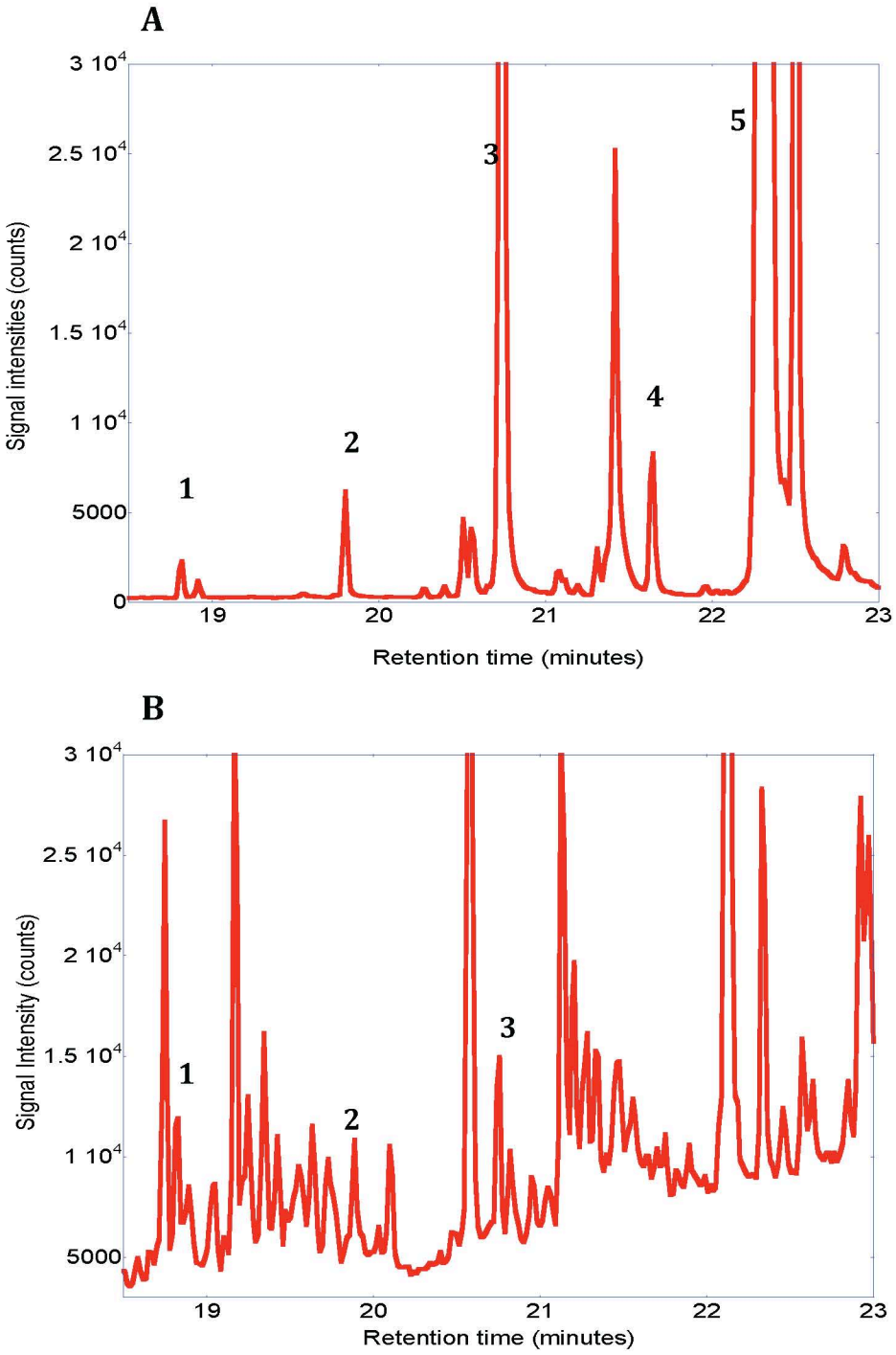


Figure 3.4 Identification of cuticular waxes for **A)** *Chlaenius cordicollis* and **B)** forked fungus beetles. Four hydrocarbons and one alcohol were identified by comparing standards to the beetle samples. **1)** Nonadecane, **2)** Eicosane, **3)** Heneicosane. **4)** Docosane and **5)** 1-Eicosanol.

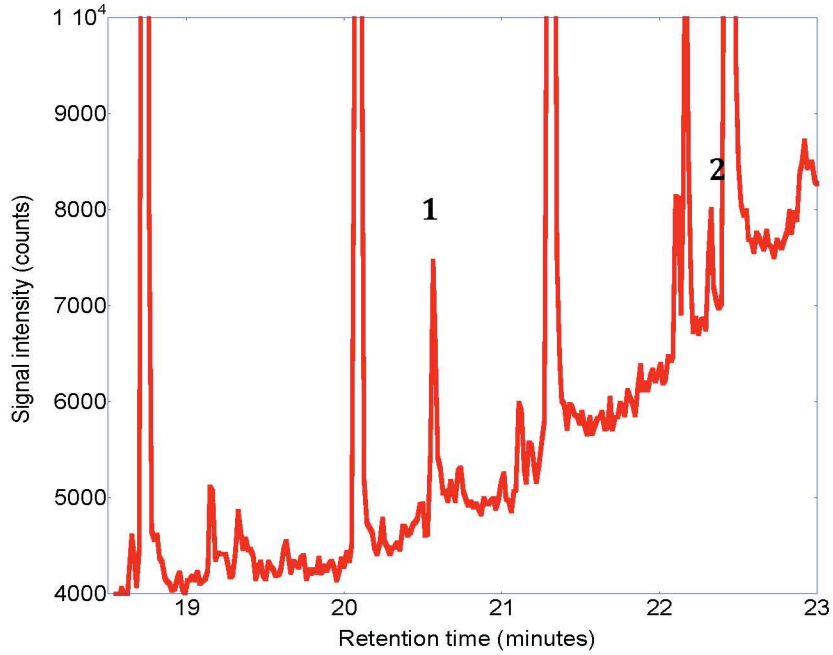


Figure 3.5 Identification of cuticular waxes for *Agonum extensicolle*. One hydrocarbon and one alcohol were identified by comparing standards to the beetle samples. **1)** Heneicosane; **2)** 1-Eicosanol.

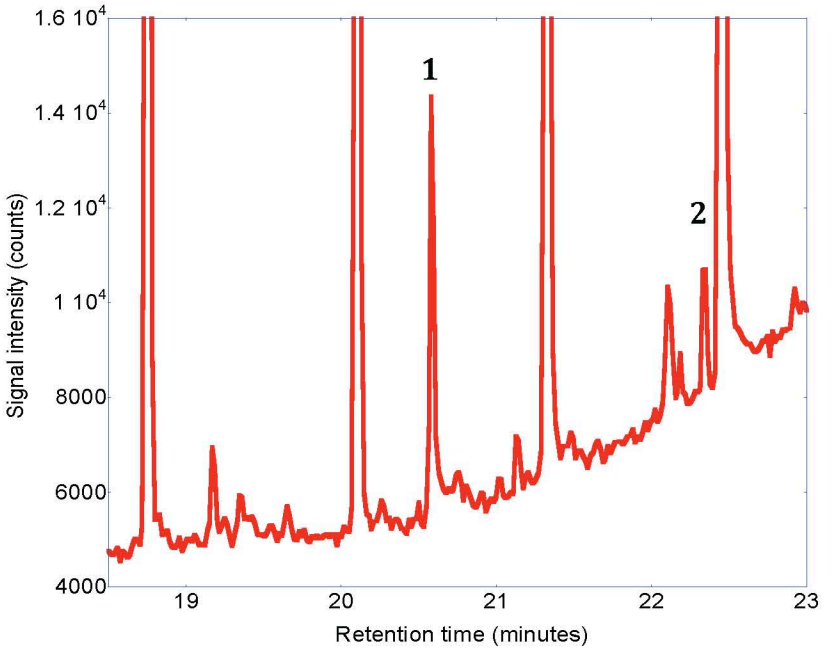


Figure 3.6 Identification of cuticular waxes for *Chlaenius tricolor*. One hydrocarbon and one alcohol were identified by comparing standards to the beetle samples. **1)** Heneicosane; **2)** 1-Eicosanol.

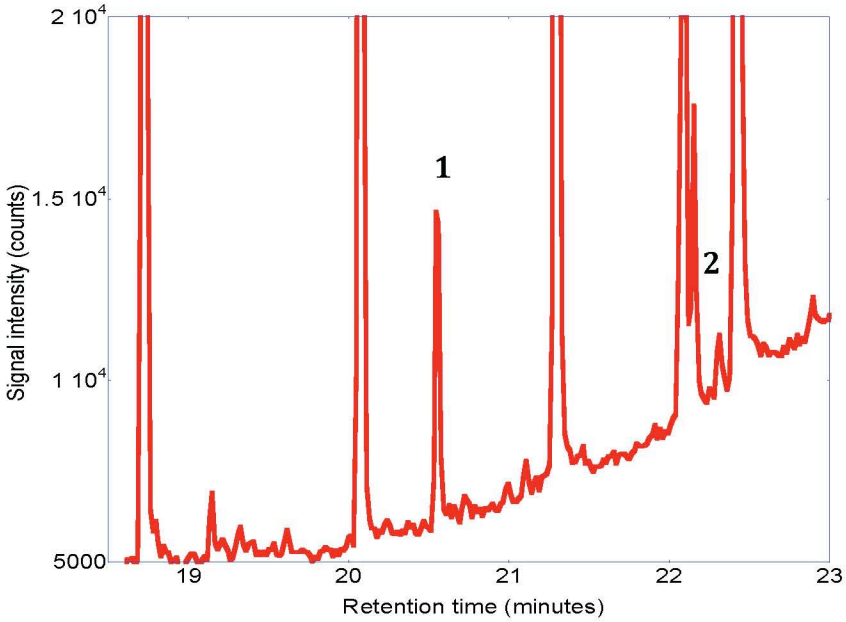


Figure 3.7 Identification of cuticular waxes for *Cheanius sericeus*. One hydrocarbon and one alcohol were identified by comparing standards to the beetle samples. **1)** Heneicosane; **2)** 1-Eicosanol.

