# MASS SPECTROMETRIC INVESTIGATIONS OF APICAL DROPLETS FROM *CULEX QUINQUEFASCIATUS* EGG RAFTS

By

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To my family and friends who have helped me along the way

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By

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*Culex quinquefasciatus*, a common mosquito in the Southeastern U.S., is a vector of pathogens that produce diseases, such as West Nile Virus, filariasis, Japanese Encephalitis Virus, and avian malaria, in animals and humans. One way of improved surveillance is through increased knowledge of the mosquito oviposition pheromones used by this mosquito. Gravid *Culex quinquefasciatus* females orient to a source of a volatile pheromone component, *erythro*-6-acetoxy-5-hexadecanolide, isolated from the apical droplets on the egg rafts. However, in order to utilize this pheromone, the chemical composition of the major components of the apical droplet needs to be known. The objective of this research is to chemically characterize the *Culex quinquefasciatus* mosquito's apical droplets by mass spectrometric (MS) methods. This includes developing a consistent method for sample introduction, separation, and identification and characterization of compounds using MS. With a better understanding of the composition of the apical droplet, one can develop strategies for larval surveillance and control which may result in a reduction of transmission of vector-borne diseases.

The first objective of this research involved using various methods for sample collection and introduction into a gas chromatogram (GC). This includes exploring

different techniques for removing the apical droplet from the *Culex quinquefasciatus* egg rafts and investigating different approaches to sample introduction onto the GC column. The next step entailed examination and identification of the different compounds within the apical droplet using GC/MS. Next, liquid chromatography/tandem mass spectrometry ($LC/MS^n$) and high resolution mass spectrometry (LC/HRMS) were used to determine and analyze the presence of less volatile compounds within the apical droplet. Nuclear magnetic resonance spectroscopy (NMR) was employed to help determine chemical structures of the compounds within the apical droplet. Finally, the influence of the apical droplet compounds on *Culex quinquefasciatus* oviposition was explored using bioassays.

In understanding the *Culex quinquefasciatus'* apical droplet composition, one may achieve improved mosquito control strategies and surveillance by controlling where the larvae will hatch.

# CHAPTER 1
# BACKGROUND AND SIGNIFICANCE

## Research Objectives

The purpose of this work is to further characterize the chemical composition of the *Culex pipiens quinquefasciatus* apical droplet to identify compounds that can be used in surveillance and control strategies for this vector of diseases. This research was accomplished using gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), high resolution mass spectrometry (HRMS), nuclear magnetic resonance (NMR), and bioassays to separate and identify chemicals that are candidate mosquito oviposition pheromones.

The first phase of this research was a comparison of sample collection and introduction methods. Different techniques for removal of the apical droplet from *Culex quinquefasciatus* egg rafts were evaluated. Several sample introduction and separation strategies were investigated to determine the best approach to minimize sample contamination and to determine the best GC column stationary-phase and parameters for the temperature programs.

The second phase entailed identification of compounds in the *Cx. quinquefasciatus* apical droplet. This was accomplished using GC or LC for separation and MS as the detection method. For GC/MS studies, electron ionization (EI) and chemical ionization (CI) modes were used on a single quadrupole MS. Precise molecular weights of compounds were determined by HRMS, and their structures were elucidated by NMR and $LC/MS^n$.

The final phase of the project involved bioassays to determine the extent each compound influenced *Cx. quinquefasciatus* oviposition behavior. The bioassay design

employed twenty gravid mosquitoes that were given the option to oviposit in two pans. One of the pans served as the neutral control, containing only well water. The second pan was a test pan that consisted of well water and one of the novel apical droplet compounds. Each of the newly identified compounds and the known pheromone, *erythro*-6-acetoxy-5-hexadecanolide, were tested.

The attraction level of test mosquitoes to the treatment compounds was expressed in terms of an "attraction ratio", which is the quotient obtained by dividing the number of egg rafts in the treatment pan minus the egg rafts in the control by the sum of the number of egg rafts in both pans. The oviposition activity is expressed as oviposition activity index (OAI) calculated as follows [1].

$$OAI = \frac{NT - NC}{NT + NC} \qquad (1\text{-}1)$$

where *NT* denotes the number of egg rafts in a treated pan and *NC* denotes the number of egg rafts in the control pan. A positive OAI represents a positive ovipositional response, i.e. attraction, whereas a negative OAI represents negative ovipositional response, i.e. repellency.

**Entomological Overview**

Each year, more than 1.5 billion people are at risk of diseases transmitted by mosquitoes. Among these diseases are Malaria, Yellow Fever, Dengue Fever, and West Nile neuroinvasive disorder. *Culex pipiens quinquefasciatus (Cx. quinquefasciatus)* is a mosquito with a large geographical distribution and is one of the most common members of the *Culex pipiens* (*Cx. pipiens*) complex [1].This species inhabits semi-tropical to warm temperature zones around the world (Figure 1-1).

Figure 1-1. *Culex quinquefasciatus* world distribution [Reprinted by permission from Larrick, S.; Connelly, R. University of Florida Department of Entomology and Nematology: *Culex quinquefasciatus* Say (Insecta: Diptera: Culicidae). *http://entnemdept.ufl.edu/creatures/aquatic/southern_house_mosquito.htm*]

*Culex quinquefasciatus* is a vector of St. Louis Encephalitis Virus, Filarial worms, West Nile Virus, Rift Valley Fever Virus, Japanese Encephalitis Virus, and other arboviruses [3,4]. It is also a vector of Lymphatic Filariasis, Wuchereria Bancrofti, and Plasmodium Relictum, an avian malaria parasite [5]. Because resistance development to pesticides can lead to reduced ability to control mosquitoes, the evolution and spread of resistance genes among members of the *Cx. pipiens* complex across continents is a topic of considerable scientific interest [6].

**Oviposition Behavior in Nature**

Pathogen infection requires transmittal of the pathogen to the host from the saliva contained in the mouthpart of a mosquito. This often occurs during the blood meal process of a female mosquito. “Disease transmission typically necessitates the completion of at least one oviposition (egg-laying) cycle before pathogen transfer can occur with a subsequent blood meal” [7]. The potential for manipulating the behavior of

infected mosquitoes in oviposition site selection presents opportunities for mosquito control [8]. *Culex* mosquitoes utilize an oviposition pheromone, (5R,6S)-6-acetoxy-5-hexadecanolide, to assess the suitability of an oviposition site [9,10]. Thus, by controlling mosquito oviposition, one may be able to influence the spread of mosquito-borne diseases when oviposition compounds are used in conjunction with larvicides or larvae-specific pathogens [11].

**Chemoreceptors**

Mosquitoes are believed to at least in part rely on the volatile semiochemicals as cues for determining preferred oviposition sites [12]. This is based on evidence that indicates that the primary sensory method used by *Cx. quinquefasciatus* and other mosquito species during long-range oviposition site seeking is olfaction [7,13]. The primary olfactory organs in mosquitoes are the antennae. Antennal trichoid and grooved peg sensilla of mosquitoes have been demonstrated to contain the olfactory receptor neurons (ORNs) that detect many of the odors involved in eliciting vector-related behaviors [13]. Mosquitoes sense olfactory cues through the activation of sensory neurons housed in hair-like structures called sensilla. Cells within the structure of the sensilla produce odorant-binding proteins. These proteins capture the chemical molecules and transport them to an extension of the neuron cell body (the dendrite). The odorant-binding proteins (OBPs) help move the semiochemicals to the dendrite [14]. Based on the interpretation of the signal from the receptor site, mosquitoes can then either not respond, or can orient themselves towards or away from the source of the semiochemical. This interpretation of chemical signals allows for orientation to occur with a potential end result of guidance of insects to their sources at which they oviposit their eggs [15].

**Physical factors**

The initiation flight to seek out a location for oviposition is linked with environmental factors, such as rainfall, relative humidity, temperature, and wind speed [7]. The *Cx. quinquefasciatus* mosquitoes typically lay their eggs on stagnant water [9]. Oviposition flights of many species occur during twilight. Mosquito species that display two crepuscular biting peaks (one in the evening followed by one in the morning) often have two peaks of ovipositional flight [7]. The *Cx. quinquefasciatus* oviposits during the early hours of the night and again after sunrise [16]. Research shows that upon touching water, gravid *Cx. pipiens* come to rest on the water surface and then lay their eggs [17]. This implies that *Cx. quinquefasciatus* prefer calm water with minimal wind speed during oviposition. Other physical factors that may influence site selection include color and optical density of the site, site texture, temperature, and reflectance. The *Cx. quinquefasciatus* prefer to lay eggs in dark colored water of increased optical density [18].

**Chemical factors**

There are three categories of influence to consider: chemicals that elicit a negative response to oviposition (repellents and deterrents), chemicals that result in a positive response (attractants and stimulants), and chemicals that may be not detected or may be detected and result in no behavioral response (change) by the target.