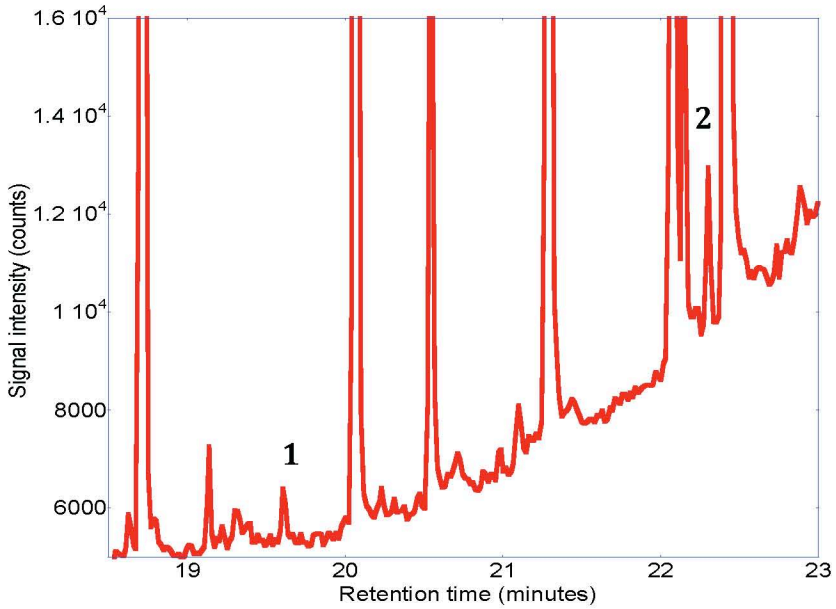
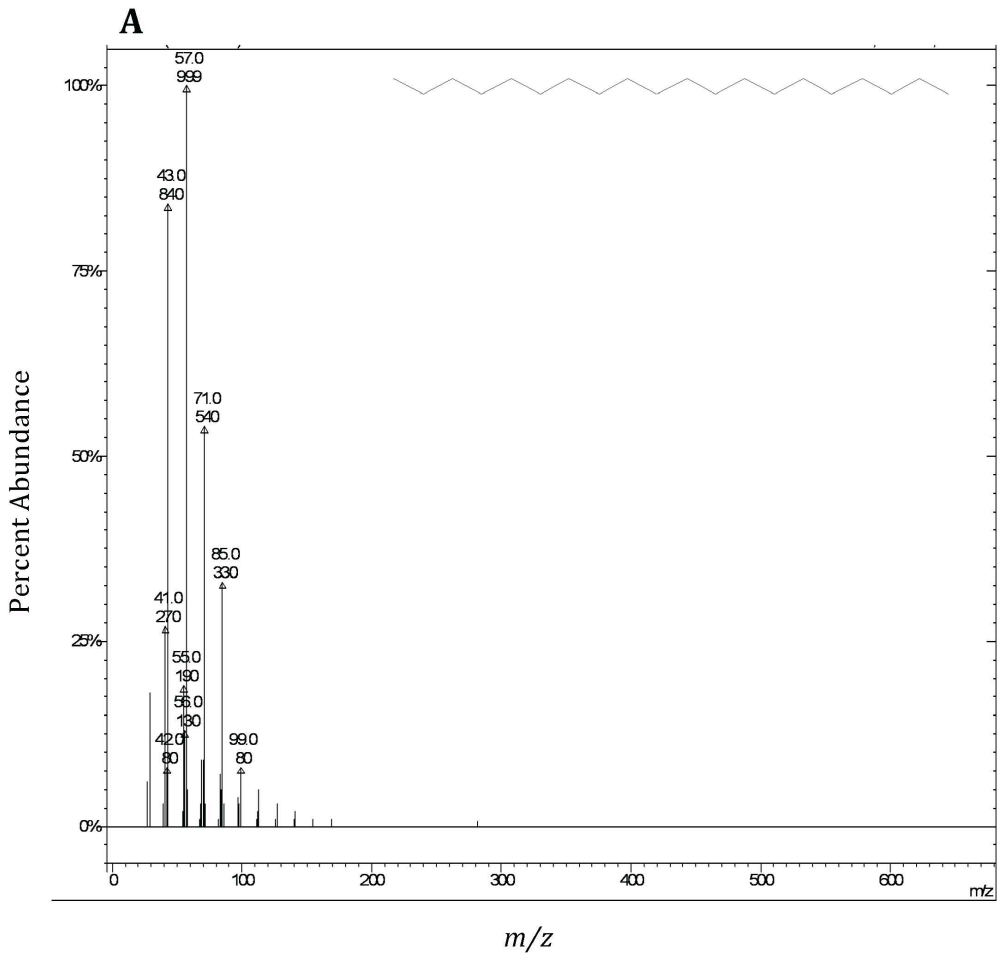
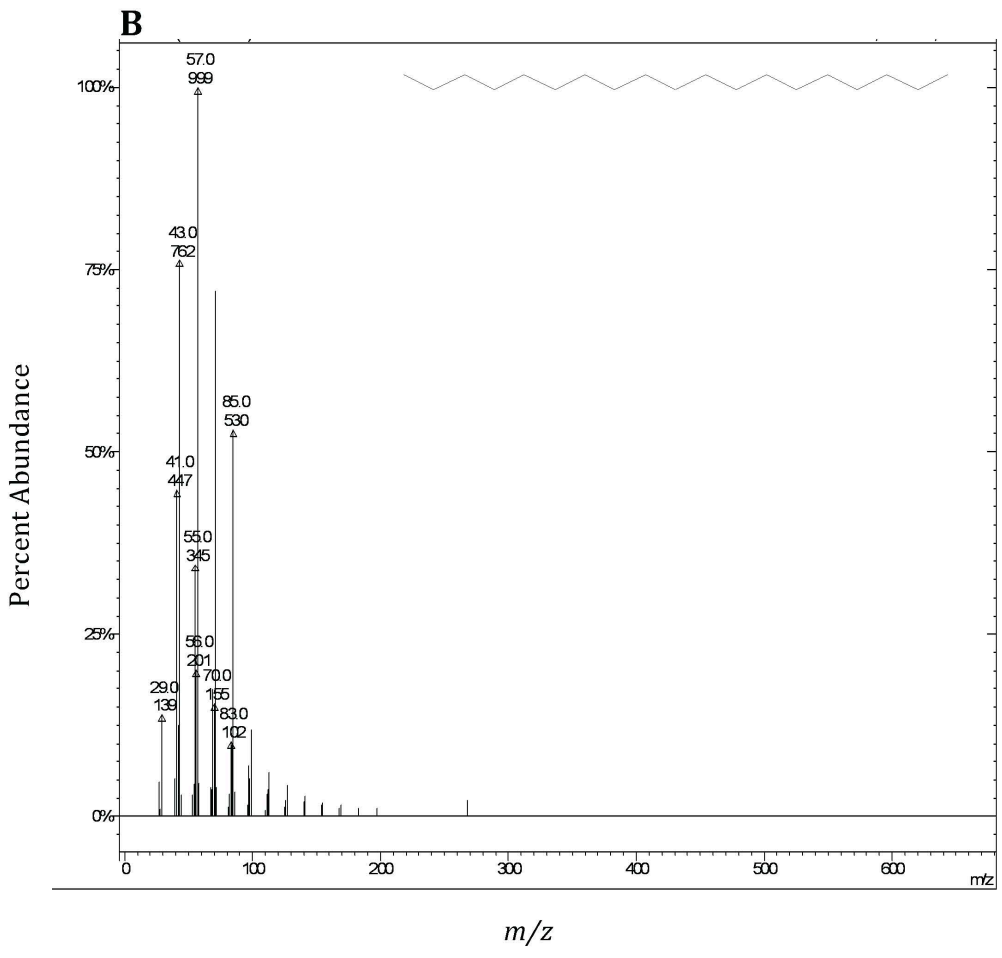


Figure 3.8 Identification of cuticular waxes for *Cheanius impunctifrons*. One hydrocarbon and one alcohol were identified by comparing standards to the beetle samples. **1)** Eicosane; **2)** 1-Eicosanol.



m/z



m/z

Figure 3.9 Mass spectra for **A)** Eicosane and **B)** Nonadecane.

Development of a Non-lethal Method to Extract Cuticular Hydrocarbons

Porapak P® extraction method

Porapak P® was the first sorbent material used in developing a non-lethal procedure to extract cuticular hydrocarbons from forked fungus and carabid beetles. Porapak P® is a styrene divinylbenzene polymer.[68] This was used because it is has similar chemical properties to Chromosorb®, which is a sorbent that was successfully used to extract cuticular hydrocarbons from *Melipona marginata*, *Apis mellifera* and *Tenebrio molitor*.[60, 70] Both are non-polar and hydrophobic and were designed for use in packed gas chromatography columns for the analysis of free fatty acids, glycols, alcohols, esters, ketones, ethers and hydrocarbons.[68] Both are able to retain small concentrations of these compounds. Chromosorb® is insoluble in most solvents allowing it to be reused.[60] Both sorbents are styrene-divinylbenzene copolymers. Styrene (C_8H_8) and divinylbenzene ($C_{10}H_{10}$) are both derivatives of benzene.[78, 79] Divinylbenzene is shown in figure 3.10. Divinylbenzene and styrene react to form styrene-divinylbenzene copolymers.

Before using the sorbent, about 5g of Porapak P® was first washed in 50 mL chloroform and then with 50 mL hexane to remove any impurities from the sorbent from the manufacturing process. The Porapak visibly degraded in the chloroform, so thereafter only hexane was used to wash the sorbent. After washing, Porapak was used to sample beetles and a control was completed in which only Porapak was put through the extraction procedure to ensure that the components are from the insect.

Benzene derivative peaks were observed in the chromatogram for the Porapak P® control (Figure 3.11 A). These peaks were originally identified using the mass spectra and the NIST library database. The retention times for these peaks were compared

between different chromatograms. To investigate if the benzene derivatives were from the Porapak P® degrading in the solvent, the sorbent was left in hexane for seven days to see if it broke down over time in hexane. Peaks for divinylbenzene and ethyl-vinyl-benzene were observed with five times greater intensity than those in figure 3.11 A, for samples immediately analyzed after sampling (Figure 3.11 B). Benzene peaks were not observed for pure hexane (Figure 3.11 C) suggesting that the Porapak P® degrades overtime in the solvent and loses some of its functionality. The intensity of the benzene peaks are large, between 5 x10^5 and 8x 10^5 counts for Porapak samples analyzed soon after sampling suggesting that the Porapak degrades quickly in hexane. However the intensities for the benzene peaks do increase with prolonged exposure to hexane, suggesting that the Porapak continues to degrade over time in hexane.

Since the sorbent loses its functionality over time, this will affect the efficiency of hydrocarbon extraction. Due to this, the results will not be consistent. It was decided that Porapak would not make a good sorbent to extract the hydrocarbons.

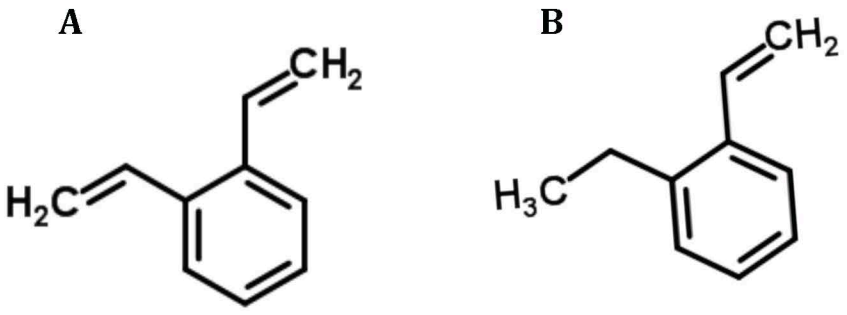


Figure 3.10 Common benzene derivatives of Porapak P®. **A)** 1,2 Divinylbenzene **B)** 1-ethyl-2-vinyl-benzene.[79]

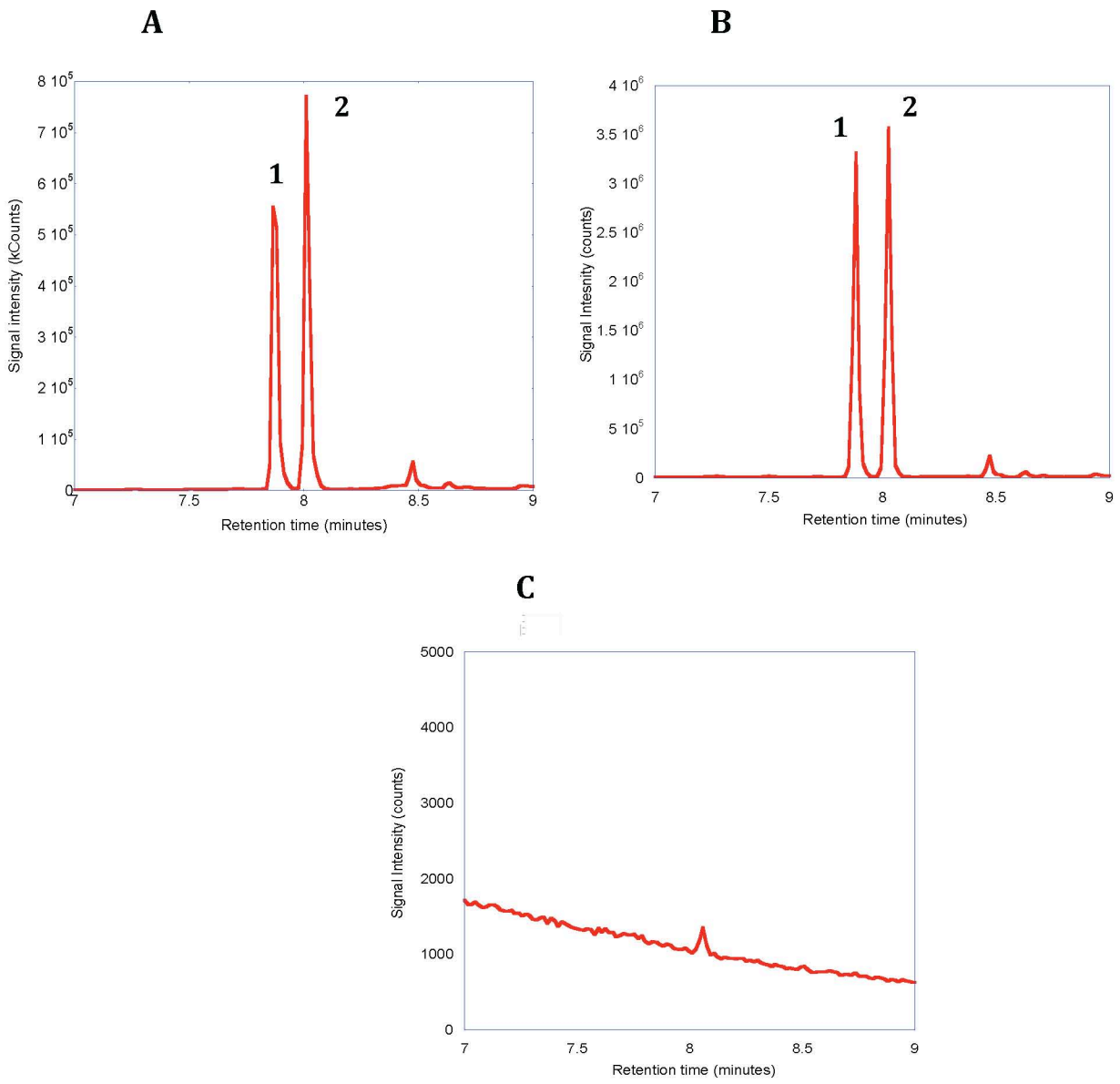


Figure 3.11 Chromatograms for the non-lethal extraction procedure using Porapak P®. Peaks labeled 1 and 2 are benzene derivatives from the Porapak, which have degraded in the solvent, hexane, used in the extraction procedure. **A)** Chromatogram for a sample of Porapak P® run through the extraction procedure. **B)** Chromatogram for a sample of Porapak P® that was immersed in hexane for a period of seven days. **C)** Chromatogram for pure hexane, the negative control. The benzene derivatives seen in **A** through **C** are not observed for pure hexane.

Sorbent: octadecyl carbon chain-bonded silica (C_{18})

C_{18} was the next sorbent used to extract cuticular hydrocarbons from the beetles. C_{18} is a porous octadecyl carbon chain-bonded silica, which was chosen because it is nonpolar and would have good affinity for the nonpolar hydrocarbons of interest (Figure 3.12).[78, 80] In addition there are negligible secondary polar interactions from the silanol groups on the surface of silica.

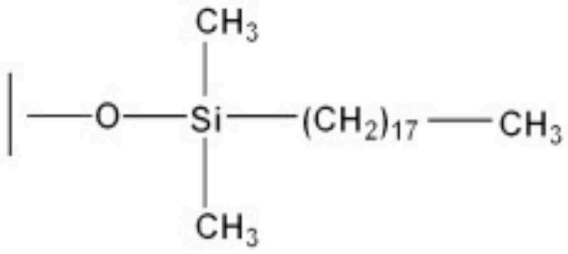


Figure 3.12 Structure of octadecyl (C_{18}). This consists of silica with an 18 carbon long chain attached.[80]

Final optimized non-lethal extraction procedure using C_{18}

The procedure was originally designed for sampling the cuticular waxes from forked fungus beetles and was later used to sample *Chlaenius cordicollis*. To remove impurities from the manufacturing process, C_{18} was washed with hexane in a 1:10 ratio prior to its use in the extraction procedure. The sorbent was dried in an oven for 30 minutes and cooled to room temperature. For each sample, 0.1 g of the sorbent was used. The beetle was gently lifted around the posterior with tweezers, taking care to avoid the legs, and placed on its back into the vial containing sorbent (Figure 3.13 A). The vial was gently rotated for five minutes, taking care that all of the sorbent came into contact with the cuticle of the beetle (Figure 3.13 B). The

beetle was then removed from the vial with tweezers and either placed onto a scoopula or held over the vial by the tweezers. To ensure that all of the sorbent used to extract the cuticular waxes was collected, the entire beetle was rinsed with water to transfer the remaining C_{18} into the vial. The end of the scoopula was held over the opening of the vial to ensure that neither sorbent nor beetle were lost. When using the scoopula, the beetle was moved between pools of water to transfer the remaining sorbent from the beetle to the water (Figure 3.13 C). All water containing sorbent was placed into the vial. A minimal amount of water was used during this process. When the beetles fell back into the vial, they were removed and re-rinsed with purified water. The beetle was then placed back into its laboratory environment.

Two milliliters of hexane was added to the vial and then the vial was capped. The vial was repeatedly inverted for one minute to transfer the extracted cuticular waxes to the organic layer. The hexane was removed with a 9" glass Pasteur pipette and placed into a 2 mL GC vial via a liquid-liquid extraction. The vial was capped and stored for analysis.

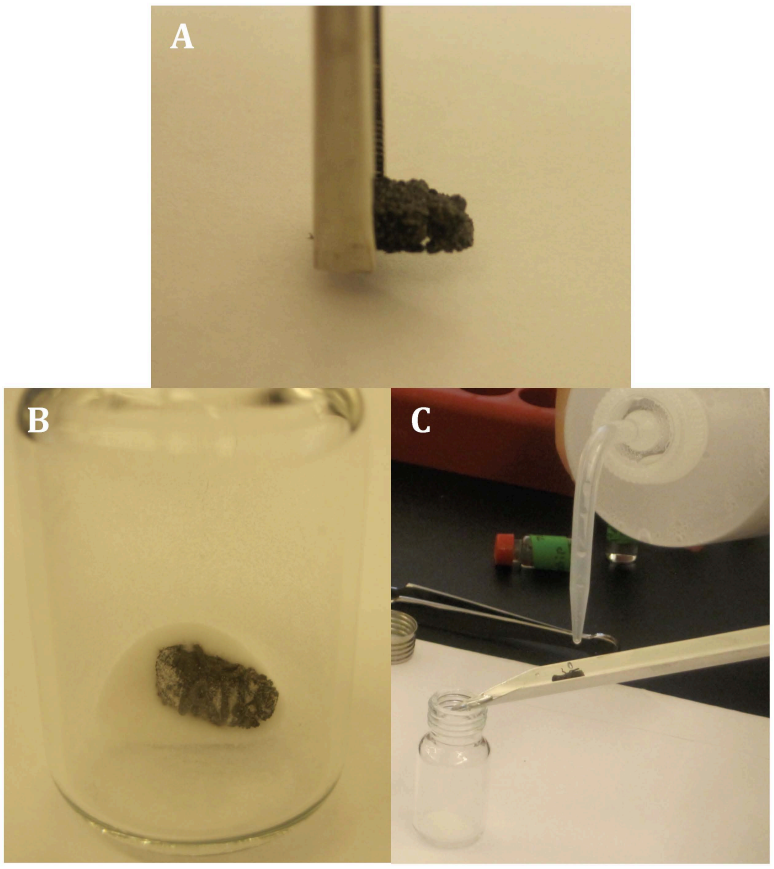


Figure 3.13 The non-lethal methods to extract cuticular hydrocarbons from forked fungus beetles and *Chlaenius cordicollis*. The procedure shown here is completed with a forked fungus beetle. **A)** The beetle is transferred from its laboratory environment to the sample vial with tweezers. **B)** The beetle was gently rotated on its back in a vial containing C_{18} to transfer to cuticular hydrocarbons to the sorbent.
C) The beetle was placed onto a scoopula and the remaining C_{18} was transferred from the beetle into the vial with purified water.