Repellents are chemicals that cause oriented movements away from its source, and a deterrent inhibits oviposition when present in a place where mosquitoes would, in its absence, oviposit [1]. Attractants are chemicals that cause oriented movements towards its source, whereas stimulants elicit oviposition [1].

**Deterrents and repellents**

Initial research into chemical influences of *Cx. quinquefasciatus* oviposition involved deterrents and repellents; however, the testing methods used did not distinguish between deterrents and repellent. In 1980, Hwang et al. reported that *Cx. quinquefasciatus* did not oviposit in water containing extracts from Purina Laboratory Chow [19]. The extracts consisted of acetic, propionic, isobutryic, butyric, isovaleric, or caproic acid. Additionally, at its extracted concentration, butyric acid produced 100% mortality in the first-instar larvae. Kramer et al. complemented these findings by reporting that lower aliphatic carboxylic acids ($C_2 – C_6$) at high concentrations were found to deter oviposition in *Cx. quinquefasciatus* [20]. Hwang et al. followed this study with the examination of longer straight-chain fatty acids [21]. Addition of $C_5 – C_{13}$ fatty acids to typical oviposition sites resulted in repellency of *Cx. quinquefasciatus*. Further investigation led to the finding that a double bond with a *Z*- configuration was required for an unsaturated fatty acid to be highly repellent to *Cx. quinquefasciatus* [22]. The authors concluded that the *E*- isomers were less repellent or inactive, and that there was no relationship between the repellency and the number of double bonds in the unsaturated fatty acid. The *Cx. quinquefasciatus* were manipulated into laying their eggs in water dishes containing a known repellent to determine if these repellencies were inherited or not [23]. Oviposition in dishes containing known repellents was not, however, carried over to the next generation. Therefore, the authors postulated that gravid female mosquitoes may have evolutionally adapted the ability to avoid oviposition in unsuitable sites which might be detrimental to the survival and development of their offspring [19].

**Attractants and stimulants**

Scientists have shown that deterrents and repellents, as well as attractants and stimulants, influence *Cx. quinquefasciatus* oviposition. Identification of oviposition attractants can aid in the development of efficient ovitraps for *Culex* vector species. Improving the trapping efficiency leads to better and more accurate information for arboviral vector surveillance [24]. The *Cx. quinquefasciatus* has been found to prefer oviposition in sites which contain decaying organic matter at low concentrations [1]. It was determined that specific acids at high concentrations led to the observed repellency. As their concentrations decreased, the ovipositional repellency gradually decreased and the compound eventually became an attractant [19]. One of the organic materials that exhibited this dose-dependent influence on oviposition behavior was hay infusions. Numerous authors reported that various hay infusions acted as oviposition attractants or stimulants to *Cx. quinquefasciatus* [7, 21]. Some authors concluded that isolated phenols induced oviposition by several *Culex* species [25]. This added to the work by Ikeshoji et al. in 1974, that determined attractive compounds were mostly nine carbons long and had an α-methyl or a γ-ethyl branch [26].

Further studies with these compounds led to the discovery that there were relational similarities between attractants [27]. Attractant molecules contained polar and nonpolar regions; often the nonpolar regions consisted of straight chain hydrocarbon sections. Many active compounds possess a carboxylic acid or a carbonyl functional group, but their activity is determined by factors such as chain length, molecular weight, presence of a methyl branch, concentration, and the species involved. It was established that presence of a methyl branching appeared to increase oviposition activity. This may explain why a fatty acid, such as capric acid ($C_{10}H_{20}O_2$), functions as

an oviposition attractant or stimulant [7, 28]. In 2008, scientists determined that an odorant-binding protein, CquiOBP1, attracted *Cx. quinquefasciatus* mosquitoes to oviposit in water containing the protein [4]. An important finding was the identification of the oviposition pheromone *erythro*-6-acetoxy-5-hexadecanolide [7,29]. This chemical influences oviposition of *Cx. quinquefasciatus* and of related mosquitoes such as *Cx. molestus* and *Cx. tarsalis* [9, 29]. The ability to increase oviposition in several mosquito species makes this compound a significant candidate for use in vector surveillance and control.

**Significance of a Natural Pheromone**

Pheromones play an important part in nature by triggering a social response in members of the same species. Pheromones can act as alarms or aggregates, mark territories or trails, or pass on information such as the availability of the female for breeding. By understanding the pheromone for a species, one may be able to manipulate their behavior [30].

In 1979, Bruno and Laurence performed an experiment concerning *Cx. pipiens fatigans* (also known as *Cx. quinquefasciatus*) females' preference for a choice of oviposition sites [31]; one site contained tap water and another site contained water which had previously contained *Cx. quinquefasciatus* egg rafts. The female mosquitoes laid more eggs in water which previously contained *Cx. quinquefasciatus* egg rafts. Further experiments demonstrated that this observed response resulted from the apical droplet material located on each egg (Figure 1-2).

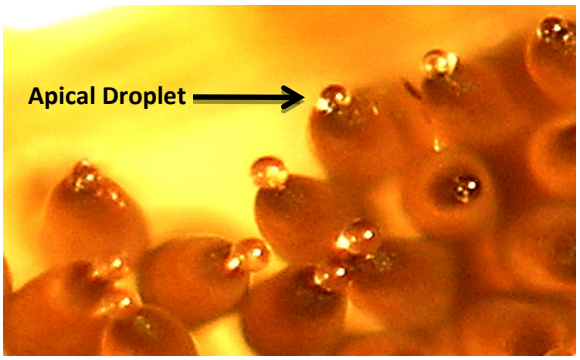



Figure 1-2. Apical droplets [Reprinted by permission from McCann, S. Apical Droplets - *Culex quinquefasciatus*. *http://bugguide.net/node/view/40388*]

**Apical droplet**

The *Cx. pipiens* apical droplet sits in a shallow, 8-10 µm wide depression at the posterior pole of the egg. In each raft, all droplets are of similar size, usually with a diameter of approximately 45 µm (volume of approximately 400 pL). The droplets appear shortly after oviposition; if removed, additional droplets appear [17].

In 1982, a series of chemical analyses, syntheses, and bioassays confirmed that the main component of the droplet was the compound, *erythro*-6-acetoxy-5-hexadecanolide [9] (Figure 1-3). The authors identified this molecule by removing the apical droplets on fine glass rods and dissolving them in hexane. They then examined the volatile components by GC/high resolution mass spectrometry (HRMS) using electron ionization (EI).

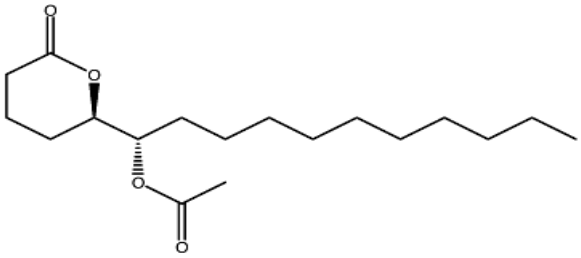


Figure 1-3. Structure of *erythro*-6-acetoxy-5-hexadecanolide

Further experimentation concluded that each egg raft from *Cx. quinquefasciatus* contained about 300 ng of 6-acetoxy-5-hexadeacnolide, with each raft consisting of 200-300 eggs [17].

**Synthetic and natural pheromone**

Analysis of mosquito oviposition pheromones provides a novel approach for surveillance and control methodologies. In 1982, scientists successfully synthesized *erythro*-6-acetoxy-5-hexadecanolide [9]. Two of the four synthetic 6-acetoxy-5-hexadecanolide enantiomers were as active an oviposition attractant for *Cx. quinquefasciatus* females as egg rafts containing an equivalent amount of natural material.

The pheromone 6-acetoxy-5-hexadecanolide is volatile but has a relatively low vapor pressure [29]. Therefore, homologues and analogues were synthesized in an attempt to produce more volatile compounds that might have a greater range of attraction. Shortening the alkyl side chain by four atoms to produce 6-acetoxy-5-dodecanolide increased volatility; however, the compound was inactive. Replacing the acetoxy group with trifluoroacetoxy yielded a more volatile, biologically active compound, but one that hydrolyzed too easily. Replacing all 17 hydrogen atoms on the terminal eight carbons of the alkyl chain with fluorine produced a volatile, active, and stable compound [32-34].

**Proposed apical droplet composition**

Prior to the identification of *erythro*-6-acetoxy-5-hexadecanolide, there were studies conducted to discover the chemical composition of the *Cx. quinquefasciatus* apical droplet. In early chemical analysis, ether washings of *Cx. quinquefasciatus* egg rafts yielded about 19 µg lipids per raft [35, 36]. Separation of the lipids via Kieselgel plates

yielded a fraction that was active in bioassays with gravid females and was shown by its NMR spectrum to comprise of 1,3-diacylglycerols at approximately 6-7 µg per egg raft [35,36]. Acid-catalyzed methanolysis of this fraction yielded the methyl esters of mono- and dihydroxy- saturated fatty acids [35, 36]. The monohydroxy-fatty acids were mainly 3-hydroxy acids. The principal diol acid was 5,6-dihydroxyhexadecanoic acid. The NMR spectrum indicated that in the diacylglycerols, the fatty acid hydroxyl groups were completely acetylated [35, 36]. Research published in 1989 suggested that the apical droplet of the *Cx. quinquefasciatus* contained volatile compounds in addition to the known fatty acids [7]. However, the ecological significance of this discovery has yet to be fully explored.