The effects of the sorbent, C_{18} on the beetles

After completing the non-lethal extraction procedure with C_{18}, the beetles were observed for one week, to see if the extraction method had an effect on the mortality of the beetles. It was suspected that the C_{18} may dehydrate the beetles, since the procedure removed some of the hydrocarbons responsible for preventing dehydration. To prevent fatalities, the beetles were kept in a moist environment.

This method was not observed to affect the mortality of either *Chlaenius cordicollis* and forked fungus beetles.

To observe if the C_{18} procedure has any physical damaging affects to the beetles, they were examined with a dissecting microscope. A *Chlaenius cordicollis* beetle sampled with the C_{18} extraction procedure was examined under a microscope and compared to a *Chlaenius cordicollis* beetle that had not been sampled. No observable differences were seen between the two beetles. Some C_{18} remained on the sampled beetle even after rinsing it with water. It is unlikely that all of the C_{18} could be removed from the beetle.

Establishing C_{18} as an effective non-lethal extraction method

To ensure that C_{18} did not degrade in hexane as Porapak P® did, the sorbent was run through the extraction procedure. Since C_{18} is an 18 carbon long chain attached to silica, if it degrades the hydrocarbons may interfere with the components extracted from the cuticular waxes. Samples were immediately analyzed by GC-MS and no hydrocarbons or any other compounds were observed. To see if C_{18} degraded over time, samples of the sorbent remained in hexane and purified water for seven days, since water is also used in the extraction procedure. No compounds were observed in these samples as well. Therefore it was concluded that C_{18} was a reliable sorbent to use for the extraction procedure.

The extraction procedure was first tested using the C7-C40 saturated alkane standard. Three microliters of the alkane standard was placed onto a folded piece of wax paper to replicate the beetle cuticle and allowed to air dry. The wax paper was

sampled following the C_{18} extraction procedure. The alkane standard sampled with C_{18} was compared to 3.0 μl of the alkane standard diluted in 2 mL hexane. This was completed to make sure that the sorbent could properly adsorb the compounds, that they would be transferred to hexane, and that no peaks were from the sorbent. The chromatograms were selected for ion *m/z* 57, which is an ion fragment formed by all alkanes in the standard. The same peaks were observed between the GC chromatograms for the two samples, confirming that C_{18} can adsorb hydrocarbons and that none of the components are from the sorbent (Figure 3.14).

The C_{18} extraction procedure was then used to sample both *Chlaenius cordicollis* and forked fungus beetles. Both the established lethal procedure, using hexane and the novel non-lethal procedure using C_{18} were observed to extract the same identified compounds (Figure 3.15). In general the intensities for the cuticular wax components and the amount of each component extracted from *Chlaenius cordicollis* were visually observed to have greater intensities than those for forked fungus beetles for both the lethal and novel non-lethal extraction methods, as shown in figures 3.16 A and B. Differences in the components extracted would be expected because these are two different species of beetle. Each species synthesizes the compounds through different mechanisms, producing different compounds and different concentrations of the components.[31, 34] One contributing factor could be the size of the beetle, with *Chlaenius cordicollis* being a larger species of beetle compared to forked fungus beetles. Since the cuticle for forked fungus beetles are smaller than those for *Chlaenius cordicollis*, it would be expected that they would have lower amounts of cuticular waxes.[7]

In an attempt to determine the effectiveness of the novel non-lethal method using C_{18}, it was compared to the established lethal method using paired t-tests. The absolute and relative abundance for each component were each statistically compared between the non-lethal and the established lethal extraction method (Table 3.3). The absolute abundance was found by integrating the peaks. The relative abundance was calculated by dividing the peak area by the total peak area of all identified components.

An example of the data set used to complete the paired t-tests is shown in table 3.4 for nonadecane. This data shows great variation in the amount of nonadecane extracted by both procedures between beetles. The absolute peak areas for nonadecane clearly differ for each beetle between the two extraction procedures, as shown in the data set (Table 3.5). Since the absolute amount of nonadecane extracted for different beetles has such great variation, the standard deviation of the difference within treatment pairs is very large and a statistical difference is not observed at the 95% confidence level (d= 30208; STD=32742; $t_{calculates}$=2.440; $t_{critical}$=2.447; DOF=6). The variation in the amount of nonadecane extracted between beetles does not follow any specific trend and is not related to the sex of the beetle.

There is great variation between within species of beetles in the amount of each identified extracted component for both procedures. Therefore, large standard deviations were obtained and almost all of the statistical tests did not show a significant difference. A significant difference at the 95% confidence level was only seen between the two procedures for the relative percent abundance of nonadecane

(d= 19.71; STD=19.03; $t_{calculates}$=2.7410; $t_{critical}$=2.447; DOF=6). Because of the large standard deviation compared to the value for the average relative percent abundance of nonadecane, this difference is not reliable. Due to the large variation amongst the beetles, the statistical tests are not meaningful.

One trend was seen in the data for 1-eicosaonal extracted from *Chlaenius cordicollis*. For both extraction methods, 1-eicosanol had the greatest absolute peak area and relative percent abundance out of the five identified components. The hexane extraction procedure looks to have extracted a greater amount of 1-eicosanol compared to the C_{18} extraction method, but this cannot be confirmed using this data.

The large standard deviations can be attributed to small data sets. While many beetles were sampled, many of the samples could not be used in the analysis due to an unknown source of contamination. Therefore, there are not enough data points to do a quantitative comparison between the hexane and C_{18} extraction procedures because of the extreme variation amongst samples. Some of the differences in the absolute and relative abundances of the components can be attributed to the beetles containing different concentrations of various compounds.[8, 60] More tests need to be completed along with other statistical studies to obtain reliable results.

While C_{18} was observed to extract all of the identified components, the data collected cannot be used to comment on any differential extraction abilities between the hexane and C_{18} extraction procedures. Therefore, it cannot be determined if the

novel non-lethal procedure can be considered an ideal replacement for the established lethal method that would provide the same information about the cuticular hydrocarbon composition. In addition, the variation in this data set made it difficult to look at biological differences between the beetles.

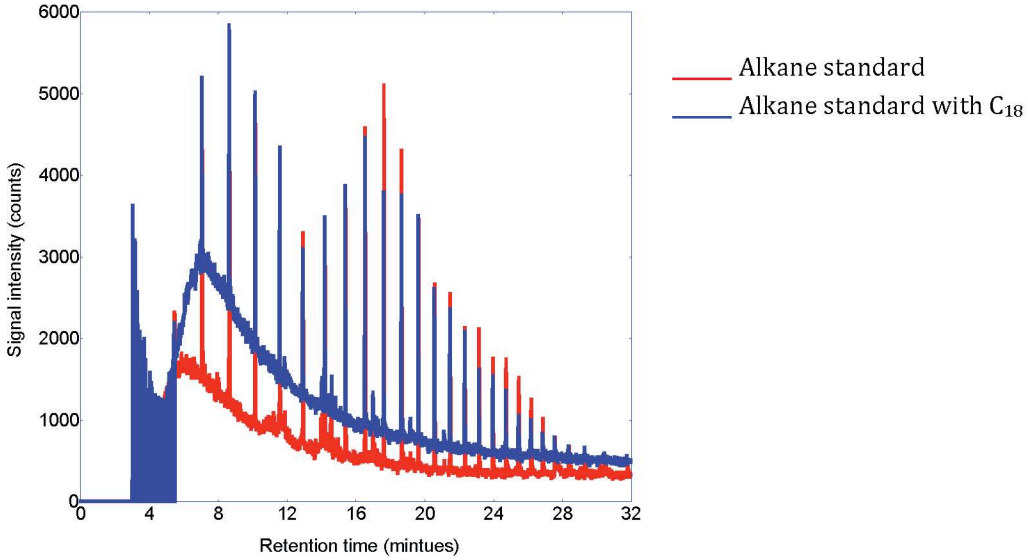


Figure 3.14 Comparison of GC chromatograms of a C7-C40 saturated alkane standard and the alkane standard sampled with the non-lethal C_{18} extraction procedure. The chromatograms are selected for ion *m/z* 57.

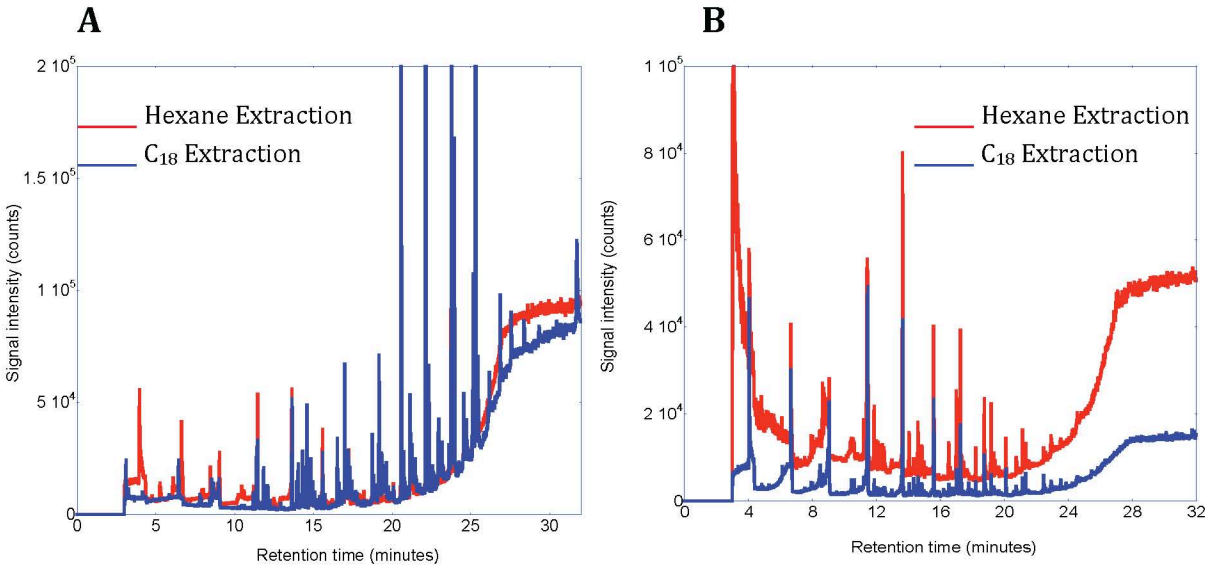


Figure 3.15. Chromatograms comparing the established lethal extraction method for cuticular hydrocarbons using hexane to the novel non-lethal procedure using C_{18}. The extractions were completed for **A)** *Chlaenius cordicollis* and **B)** forked fungus beetles. Both procedures are observed to extract the same cuticular wax components. The extraction procedure using hexane has greater intensities for the majority of the compounds compared to the C_{18} extraction procedure.

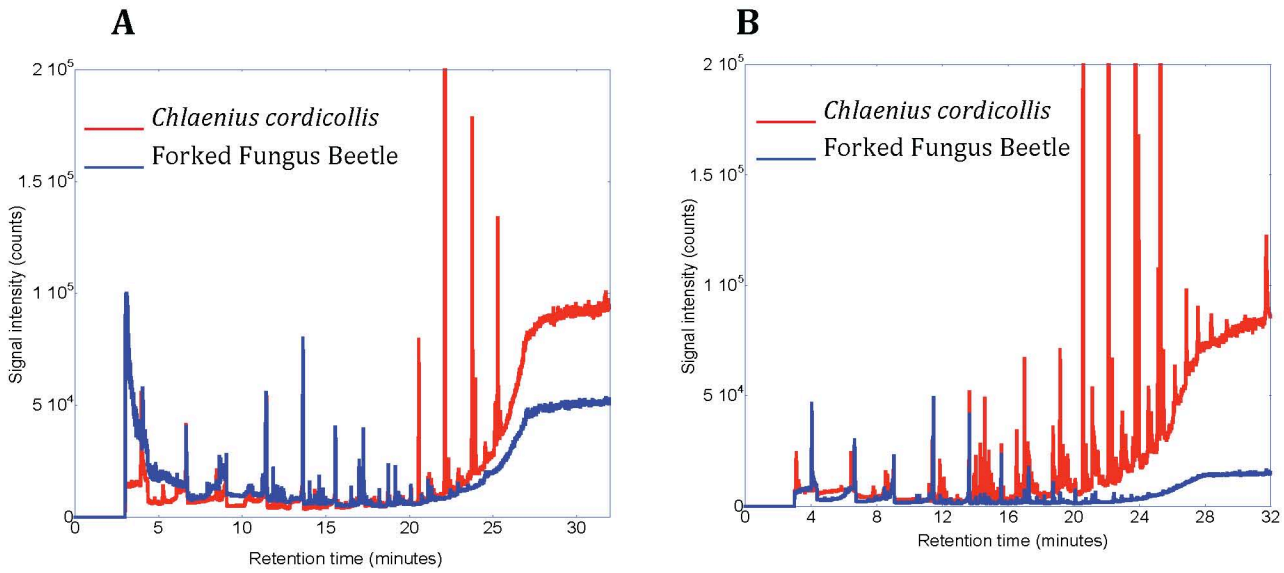


Figure 3.16 GC chromatograms comparing the intensities of the peaks between *Chlaenius cordicollis* and forked fungus beetles for **A)** the lethal extraction using hexane and the **B)** novel nonlethal extraction using C_{18}. For both the lethal and novel non-lethal method, the majority of the peaks have greater intensities for the cuticular wax components extracted from *Chlaenius cordicollis* versus those extracted from forked fungus beetles.

Table 3.3 The average absolute peak areas and the average relative percent abundances of the five identified peaks for *Chlaenius cordicollis* are reported. In addition, the average standard deviation for the absolute peak area and the percent standard deviation for the relative percent abundance are reported. Values are reported for the components extracted with both the lethal (hexane) and non-lethal (C_{18}) extraction procedures. The relative percent abundance for each component was found relative to the other identified components. N=5

Chlaenius cordicollis								
	Hexane Extraction				C_{18} Extraction			
Cuticular wax Components	Absolute peak area	Standard Deviation (Mean)	Relative percent abundance	Percent Standard Deviation (Mean)	Absolute peak area	Standard Deviation (Mean)	Relative percent abundance	Percent Standard Deviation (Mean)
Nonadecane	13777	87352	4%	4%	53951	94549	20%	22%
Eicosane	55950	68239	17%	14%	75992	75508	26%	22%
Heneicosane	58961	56046	18%	8%	25915	47969	10%	5%
Docosane	70346	22612	7%	5%	21312	10909	8%	2%
1-Eicosanol	544118	94548	54%	13%	96722	21946	36%	18%

Table 3.4 The average absolute peak areas and the average relative percent abundances of the three identified peaks for forked fungus beetles are reported. In addition, the average standard deviation for the absolute peak area and the percent standard deviation for the relative percent abundance are reported. Values are reported for the components extracted with both the lethal (hexane) and non-lethal (C_{18}) extraction procedures. The relative percent abundance for each component was found relative to the other identified components.

Forked Fungus Beetles								
	Hexane Extraction				C_{18} Extraction			
Cuticular wax components	Absolute peak area	Standard Deviation (Mean)	Relative percent abundance	Percent Standard Deviation (Mean)	Absolute peak area	Standard Deviation (Mean)	Relative percent abundance	Percent Standard Deviation (Mean)
Nonadecane	33004	32949	46%	18%	3491	1649	18%	5%
Eicosane	28769	28405	41%	17%	11484	8413	59%	20%
Heneicosane	9528	9965	13%	19%	4433	2797	23%	21%

Table 3.5 The absolute peak areas and relative percent abundances of nonadecane extracted from seven individual forked fungus beetles. Values are reported for the components extracted with both the lethal (hexane) and non-lethal (C_{18}) extraction procedures. The relative percent abundance for each component was found relative to the other identified components. Large variation is observed between beetles in both the absolute peak areas and the relative percent abundance for nonadecane. A paired t-test showed that there is no statistical difference in the absolute peak areas between the two methods (d= 30208; STD=32742; $t_{calculates}$=2.440; $t_{critical}$=2.447; DOF=6) but that there is a significant difference in the relative percent abundance of nonadecane at het 95% confidence level (d= 19.71; STD=19.03; $t_{calculates}$=2.7410; $t_{critical}$=2.447; DOF=6).

	Hexane extraction		C_{18} extraction		
Beetle	Absolute peak area	Relative % abundance	Absolute peak area	Relative % abundance	Sex
1	10016	29%	5294	27%	Male
2	43654	57%	1025	16%	Male
3	20680	28%	2314	18%	Male
4	9847	35%	1089	21%	Female
5	50813	54%	5195	16%	Female
6	95003	53%	2888	15%	Female
7	1018	8%	1766	13%	Female

SPME Extraction

A known non-lethal procedure using SPME fibers coated with polydimethylsiloxane was used to sample the cuticular waxes from both *Chlaenius cordicollis* and forked fungus beetles.[38, 64, 65] The fiber was gently rubbed on the cuticle of the beetle for 20 seconds, rotating the fiber so the entire circumference of the fiber came in contact with the cuticle (Figure 3.17). The GC chromatograms for the SPME extraction were compared to the chromatograms for the hexane extraction. The chromatograms were selected for ion *m/z* 57, a common ion fragment formed by hydrocarbons. This is to focus on the peaks for the components of interest.

For both forked fungus beetles and *Chlaenius* cordicollis, the SPME fiber extracted most of the cuticular waxes but not all of the components shown in the hexane extraction. The intensities of the peaks for the SPME extraction are between two to three times less than those for the hexane extraction (Figure 3.18 A and B). This suggests that the fiber may have a greater affinity for certain components of the cuticular waxes. In addition the fiber does not come in contact with the entire cuticle as the hexane does, so this does not provide a full representation of the cuticular waxes.

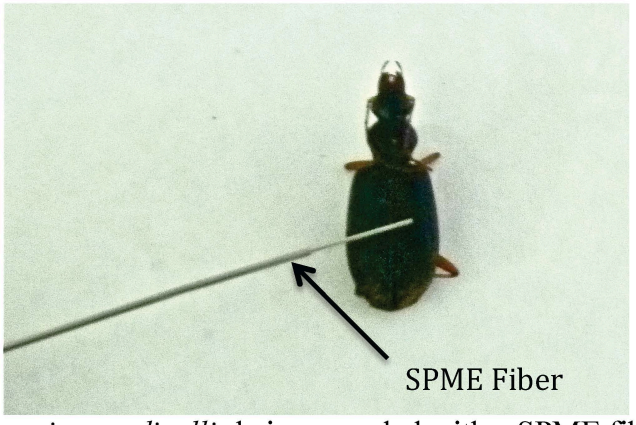


Figure 3.17 *Chlaenius cordicollis* being sampled with a SPME fiber to extract the cuticular waxes.