**Current State of Mosquito Research**

GC analysis performed on the acidic hydrolysates of the *Cx. quinquefasciatus* apical droplet, in 1973 by Aharoni et al. identified the compounds correlated with chromatographic peaks by comparing them to known standards [37]. As previously stated, Laurence et al. performed GC/MS analysis on the droplets in 1982 [9], but this only confirmed the identification and structure of *erythro*-6-acetoxy-5-hexadecanolide. This type of analysis has not been extended to the entire apical droplet. Experimentation shows that often a combination of compounds in the appropriate ratio attracts mosquitoes to oviposit [38]. To optimize the use of *erythro*-6-acetoxy-5-hexadecanolide, the correct proportion of it and the potential other pheromone compounds should be quantitatively determined.

## Overview of Analytical Methods

Mass spectrometry (MS) is an analytical technique used for determining masses of molecules, the elemental composition of a molecule, and for elucidating the chemical

structures of molecules. The MS method consists of ionizing chemical compounds to generate charged molecules (ions) or molecule fragments and measuring their mass-to-charge ratios [39]. Identification and trace detection of volatile compounds is commonly performed by GC/MS; in contrast, LC/MS is typically employed for the identification of compounds that are not volatile or thermally stable enough for GC analysis. This dissertation employs both GC/MS and LC/MS. The most common ionization processes in mass spectrometry are electron ionization (EI) for GC/MS and electrospray ionization (ESI) for LC/MS. These injection methods, as well as chemical ionization (CI), will be addressed in this chapter. Tandem mass spectrometry (MS/MS) allows for the analysis of more complex mixtures with less need for prior clean-up of the sample and matrix. MS/MS was employed in this dissertation for structural identification via fragmentation patterns. Nuclear magnetic resonance spectroscopy (NMR) was also employed to help confirm possible structures of the apical droplet compounds.

**Separation Methods**

The complexity of a sample may dictate the need to employ multiple separation methods to adequately transfer all compounds of interest to the mass spectral detector for analysis. The major focus of this work is on the identification and biological influence of volatiles and non-volatiles compounds in the apical droplet. Therefore, gas chromatographic and liquid chromatographic separation methods were chosen.

**Sample Ionization**

Mass spectrometric analysis requires that compounds of interest be converted to gas-phase ions. Ionization can be accomplished in a number of ways. For the work in this dissertation, samples were ionized either by electron ionization (EI) or chemical ionization (CI) for GC/MS and electrospray ionization (ESI) for LC/MS.

**Electron ionization (EI)**

Electron ionization produces molecular ions $[M]^+$ from gas-phase molecules [M]. A metal filament is heated to a temperature at which it emits electrons; they are accelerated to 70 eV. The sample, which contains the neutral molecules, is introduced to the ion source, where the electrons can collide with the sample molecules, producing ionization and fragmentation of the neutral molecules. The ionization process often follows predictable cleavage reactions from which structural information of the analyte molecule can be deduced.

**Chemical ionization (CI)**

Chemical ionization is a “soft” ionization technique. This ionization process results in decreased fragmentation of molecules compared to EI, and it can produce ions based on proton affinities; i.e. results in an ion representing the intact molecule such as $[M+H]^+$ ion.[40]. Chemical ionization is accomplished via a reagent gas. Methane is a common reagent gas used for positive ion chemical ionization (PICI); however, methane has a low proton affinity which would cause molecules with high proton affinity to fragment more. Therefore, isobutane and ammonia are employed as CI reagent gases for compounds with high proton affinity. The reagent gas is in greater abundance in the ion source than the sample, and at a pressure high enough to favor ion-molecule reactions [40]. Electrons ionize the reagent gas, and the resultant reagent ions react with sample molecules via ion-molecule reactions. One of the more common reactions is proton transfer. Decreased fragmentation of the molecular ion occurs due to the transfer of a decreased amount of energy from the ionized reagent ions compared to EI where the electrons given off by the filament directly bombard the sample molecules. The greater abundance of intact molecular ion species relative to the fragment ions and the

diagnostic $[M+H]^+$, $[M+29]^+$, and $[M+41]^+$, in the case of methane, allows for molecular weight determination. In addition to positive ion CI, negative chemical ionization (NCI) can also provide confirmation of molecular weight for compounds that have a high electron affinity.

**Electrospray ionization (ESI)**

Electrospray ionization produces ions from nonvolatile, thermally labile compounds in solution. ESI involves forcing a solution of the analyte through a small capillary held at a high electric field so that the fluid sprays, generating very fine droplets [40]. The electric field is imposed between the tip of the spraying capillary and a counter electrode. The droplets decrease in size due to evaporation of the solvent in a drying gas at atmospheric pressure or in a heated chamber. As the solvent evaporates, the analyte molecules retain a charge.

**Single Quadrupole Mass Spectrometry**

This nonmagnetic mass-to-charge (*m/z*) analyzer employs a combination of direct current (DC) and radio frequency (RF) fields as a mass “filter”. The quadrupole consists of four parallel surfaces with a hyperbolic cross section. Opposing surfaces are connected together electrically and to RF and DC power sources. Extracted ions from the ion source are accelerated by lenses into the central space that constitutes the quadrupole electric field along the longitudinal axis towards the detector. Ions of a particular *m/z* are passed through the mass analyzer by increasing the magnitude of the RF amplitude and DC potentials at a fixed ratio. For any given set of DC and RF potentials, only ions of a specific *m/z* value avoid collision with the poles and successfully pass through the quadrupole filter along the z-axis to the detector; all other

ions collide with the quadrupole's surfaces [40]. The GC/MS analyses described within this dissertation employed single quadrupole mass spectrometry.

**Quadrupole Ion Trap Mass Spectrometry**

The LC/MS analyses described within this dissertation employed QITMS. A 3D quadrupole ion trap (QIT) consists of two hyperbolic metal electrodes with their foci facing each other and a hyperbolic ring electrode halfway between the other two electrodes. Ions are trapped in the space between these electrodes by an RF potential applied to the ring electrode. This produces a 3D quadrupolar potential field within the trap. The field traps the ions in stable trajectories, with the high *m/z* ions closer to the center and the lower *m/z* ions towards the outer part of the field. For detection of the ions, the RF potential is altered to destabilize the ion motions resulting in ejection of the ions through a hole in the exit endcap. The ions are ejected in order of increasing *m/z* by a gradual ramping of the RF potentials. This 'stream' of ions is focused onto the detector of the instrument to produce the mass spectrum.

Tandem mass spectrometry (MS/MS) is used to assist in understanding the fragmentation pathway of a molecule to gain knowledge regarding the molecular structure. This technique is accomplished by fragmentation of a mass-selected precursor ion which is allowed to collide with neutral gas atoms or molecules [40], followed by mass analysis of the product ions. The products of the dissociation process, which characterize the precursor ion, are then analyzed according to the *m/z* values.

A QIT mass spectrometer can perform tandem mass spectrometry by isolating an ion of a specific *m/z* value (the precursor ion) during one time interval, and collisionally activating the precursor ion so that dissociation takes place during a second time

interval. A product-ion mass spectrum is obtained by scanning the product ions out of the device during a third time interval [40].

**Nuclear Magnetic Resonance Spectroscopy**

NMR is an analytical technique that applies a magnetic field to the nuclei in a molecule, which then absorb and re-emit electromagnetic radiation. This energy is at a specific resonance frequency which depends on the strength of the magnetic field and the magnetic properties of the isotope of the atoms, specifically $^{1}H$ and $^{13}C$, and their chemical environment. This phenomenon is called 1D NMR when analyzing only one isotope at a time.

2D NMR is a set of NMR methods in which data are plotted in a space defined by two frequency axes rather than one. Correlation spectroscopy (COSY) was performed in this dissertation. COSY spectra show the frequencies for a single isotope, most commonly hydrogen ($^{1}H$), along both axes. Diagonal peaks have the same frequency coordinate on each axis and appear along the diagonal of the plot, whereas cross peaks have different values for each frequency coordinate and appear off the diagonal. Cross peaks indicate $^{1}H$ protons which are coupled to each other, that is, on adjacent carbons.

## Organization of Dissertation

This dissertation consists of seven chapters; the overall emphasis is on a combinatorial approach, involving chemistry and entomology, to better understand the chemical compounds in *Cx. quinquefasciatus* apical droplets and their influence on oviposition. The first chapter covers the objectives of this work, an introductory overview of entomological fundamentals concerning the mosquito, the relation of this work to previous studies of the *Cx. quinquefasciatus* apical droplet, and an overview of the analytical methods of sampling and detection by mass spectrometry and NMR

employed in this dissertation. Chapter 2 summarizes a comparison of various methods of sampling apical droplets. Chapter 3 focuses on GC/MS identification of compounds in the *Cx. quinquefasciatus* apical droplet, and the confirmation of previous apical droplet analyses via GC/MS. Chapter 4 concentrates on the utility of $LC/MS^n$ to this project, and addresses the identification of pheromone-like compounds found within the droplet. Chapter 5 discusses the NMR analysis of the compounds individually separated from *Cx. quinquefasciatus* apical droplets using LC. Chapter 6 addresses the biological impact of these findings. This chapter reports studies determining the influence the identified compounds have on *Cx. quinquefasciatus* oviposition. The seventh and final chapter of this dissertation contains the conclusions and suggested future experiments.

# CHAPTER 2
# SAMPLING METHODS

## Introduction

Sampling is of significant concern when it comes to explorative research into the chemical cues that mediate insect behavior. The sampling method can impart biases that can reduce or remove compounds that may be important. They can enhance collection relative to others, but not actually enhance the abundance above the level of compound that is already there. In studies aimed at discovering biologically active compounds, there is a risk that compounds of interest will be collected at levels below the limit of detection of the instrumentation, which will clearly result in the answers remaining undiscovered. This chapter covers the sampling methods, the sample introduction methods used for this study, and the results from the sampling techniques used to collect chemical compounds from apical droplets.

## Experimental

### Direct Sampling

In order to oviposit, *Cx*. *quinquefasciatus* female mosquitoes must consume a bloodmeal [17]. The mosquitoes were blood fed every seven days, and were offered a pan for oviposition five days after feeding. The egg rafts were collected about 12 h after being laid. All analyzed apical droplets were collected from a single raft to minimize sample bias. The average droplet size was 45 μm in diameter, which corresponded to 400 pL at the droplets' average size [17]. Three collection techniques were used to remove the apical droplets from the egg rafts. These techniques included glass rod collection followed by sample dissolution, micropipette sampling, and SPME sampling.

**Micropipette sampling**

This technique involved the use of tapered capillary collection tubes prepared by heating and pulling glass capillary tubes (World Precision Instruments, Sarasota, FL, 1.5 mm outer diameter, 1.12 mm ID, 100 mm length) using a PUL-1 (World Precision Instruments) micropipette puller. The final dimensions of the micropipettes were 66 mm long with an outer diameter (O.D.) of 50 µm. The I.D. was approximately 7.5 µm.

The egg rafts were placed under a microscope and the apical droplets removed through capillary action by touching them with the tip of the tapered capillary collection tube (Figure 2-1). The micropipette was inserted into the GC inlet by removing the septum nut, dropping the micropipette into the GC liner, and replacing the spectrum nut

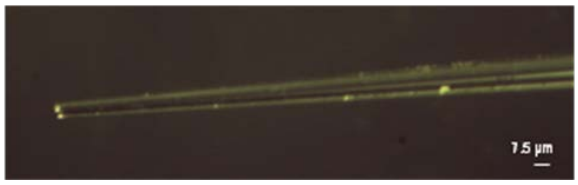



Figure 2-1. Enlarged image of micropipette

**Sample dissolution**

This approach involved collecting the droplets using the micropipette method. The samples were then dissolved in n-hexane, and manually injected into the GC.

**SPME sampling**

Sample acquisition with solid-phase microextraction (SPME) was also conducted. SPME is a sample collection/preparation technique for GC, based on the adsorption of analytes directly from a sample onto a solid, coated, fiber of various compositions [41]. The analytes desorb from the fiber when introduced in the GC inlet (Figure 2-2). This technique is fast and easy to use compared to the glass rod and micropipette techniques. The SPME fiber had a film thickness of 7 µm and was approximately 1 cm

long. The apical droplets were collected by touching the SPME fiber to the apical droplets, which were placed under a microscope, and adsorbing them onto the fiber.

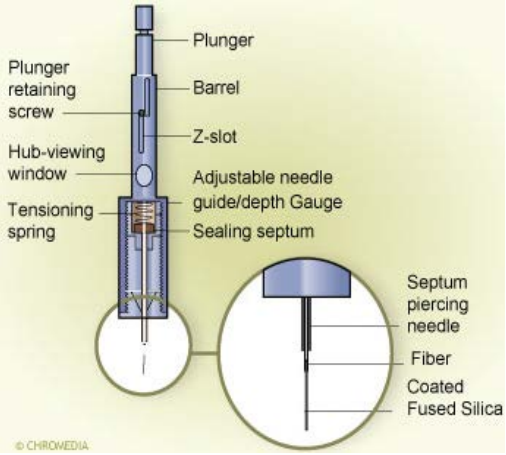



Figure 2-2. SPME commercial device similar to that used in this research [Reprinted by permission from Chromedia; Tuglea, A. Principles of SPME. *http://www.chromedia.org/chromedia?waxtrapp=npuhcHsHqnOxmOlIEcCxBWeB&subNav=abffyDsHqnOxmOlIEcCbCuEnEL*]

To adequately compare the micropipette to the SPME fiber collection methods, the fused silica fiber was removed from the SPME holder and a hollow glass fiber was put in its place. The hollow glass fiber was approximately 1 cm long with approximately a 5 µm I.D. and a 7 µm O.D. This allowed for a comparison between a micropipette dropped into the GC inlet and a hollow glass fiber and the coated, solid, fused silica SPME fiber inserted into the inlet.

**Gas Chromatogram Set-Up**

Below are descriptions of how the gas chromatogram was set-up.

**Solventless injection**

A PTV (programmed temperature vaporizing) injection port was used for analysis of the *Cx. quinquefasciatus* apical droplets (Figure 2-3). This inlet allowed for ramped heating for the solventless injection method.

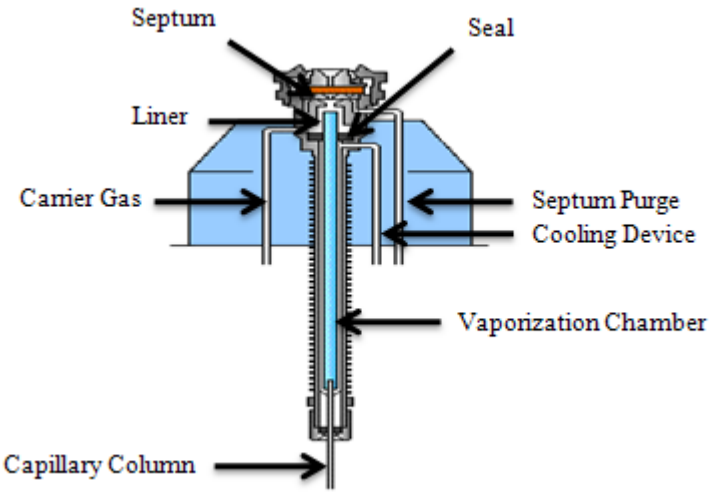



Figure 2-3. PTV injection port set-up

The solventless (direct) injection method involved placing the micropipette or the SPME fiber into the GC inlet. For the micropipette, the PTV inlet was opened, and the entire micropipette dropped directly into the GC liner. The SPME fiber, on the other hand, retracts into a holder that acts the same as a GC syringe. The holder was inserted through the septum into the GC inlet, and the fiber extended during the entire analysis to permit desorption of all compounds.

In the solventless injection method, the analytes from either the SPME fiber or the micropipette volatilize and are transported by the carrier gas into the GC column. Since nearly the entire sample is transferred to the column, little sample discrimination is expected. There is no sample acquisition delay and early eluting peaks can be viewed without interference of co-elution with a solvent peak. Thus, a solventless injection minimizes interferences associated with large solvent fronts.

**GC liners**

GC liners are composed of various materials; the choice of material is linked to heat transfer, and this determines the speed of vaporization [42]. While the glass rod

sampling technique using a solvent injection or the SPME device does not require a specific liner, placing the micropipette directly into the injection port requires a tapered liner to prevent it from breaking or jamming into the end of the capillary column.

**Inlet temperature programming**

The *Cx*. *quinquefasciatus* typically oviposit at ambient temperature [43]. Therefore, the initial injection inlet temperature was initially set to 35 °C to prevent volatile compound loss and to simulate a temperature typical for oviposition by this species. The injection inlet temperature was then ramped heated to vaporize the apical droplet compounds. However, there was an issue of not all the compounds desorbing from the sampling devices. Thus, an elevated isothermal injection inlet temperature was also evaluated.

**Oven temperature programming**

The GC oven temperature programming for the analysis of apical droplets consisted of an initial temperature of 60 °C. After injection, there was an immediate ramp of 3 °C/min to 260 °C, and a final hold for three minutes. This oven temperature program closely matched the protocol of Adams which is used for identifying essential oil compounds by GC/MS [44]. The intent was to correlate observed peaks with those previously reported. However, the temperature program of Adams is used to characterize floral compounds (terpenes, etc.), rather than animal based compounds (fatty acids).Thus, the oven temperature program was amended accordingly, as described later in this chapter.