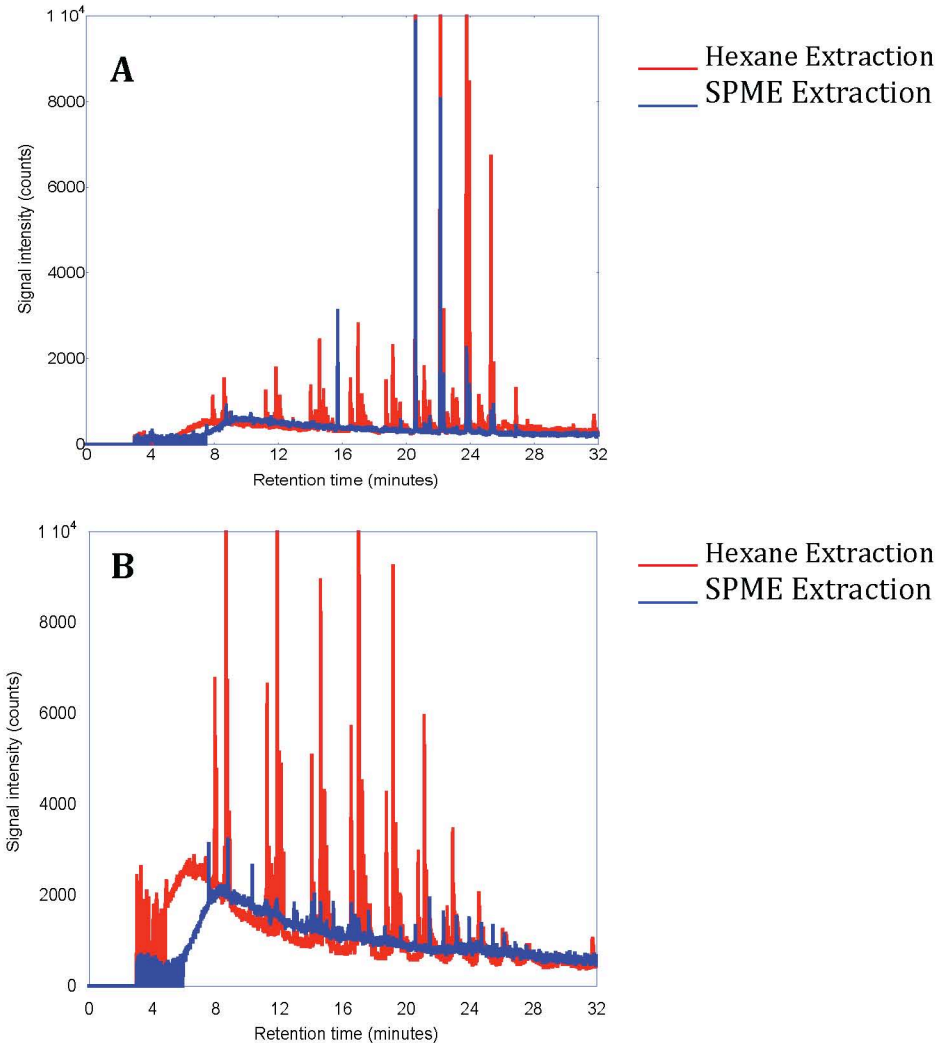


Figure 3.18 The cuticular waxes of **A)** *Chlaenius cordicollis* and **B)** a forked fungus beetle extracted with a SPME fiber and with hexane. The chromatograms are selected for ion *m/z* 57.

Sampling Fungus

Forked fungus beetles inhabit and feed on bracket fungi. Since they are continuously in close contact with the fungi, fungal samples were taken to ensure that the extracted hydrocarbons were synthesized by the beetles and not components that they picked up from the fungus. Uninhabited fungus could not be obtained, so fungus that had been inhabited by forked fungus beetles was sampled.

The cuticular waxes of forked fungus beetles and the fungus they inhabited were both sampled using C_{18} and the resulting chromatograms were compared. Ion *m/z* 57 was selected for the chromatograms to focus on the compounds extracted.

The chromatograms for the fungus and the beetle that inhabited it have very similar profiles (Figure 3.19). The chromatogram for the fungus has much lower intensities than those for the beetle. This may be attributed to the beetle transferring its hydrocarbons onto the surface of the fungus; the components would be the same and the abundance of the compounds on the fungus would be lower than the beetle, resulting in a lower intensity. Uninhabited fungus needs to be sampled to confirm that the extracted hydrocarbons are from the beetles.

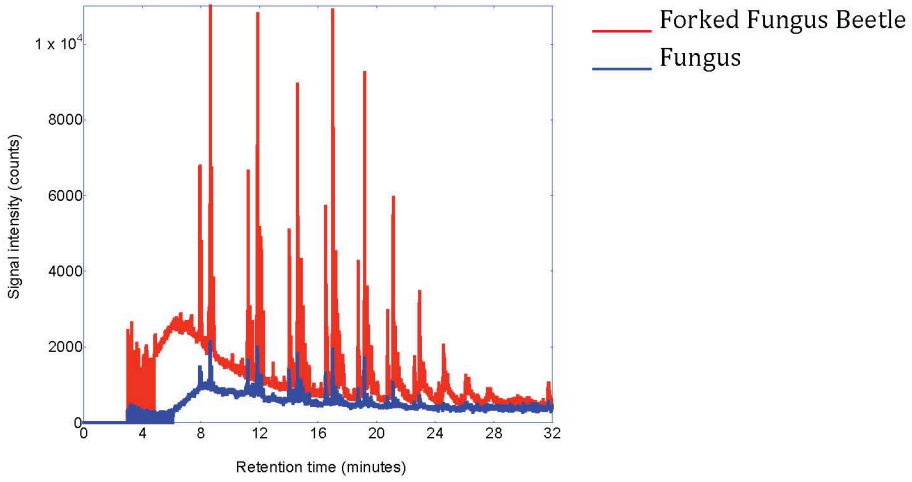


Figure 3.19 The cuticular waxes of a forked fungus beetle extracted with C_{18} and the fungus the beetle inhabited sampled using C_{18}. The chromatograms are selected for ion *m/z* 57.

Methods to achieve greater signal intensities

The signal intensities for the peaks of the compounds of interest are low. This can be attributed to there being a low concentration of the compound extracted from the beetle. The absolute amount of cuticular waxes depends on the beetle species and the size of the cuticle. A larger cuticle will contain a greater amount of cuticular waxes.[2, 10] Low peak intensities make it difficult to clearly observe and analyze the peaks. A few different techniques have been applied to try to obtain greater signal intensities for the components of interest. Some samples were concentrated, some were filtered, and composite sampling was also completed. All of the samples referenced in figure 3.20 were completed with a forked fungus beetle using the C_{18} extraction procedure.

Some of the samples were concentrated by reducing the amount of solvent, so that there was a greater abundance of the compounds in the sample. Samples were fully evaporated using a flow of air. The samples were then dissolved in 30 μl hexane. The signal intensities did increase by about 1.5 times compared to the signal intensities of the original sample (Figures 3.20 A, B). The samples were concentrated by more than two times the original concentration and the signal intensities were therefore expected to have increased more. Since the signal intensities only increased by about 1.5 times, the intensities were still difficult to analyze.

Samples were also filtered with 0.45 μm PTFE syringe filters and 3mL BD Luer-Lok Tip syringes, which were noted to have limited compatibility with hexane.

Therefore, the samples were filtered immediately and not left in the syringes to prevent the hexane from degrading the syringe. Filtering was completed to eliminate contaminates from residual particles of the sorbent and to better concentrate the samples. After filtering the samples had 1.5 times greater intensity than the original sample (Figures 3.20 A,C). This increase in the intensity of the peaks was more than expected. The increase in intensity may be due to all of the analytes being filtered through and some of the solvent being lost, concentrating the samples. In addition, there is a better peak resolution, suggesting that the filter eliminated contaminants contributing to background noise and contaminants that may overlap with the peaks of interest.

In some cases the samples were filtered and concentrated. The samples were first filtered to eliminate any contaminates and then concentrated to increase the abundance of the extracted compounds in the sample. These samples were shown to have peak intensities 2.5 times greater than the original sample (Figures 3.20 A, D).

Composite sampling was completed for both *Chlaenius cordicollis* and forked fungus beetles. Composite sampling has been completed for smaller insects such as ants and bees that have low abundances of cuticular waxes.[41] Multiple insects are used to complete one sample, resulting in a greater amount of components collected and therefore greater peak intensities.[38, 40]

Six individual male *Chlaenius cordicollis* beetles were sampled in hexane. Each beetle was sampled one at a time in the same 2 mL hexane. Most of the peaks for the composite sample have intensities 1.5 to 2 times greater than the sample with one beetle (Figure 3.21 A). A few of the peaks for the composite sample have

similar intensities to the single beetle sample. Since the beetles synthesize these components, there may be variation between beetles. The beetles used in the composite sample may not contain high abundances of certain components. Since some components were shown to have a much greater intensity for the composite sample, this suggests that composite sampling may aid in achieving greater signal intensities.

Composite sampling was completed with forked fungus beetle using C_{18}. Two male forked fungus beetles were sampled one at a time in the same portion of C_{18}. More beetles were not used in the sample because those were the only available. Some of the peaks were observed to have slightly greater intensities for the composite sample compared to a sample with only one beetle, which was used in the composite sample (Figure 3.21 B). Since forked fungus beetles are small, smaller than *Chlaenius cordicollis*, more beetles may need to be used to achieve greater signal intensities.

Each of these methods showed some improvement in the signal intensities. For each procedure used to improve the signal intensities of the peaks, all of the chromatogram peaks were observed as in the original samples. All of the procedures did help in making the peaks more visible to observe and analyze. Many samples were concentrated and the signal intensities were consistently seen to increase by 1.5 times the original intensities. More trials need to be completed for each method to observe which is most effective at improving signal intensities.