**Cryofocusing**

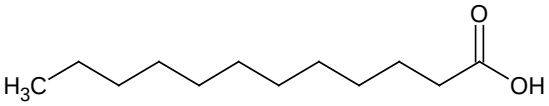
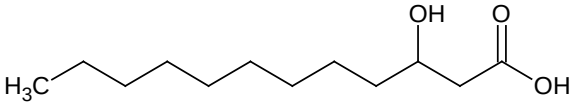
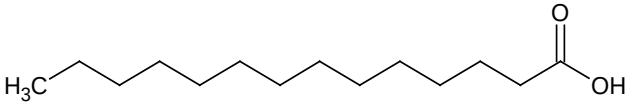
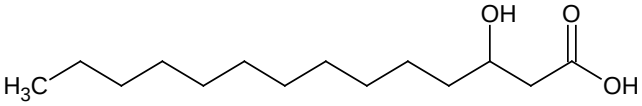
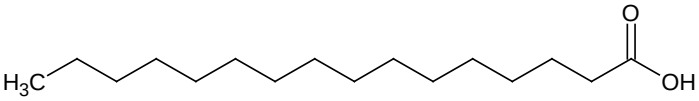
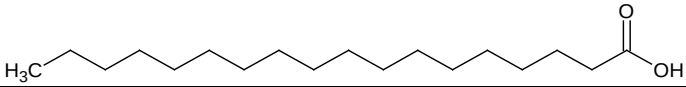
Cryofocusing was used to concentrate early eluting compounds near the head of the GC column. This was accomplished by immersing a loop of the column in a mixture

of liquid nitrogen (-78 °C). This greatly reduces the migration of the injected compounds at the immersed section, which allows for the trapping and refocusing of volatile organic compounds prior to analysis [45].

**Previously identified compounds and columns used to identify those compounds**

Below is a list of compounds previously determined to be in the acidic hydrolysate of the *Cx. quinquefasciatus* apical droplet [37] (Table 2-2)*:*

Table 2-1. Previously identified compounds in the acidic hydrolysate of apical droplets analyzed by GC as their methyl ester [37]

| Compound | Structure | B.P. (° C) | M.W. (g/mol) |
|---|---|---|---|
| Lauric Acid |  | 225 | 200.18 |
| β-OH Lauric Acid |  | | 216.32 |
| Myristic Acid |  | 250 | 228.21 |
| β-OH Myristic Acid |  | | 244.37 |
| Palmitic Acid |  | 272 | 256.24 |
| Stearic Acid |  | 361 | 284.27 |

Aharoni et al. obtained their data using a 5 ft stainless steel GC column, 1/8” OD, packed with 20% (w/w) glycol adipate polymer (LAC 446) on 30/60 mesh Chromosorb. Packed columns produce poor results for trace analysis because of poor resolution due to fewer theoretical plates, which gives poor separation power.

The fatty acids listed in Table 2-2 could be separated equally well using a 30 m DB-5MS capillary column with a phenyl arylene polymer stationary phase. The 30 m length provides better separation than a packed column. The bonded stationary phase

allows the column to operate at high temperatures (up to 325 °C) which can aid in separating less volatile compounds and shorten analysis time. Finally, the narrow ID (0.25 µm) produces narrower peaks, resulting in an increased GC signal-to-noise ratio.

In conclusion, neat sampling will provide analysis of only the compounds in the *Cx. quinquefasciatus* apical droplets and not any solvent. A temperature program ramp will result in improved separation of compounds with a wide range of boiling points, and the capillary column will provide narrower peaks and better resolution of compounds over the packed column because a capillary column, per length, has a far greater number of theoretical plates.

## Results and Discussion

### Direct Sampling

This section presents and discusses the results from direct sampling of *Cx. quinquefasciatus* apical droplets.

### Sample dissolution

Transfer of the droplets from the micropipette to a solvent was not accomplished because the micropipette collected the solvent via capillary action rather than the droplets dissolving in the solvent. One approach to overcome this problem consisted of breaking the micropipette in a GC vial containing the solvent, vortexing the vial, and then injecting 1 µL of solution into the GC. Problems arose that peaks which eluted prior to six minutes could not be recorded with the mass spectrometer since the initiation of data collection had to occur after most of the solvent had eluted to preserve the lifetime of the filament (Figure 2-4).

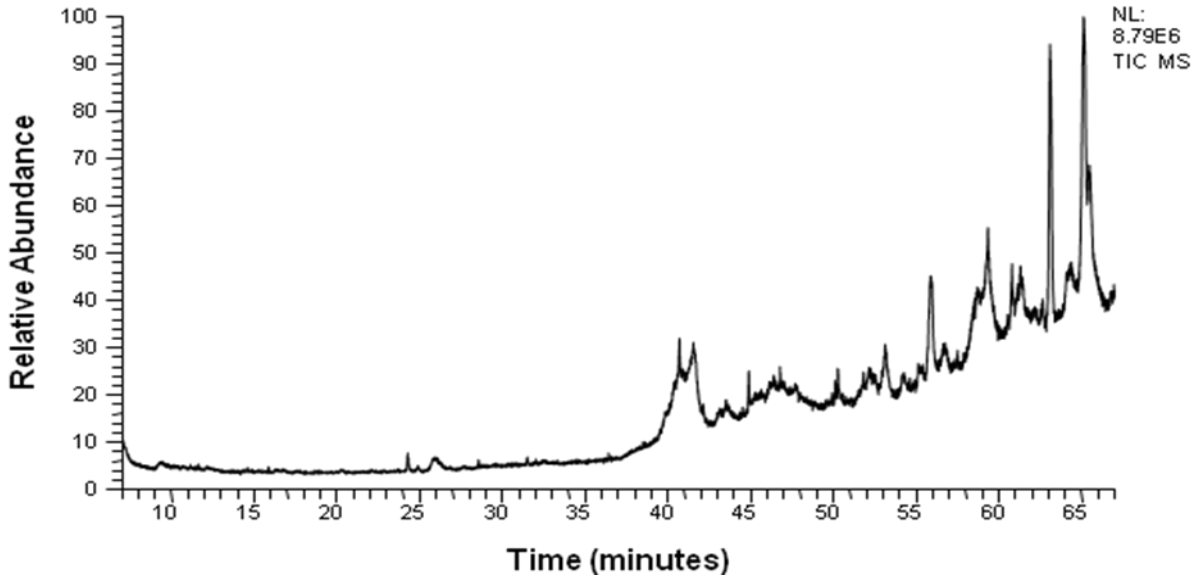



Figure 2-4. GC/MS total ion chromatogram of *Cx. quinquefasciatus* apical droplets in hexane using broken micropipette method

The poor peak separation of this sample collection and dissolution technique was the reason that this approach was not investigated further. Instead, subsequent experiments involved solventless introduction of apical droplets directly into the GC injection port.

**Micropipette sampling**

The second technique tested for sample collection involved tapered capillary collection tubes. This also was not facile due to problems that arose from the use of the PUL-1. The instrument did not uniformly heat the micropipettes prior to pulling, which produced inconsistent dimensions of the manufactured micropipettes. In addition, the heating also sealed the tips of the micropipettes. Even after the rate of heating was adjusted, both issues remained. To overcome these limitations, the sealed ends of the micropipettes were broken using a razor blade.

A comparison of the micropipette to the SPME fiber collection techniques was accomplished by removal of the fused silica SPME fiber from the SPME holder and insertion of a hollow glass fiber in its place. Ten apical droplets were collected using all three collection media (micropipette, hollow glass fiber, and PDMS SPME fiber). All collections were made on the same day, and analyzed using the same GC method. The analysis method consisted of placing the sample in the inlet under low temperature conditions, then rapidly heating the GC inlet temperature to better load volatiles onto the column. The oven temperature was ramped at 5 °C per minutes, after a one minutes hold, from 35 °C to 260 °C, and then held for fifteen minutes. The results are shown below. Crofocusing was not performed.

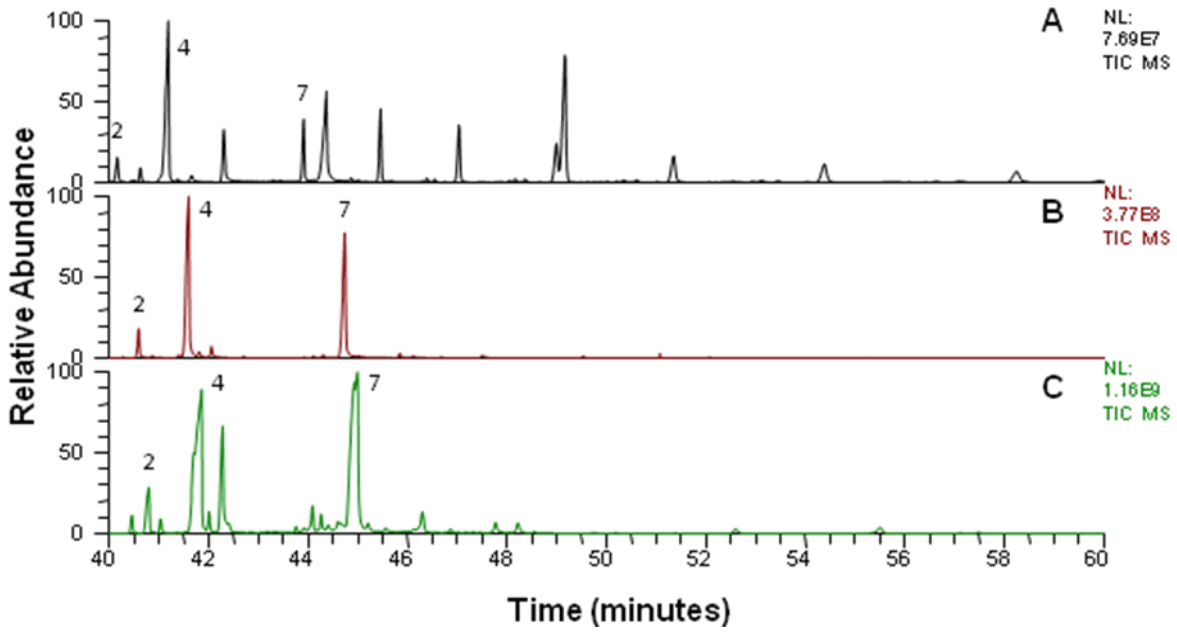



Figure 2-5. GC/MS total ion chromatograms of *Cx. quinquefasciatus* apical droplets collected with (A) a micropipette, (B) a hollow glass fiber, (C) a solid PDMS SPME fiber. Note that the retention times shifted based on the sample introduction technique (peak numbers correspond to those used in Figure 3-1).

Figure 2-5 illustrates that the chromatogram from the micropipette collection contained a large number of peaks. It was determined from the EI mass spectra that most of these contaminates were hydrocarbons. These hydrocarbons were only detected with a sample loading method that involved opening of the GC injection port and insertion of the micropipette directly into the glass liner. The problem may arise from atmospheric hydrocarbons which could enter the GC when the inlet was opened. A benefit of this using the micropipette technique was that droplets were easily visible within the micropipette; therefore confirming that a sample had been collected appropriately. Droplets were not observable in the hollow glass fiber or on the PDMS SPME fiber once they were collected.

To determine the amount of sample carryover for each technique, a blank sample was injected in between each injection of a sample. Prior to collecting any samples, the micropipette, hollow glass fiber, or PDMS SPME fiber was baked out in the injection port at 300 °C overnight. The sample collection medium was removed from the inlet, used to collect ten apical droplets, reinserted into the inlet, and then remained there for the entire acquisition period. For sample analysis, the micropipette, hollow glass fiber, or PDMS SPME fiber was inserted into a 0.05% v/v solution of the pheromone standard in acetone, and then reinserted into the inlet.

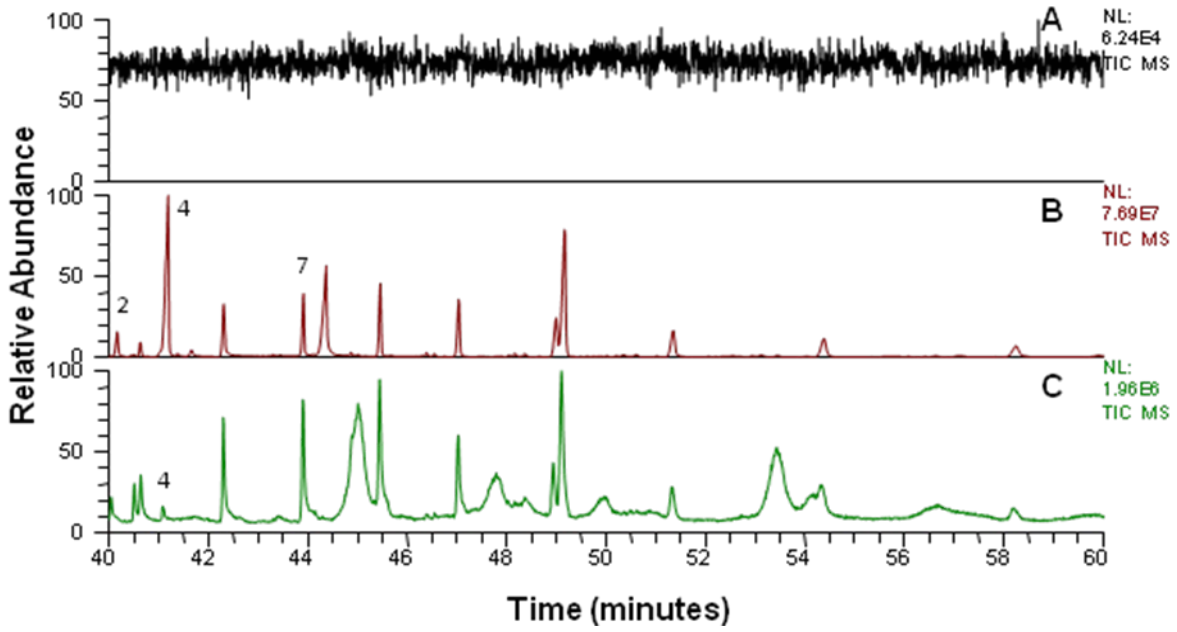



Figure 2-6. GC/MS total ion chromatograms of (A) a blank micropipette, (B) a micropipette with apical droplets, and (C) a blank micropipette

Figure 2-6 indicates that there is significant contamination carryover (B and C) following analysis of a sample that contains apical droplets when a micropipette is used for collection.

The removal of the micropipette from the GC inlet was also difficult. To remove the micropipette, the liner had to be extracted from the GC inlet, inverted, and forcefully tapped to dislodge the micropipette from the liner. This had to be performed after each run, and each time there was a chance of chipping the liner, which translated to reduction of the lifetime of the liner. The extraction process could also break the micropipettes so that they could not be reused. Thus, new micropipettes were sometimes needed for each analysis.

**Hollow glass fiber and SPME sampling**

One difficulty with collecting samples with the SPME fiber and the hollow fiber was that there was no visual confirmation of any sample being transferred to either fiber.

Another complexity was the fragility of the fibers; additional care needed to be exerted when handing the hollow glass fiber and the SPME fiber during sample collection.

Figure 2-7 illustrates the amount of carryover observed using the hollow glass fiber technique for apical droplet analysis. Fewer contaminants are present in the analysis of the hollow glass fiber than for the micropipette (Figure 2-6). However, sample carryover from the previous sample is still evident. The carryover may be the result of overloading the hollow glass fiber

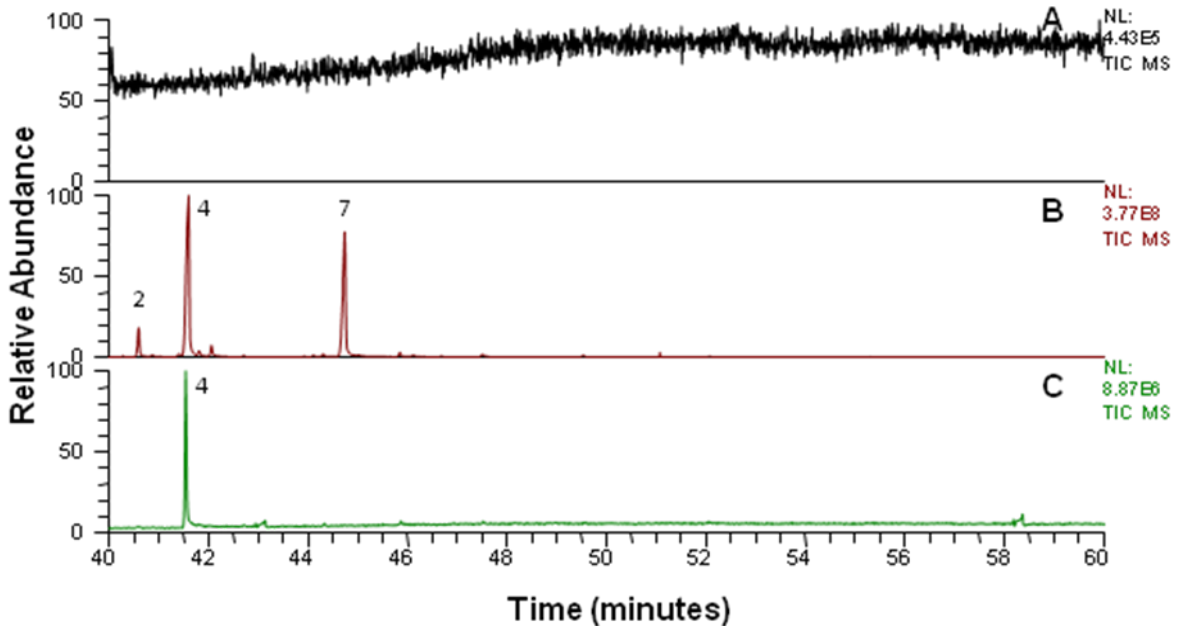



Figure 2-7. GC/MS total ion chromatograms of (A) a blank hollow glass fiber, (B) hollow glass fiber with apical droplets, and (C) a blank run of the hollow glass fiber

The PDMS SPME fiber collected a greater amount of sample than the hollow glass fiber, resulting in broadened GC peaks; nevertheless no contamination carryover was observed (Figure 2-8), though there was carryover with the pheromone standard. This indicates that the compounds more readily desorb from the solid SPME fiber than from the hollow glass fiber. The carryover is due to the overloading of the sample.