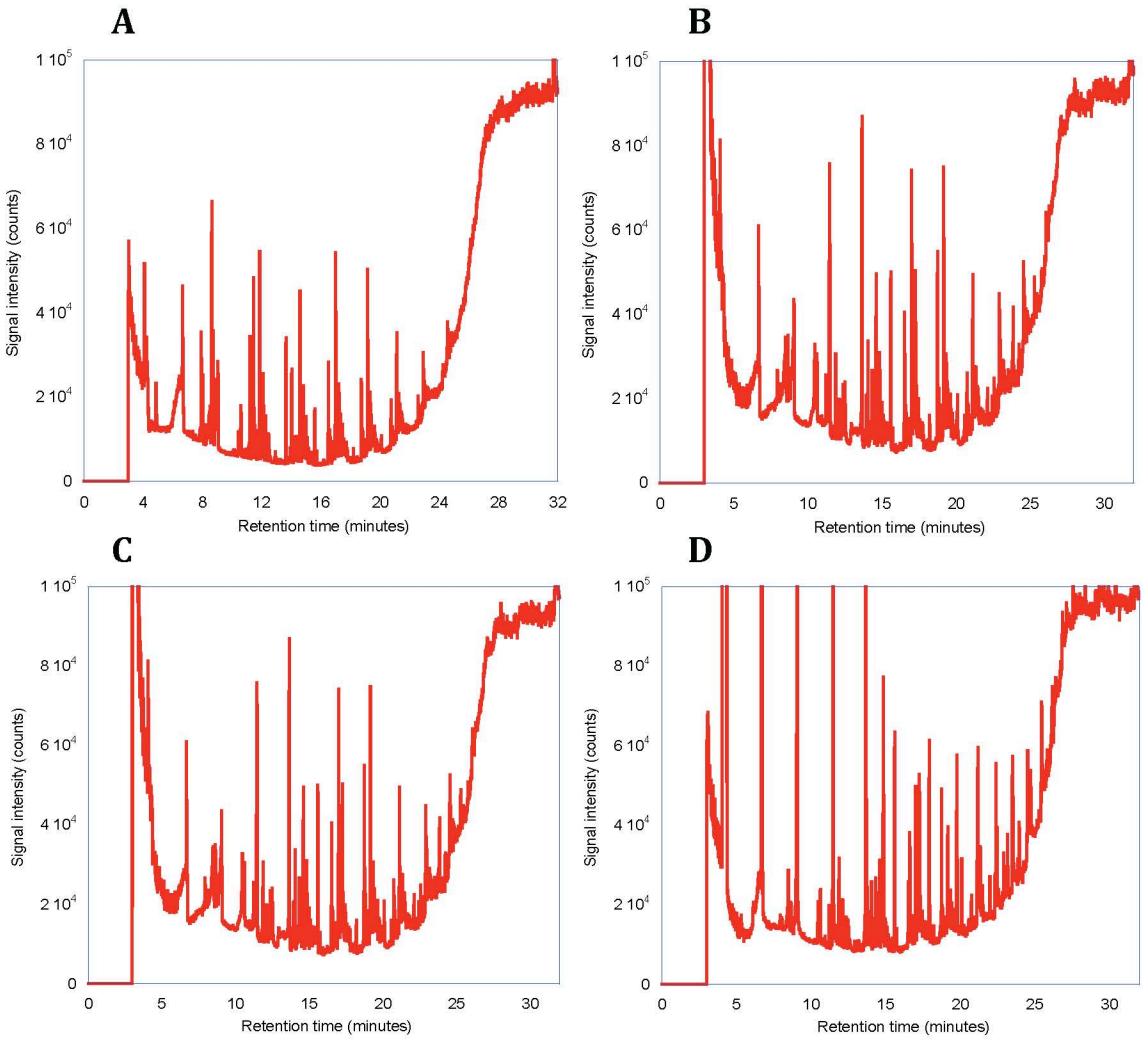


Figure 3.20 Methods to improve the signal intensities of the peaks in the GC chromatograms. All samples were completed with the C_{18} procedure using a forked fungus beetle. **A)** The original sample in which the normal C_{18} extraction procedure was completed can be compared to **B)** the original sample concentrated, **C)** the original sample filtered and **D)** the original sample filtered and concentrated.

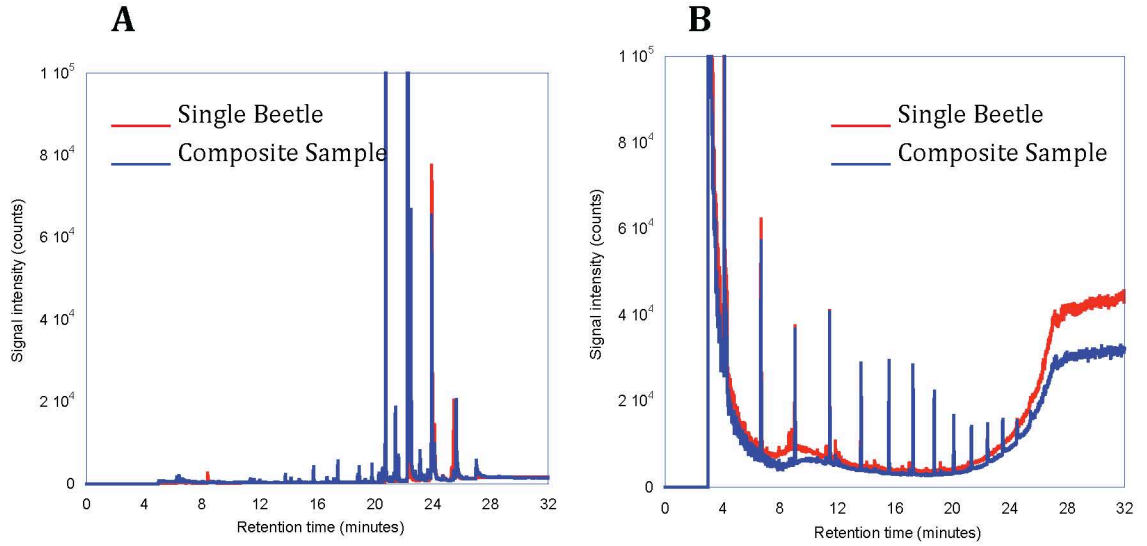


Figure 3.21 Composite sampling was completed with **A)** six male *Chlaenius cordicollis* in hexane and **B)** two male forked fungus beetles in C_{18}. Each is compared to a sample with a single beetle. This method was completed to improve the signal intensities in the chromatogram. The majority of the peaks with greater intensities are siloxanes.

Contamination

A few sources of contamination were observed during analysis of the samples, which are attributed to the instrumentation. In all of the chromatograms siloxane peaks are observed, which can be attributed to various sources of bleeding. In addition, part way through this experiment the GC-MS was found to have multiple contamination peaks, which had similar retention times and intensities to the peaks of interest in this study. These peaks interfered with the analysis of the samples and these samples were disregarded.

Siloxanes

The peaks with the largest intensities in all of the chromatograms are siloxanes and are not from components the sample. Siloxanes have a backbone structure alternating of silicon and oxygen and have hydrocarbons attached to the silicon side chain (Figure 3.22).[81] The siloxane peaks are attributed to bleeding, likely from the column, septum and the o-ring in the inlet port, which all contain siloxanes. As the column and septum degrade overtime due to exposure to the high temperatures of the oven, the components from these elute with the sample when the carrier gas flows through the column.[74]

There are multiple siloxane peaks due to different siloxane components and the intensities of these peaks change due to the amount of bleeding that occurs. To avoid bleeding the o-ring and septum were frequently changed. The column was not kept at high temperatures for extended periods of time as the stationary phase will degrade and siloxanes will be released. In addition, SPME fibers used in other

research, which are placed in the GC inlet port, contain siloxanes. SPME fibers are fragile and degrade at temperatures of 280 °C and above. Therefore some of the siloxane peaks may be due to the SPME fibers as well.

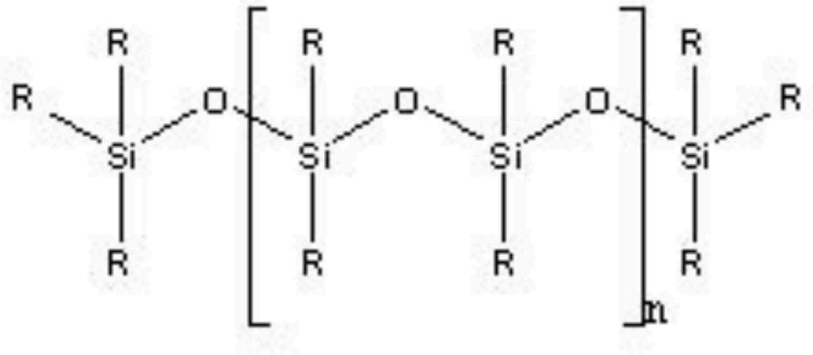


Figure 3.22 Structure of siloxanes. The backbone of siloxanes consist of silicon and oxygen which alternate and the silicon side chains have hydrocarbons attached = R.[81]

GC Contamination

Each time the beetle samples were run on the GC-MS, pure hexane was run as a negative control to ensure that the components were only from the sample. The chromatogram for the pure hexane was observed to contain some of the same peaks as one set of the beetle samples. The beetle samples and additional samples of pure hexane were run to confirm the contamination. The contamination peaks were consistently at the same retention time. Therefore, the same compounds are responsible for the contamination seen with each run. To find the source of the contamination three tests were completed: 1) manual injection with pure hexane, 2) manual injection with no solvent and 3) pressing the injection port with no injection. For analysis, each chromatogram was selected for ion *m/z* 57, which is a common ion

fragment that hydrocarbons form. This is to see if there were contamination peaks that would interfere with the components being analyzed in the samples.

The solvent, hexane, was considered as a source of contamination. It is also possible that the compounds are desorbed from the start of the column when solvent is vaporized.[74] To rule out the solvent as a source of contamination, manual injections with hexane that had been used to make the previous contaminated samples were compared to manual injections of a new source of hexane and a different solvent, dichloromethane. The chromatograms for both sources of hexane had the same contamination peaks (Figures 3. 23 A, B). Dichloromethane had contamination peaks but not all of the peaks shown for the pure sample of hexane (Figure 3.23 C). Though the hexane is 99.5% pure, this suggests that some of the contaminants may be from the manufacturing process of hexane. In addition the contaminants could be in the injection port and solvent would dissolve contaminants from the inlet.[74, 75] Some of the compounds may not be soluble in dichloromethane and therefore would not be brought onto the column. Since the chromatograms for all three solvents contained contamination peaks, this suggests that the hexane used may not be the only source of the contamination.

The needle and septum were considered to be an additional potential source of contamination. To check if contamination could be attributed to the needle, a previously used needle containing no sample was put into the injection port. Nothing was injected from the needle when doing so. This was compared to a new, unused needle injected in the same way. Over time the septum can degrade and release components, therefore the septum was changed multiple times. The same

contamination peaks were observed in the chromatograms for both needle injections and with every septum (Figure 3.23 D). In addition the same contaminant peaks were seen as those for the pure hexane. This suggests that the contamination was not due to the needle used and that the contamination occurs without solvent. Therefore it is unlikely that the contaminants are due to the solvent dissolving contaminants from the inlet.

Another source from which the contamination could have originated from was contaminants adhering to the start of the column. To rule out the column as the source of contamination, the injection port was pressed down, without any injection to begin the temperature program. This was to investigate if the carrier gas, helium, flowing through the column, brings contaminants from the column.[74] Since nothing was placed into the injection port, it is unlikely that the contaminants would be from compounds desorbing in the injection port and therefore they would be from the column. With the exception of a transient species found immediately after changing columns, contaminant peaks were not seen in these runs, suggesting that the majority of the contamination is not from the column.