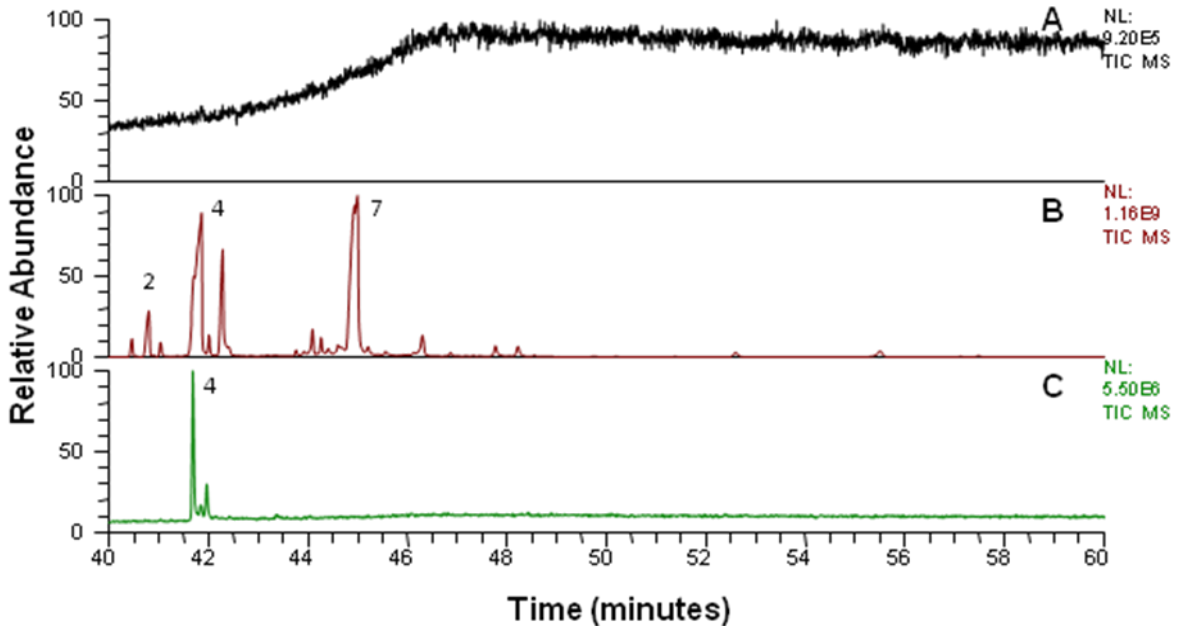



Figure 2-8. GC/MS total ion chromatograms of (A) a blank SPME, (B) SPME with apical droplets, and (C) a blank SPME run

The SPME fiber apical droplet chromatogram contains peaks that were not observed in chromatograms of compounds collected with the hollow glass fiber. This may result from overloading the SPME fiber. One available option is to investigate an alternative SPME stationary phases to ascertain if improved sample collection and reduced sample bias will result. For instance, Carson et al. used stir bar sorptive extraction (SBSE) to extract behaviorally active small lipophilic molecules (SLMs) from water samples collected at *Cx. quinquefasciatus* breeding sites [46]. The stationary phase coating was a polydimethylsiloxane (PDMS) polymer, and SLMs were redissolved in ether. Therefore, a PDMS polymer for the SPME stationary phase is believed to be a suitable choice for sample collection of similar compounds from apical droplets. Fibers were used and cleaned for multiple analyses.

**Gas Chromatogram Operational Parameters**

The GC parameters are presented in this section.

**Inlet temperature programming**

For sample introduction with the micropipette technique, once the septum nut was replaced, the GC inlet program consisted of an initial temperature of 35 °C until injection, followed by a ramp to 260 °C at 14.5 °C/min. The rapid heating of the inlet was performed in an effort to minimize loss of volatile compounds. The ramp rate employed was the maximum ramp rate of the GC inlet. Because under the selected conditions and with the chosen GC column, squalene (the least volatile compound previously observed) elutes off the GC column around 250 °C on a DB-5MS column, the final injection inlet temperature was set to 260 °C and held for the duration of the chromatographic analysis. For analysis of compounds collected by hollow glass fiber and SPME fiber, the injection port temperature was either ramped at 14.5 °C from 35 °C to 260 °C or operated isothermally at 260 °C.

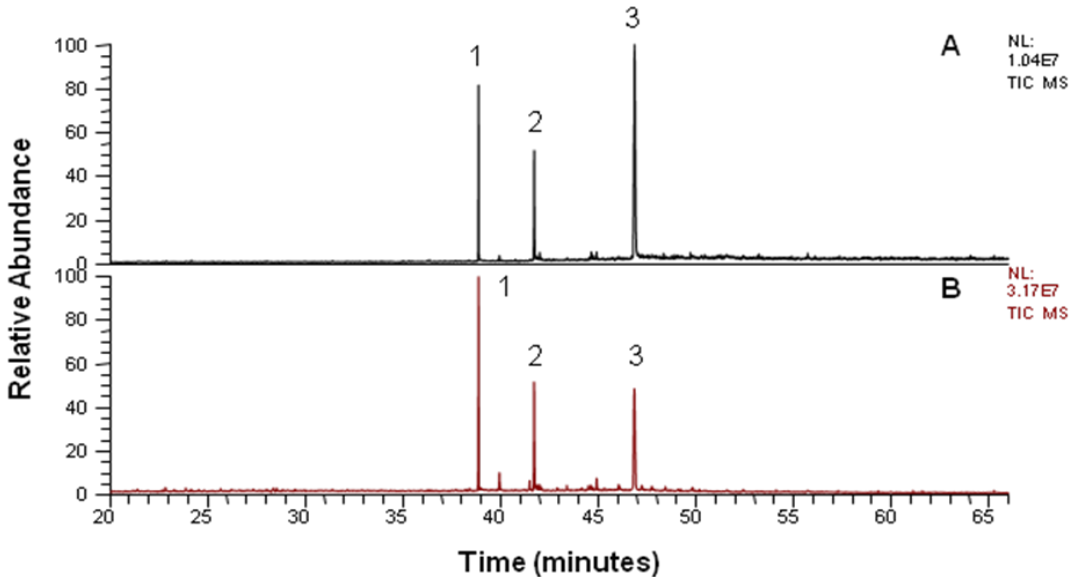



Figure 2-9. GC/MS total ion chromatograms of 1 uL injection volumes of 0.05% v/v nonanal, mosquito pheromone, and squalene in hexane acquired with the injection port (A) heated at 14.5 °C from 30 °C to 260 °C and (B) isothermal injection port temperature of 260 °C. The peaks are labeled 1 (Nonanal), 2 (pheromone), and 3 (squalene)

Comparison of chromatograms in Figure 2-9 indicates a high level of similarity of the peaks in the chromatograms. However, differences exist in the peak heights and areas. The isothermally operated injection port demonstrates a bias in later eluting peaks; their abundance is reduced compared to the amount recorded in the chromatogram with the temperature-ramped injection port. Notwithstanding the sample bias observed in the chromatogram with isothermal injection port operation, either method is suitable for a purely qualitative analysis of *Cx. quinquefasciatus* apical droplets.

**Programmed oven temperature ramp**

Figure 2-10(A) contains a chromatogram from the analysis of apical droplets. These data were acquired using the Adams GC oven ramp temperature program: an initial temperature of 60 °C, with a ramp of 3 °C/min to 250 °C post-injection, followed by a final hold at 250 °C for three minutes. Band broadening in some of the later eluting peaks are apparent. Attempts to minimize this broadening in later eluting peaks were made by modifying the oven temperature program. Modifications consisted of an increased ramp rate and a higher temperature that was hypothesized to lead to more rapid elution and sharper peaks from compounds in the apical droplet. A modified temperature program with an initial temperature of 35 °C with a one minute hold followed by a ramp of 5 °C/min to 260 °C and a five minute hold at 260 °C was executed. Figure 2-10(B) was acquired using the modified program. Comparison of the chromatograms indicates the superior peak shape from the modified program.