The contamination peaks have consistent retention times and relatively consistent intensities, therefore it is likely that the contamination is due to a component of the injection or compounds at the start of the column, and not a contaminant permanently throughout the column.[75] If the contaminants were found throughout the column, the retention times and intensities of the peaks would be expected to differ between runs because the amount and type of components coming off the column would also be different.[74, 75] In addition, some of the

contamination peaks have greater intensities than others. This suggests that certain components of the contamination are consistently present in a greater abundance.

After continuous contamination, the GC-MS was cleaned and serviced. After changing the septum, cleaning the injection port and changing the column, the contamination peaks still appeared. The GC-MS was then serviced and the Agilent support person was unsure of the source of contamination. After the GC was serviced the contamination peaks were no longer present. The source of contamination is still unclear. All of the samples run when contamination peaks appeared were disregarded during analysis. As a result, there was a period of time in which data could not be analyzed and there is a large amount of unusable data.

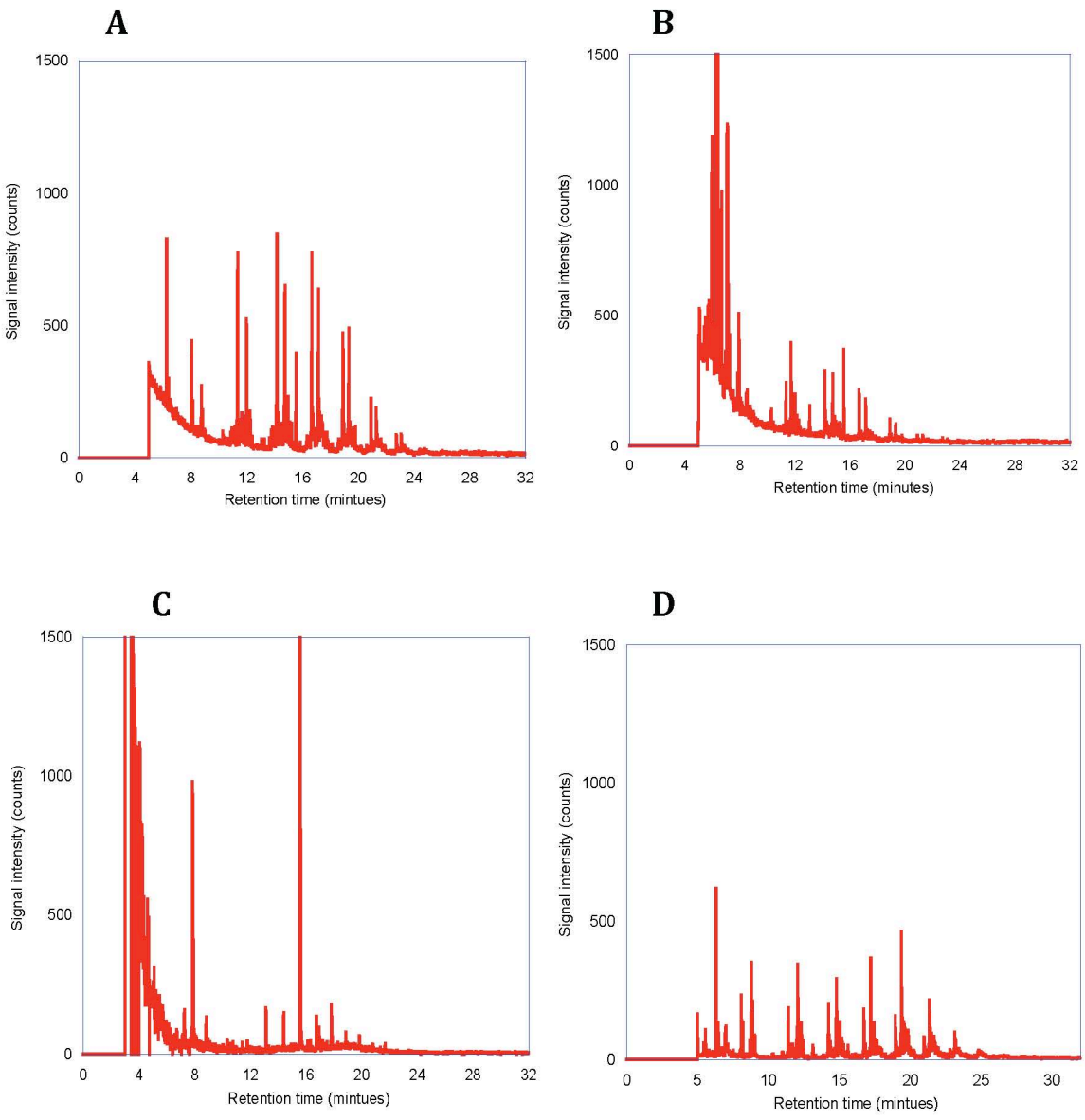


Figure 3.23 Tests for the source of contamination in the GC chromatograms. Each chromatogram is selected for ion *m/z* 57. **A)** Pure hexane using the original source of hexane used to make the contaminated samples, **B)** Pure hexane from a new source of hexane, **C)** Pure dichloromethane, and a **D)** Needle injected with no sample.

Chapter 4: Conclusion

A non-lethal procedure using C_{18} was described and established to extract all of the identified components for both *Chlaenius cordicollis* and forked fungus beetles. This procedure was established to be compatible with sampling live *Chlaenius cordicollis* and forked fungus beetles. There is no consensus on the efficiency of this method. The samples with C7-C40 saturated alkane standard and the C_{18} extraction procedure showed that the sorbent could efficiently adsorb hydrocarbons. Further tests need to be completed to see if this procedure effectively extracts the cuticular wax components and clearly provides the relative concentrations of the components found on the cuticle of the beetle. The sorbent was shown to be reusable after washing with hexane and therefore is a cost effective method.

Five components in the cuticular waxes of the beetles were identified overall, including nonadecane, eicosane, heneicosane, docosane, and 1-eicosanol. All five components were found in the profile for *Chlaenius cordicollis* and three of the n-alkanes, nonadecane, eicosane and heneicosane, were found to be cuticular wax components for forked fungus beetles.

Qualitative results obtained for the lethal hexane extraction and the non-lethal C_{18} extraction showed that the two methods extracted the same components, although the hexane extraction showed greater intensities compared to the C_{18} extraction. Since hexane fully strips the insect of its cuticular hydrocarbons, a greater amount of each component was expected to be extracted with this procedure compared to the C_{18} procedure. Quantitative results showed a large amount of variation of absolute proportions in all of the compounds for the studied species as shown in the standard deviations (Tables 3.3

and 3.4). Cuticular hydrocarbon variation among beetles is expected as they also play a role as individual chemical signatures for the insects.[60]

The intensities of the GC chromatogram peaks were small, making the components difficult to identify. While different methods were used in an attempt to increase the intensities of the peaks, including concentrating and filtering the sample and composite sampling, none of them resulted in a significant increase in the intensities. Different techniques need to be used to analyze the sample to achieve better signal intensities. In addition, a greater number of beetles can be used in composite sampling to see if an increased concentration of extracted components would result in greater signal intensities.

When sampling the live beetles with the C_{18} extraction procedure, the beetles sometimes released defensive secretions that would contaminate the samples. While these peaks were noted and not taken into consideration when identifying cuticular wax components, the hexane extraction procedure is also known to introduce contamination. The solvent extracts of freeze-killed insects have been found to contain glandular compounds and alkaloids from venom gland.[66] Therefore, there is risk of contamination with both extraction procedures.

Four of the five identified components of the cuticular waxes for these beetles were n-alkanes. N-alkanes have been shown to play a key role in controlling trans-cuticular water movement, mainly being involved in preventing desiccation of the beetles.[3] One alcohol was identified for carabid beetles; alcohols are often found as minor constituents in the cuticular waxes of insects and have been found to be sociochemicals

involved in communication.[12, 21, 23] While n-alkanes play an important role as a component of the cuticular profile, most of the components identified are not considered to be sociochemicals. Biological studies would be needed to determine the role of the identified components as sociochemicals.

In addition, behavioral responses were observed while sampling the beetles. While completing the novel non-lethal procedure with forked fungus beetles, the females were noted to be very inactive and non-responsive. The male forked fungus beetles were clearly observed to respond during the sampling procedure. Upon rotating the vial with C_{18}, males on their backs would kick their legs in what looked like an attempt to turn onto their abdomens and stand up. This response did not interfere with the sampling procedure.

Future Directions

Peak Identification for the cuticular waxes of carabid and forked fungus beetles

There are many peaks in the cuticular profiles of carabid and forked fungus beetles that have not been identified. More cuticular hydrocarbon components could be classified, which will help to further elucidate which components play a role in intraspecific communication. Further identifying the key components in forked fungus beetles and multiple species of carabid beetles will help determine whether these different species of beetles share components that are key to their communication, and if there are common components for intraspecific communication for beetles in the carabid family.

As of now, only straight-chain alkanes and a straight-chain alcohol have been identified for forked fungus and carabid beetles. Searching for other chemical classes in the cuticular wax profiles that are common components of cuticular waxes, such as branched-alkanes, should be completed to identify all of the contributing chemical classes to the profile. Identification of other chemical classes will also provide insight into the complexity of the chemicals synthesized by the beetles. Further studies to achieve greater signal intensities should first be completed, allowing for the compounds to be more easily observed and identified. Other instrumentation such as MALDI could be used to look at longer carbon chains and hopefully achieve greater signal intensities.

Biological Implications of the cuticular waxes

Once more peaks are identified and the key components of the cuticular waxes are determined, cuticular wax profiles can be further examined to identify any biological implications based on the beetle's sex, habitat, food sources, and colonies. Preliminary comparisons were made between male and female forked fungus beetles and beetles inhabiting different species of fungus. No clear differences in the components were observed between the groups of forked fungus beetles compared. Further identifying more of the hydrocarbons will provide a clearer focus on which components to compare.

Biological studies

Biological studies with the beetles, such as dose-response tests, can be completed once the main components of the cuticular waxes are further classified. Studies can be completed to observe how the beetles respond to different components and different

concentrations of the components. This will provide further information on the biological significance of these compounds, including their roles as sociochemicals. Specific behavioral interactions between these beetles have been observed, such as aggression between male forked fungus beetles, mating rituals for forked fungus beetles and aggregation for carabid beetles.[54, 56] These observations suggest that the beetles may have specific behavioral responses to different cuticular waxes. Biological studies could confirm these observations.

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