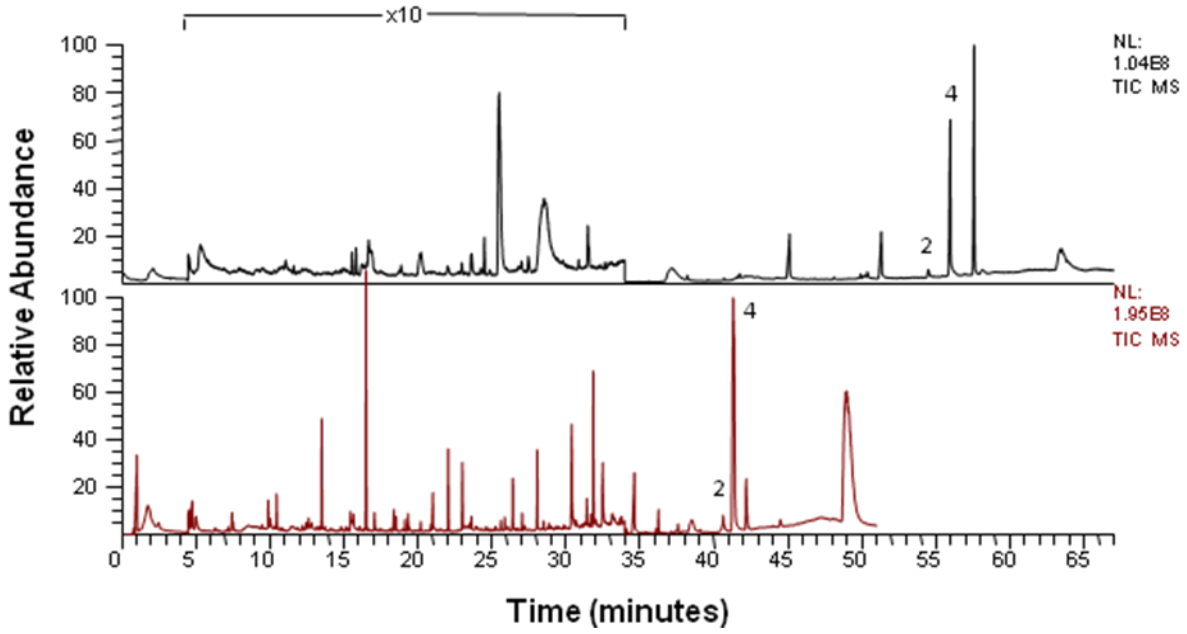



Figure 2-10. GC/MS total ion chromatograms of apical droplets collected by the micropipette sampling method and run with the (A) initial temperature program and (B) adjusted temperature program

The modifications resulted in sharper early eluting peaks along with a reduced run time.

**Cryofocusing of compounds near the column entrance**

The chromatogram of Figure 2-11 was acquired with cryofocusing by liquid nitrogen performed just after the injection port.

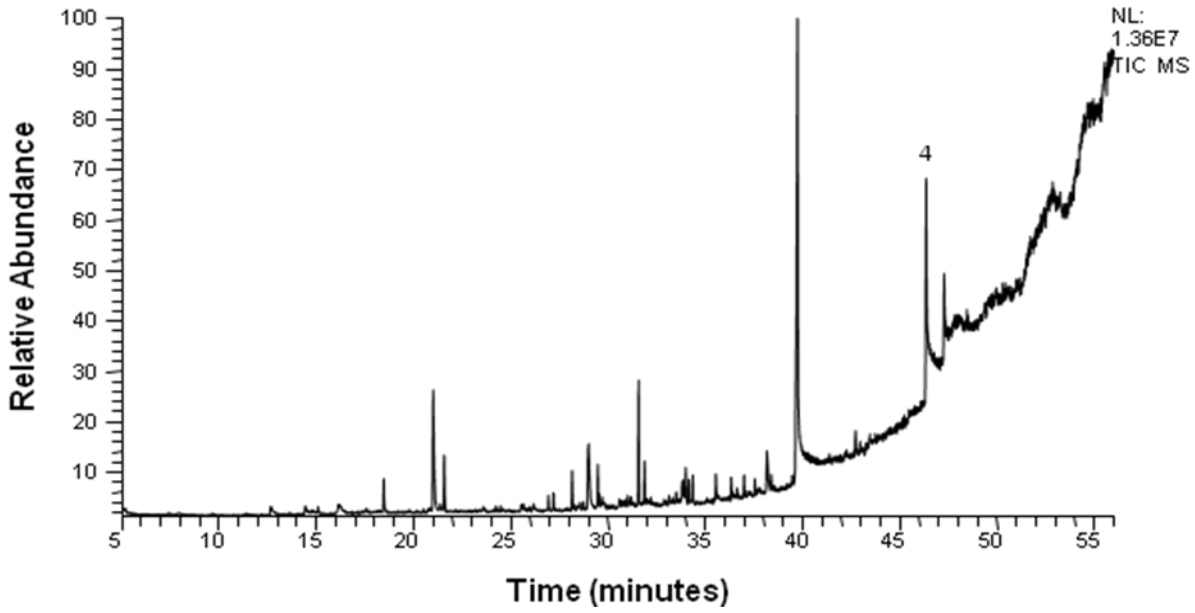



Figure 2-11. GC/MS total ion chromatogram of apical droplets collected by the micropipette sampling method and run with the cryofocused temperature program

The gradual upward drift in the baseline was expected in temperature-programmed situations since column bleed typically increases along with column temperature [47]; though this is observed less frequently with bonded columns. In this experiment, the baseline rise is due to destruction of the stationary phase when the column exceeds the low end temperature capacity [47]. The DB-5MS column has a low temperature limit of -60 °C and cryofocusing was performed with liquid nitrogen, which has a temperature of -78 °C. The increase of baseline intensity due to cryofocusing also makes later eluting peaks difficult to detect and impairs mass spectral identification of the compounds that produce those peaks. Thus, if cryofocusing is to be used, a more suitable column stationary phase will have to be selected.

**Column composition**

Figure 2-12 is the chromatogram obtained in the study of Aharoni et al. from the separation of acid hydrolysate of apical droplets on a 5 ft stainless steel GC column with 1/8" OD, packed with 20% (w/w) glycol adipate polymer (LAC 446) on 30/60 mesh Chromosorb [37].

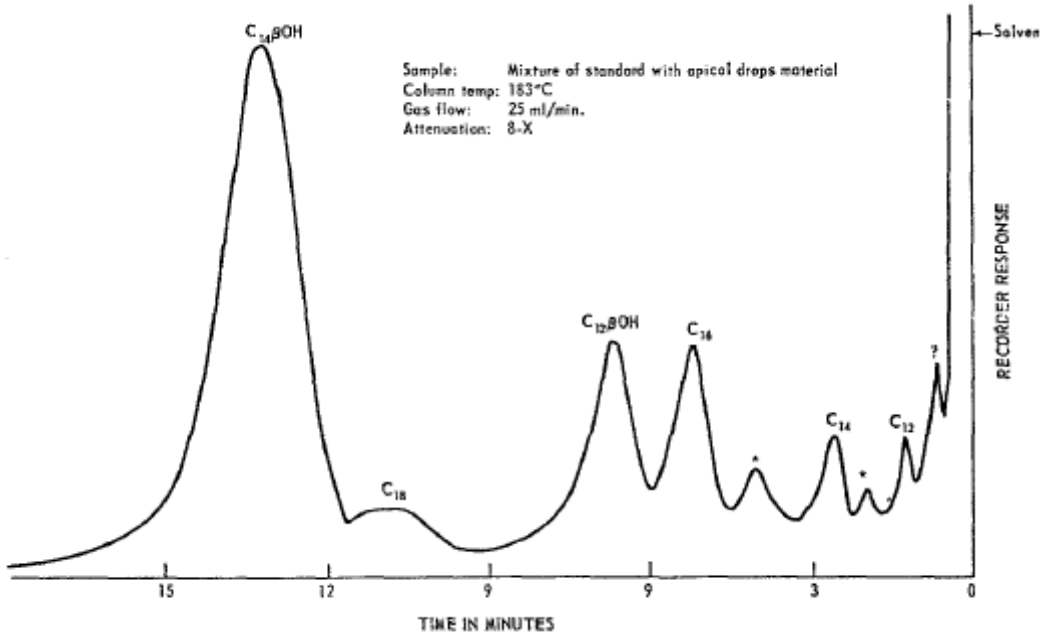



Figure 2-12. GC/FID chromatogram obtained by Aharoni et al. by injection of 1 µL of methanol hydrochloride esterified *Cx. quinquefasciatus* apical droplets on a packed column. [Reprinted by permission from Aharoni, A.; Zweig, G. Chemical Structure of Apical Droplet from Eggs of *Culex-pipiens*. *J. Insect Physiol.* **1973***, 19*, 861-875]

The identified compounds that contribute to the observed peaks in Figure 2-12 are listed in Table 2-2. In the 40 years since this study, fused silica open tubular (FSOT) columns were invented. These columns have improved separation power due to the increased number of theoretical plates. Separations of complex samples, such as apical droplets, on these newer columns will allow separation of a greater number of compounds through improved resolving power.

Figure 2-13 is a chromatogram of *Cx. quinquefasciatus* apical droplets obtained using a 30 meter DB-5MS capillary column with a 0.25 mm I.D. and 0.25 µm film thickness.

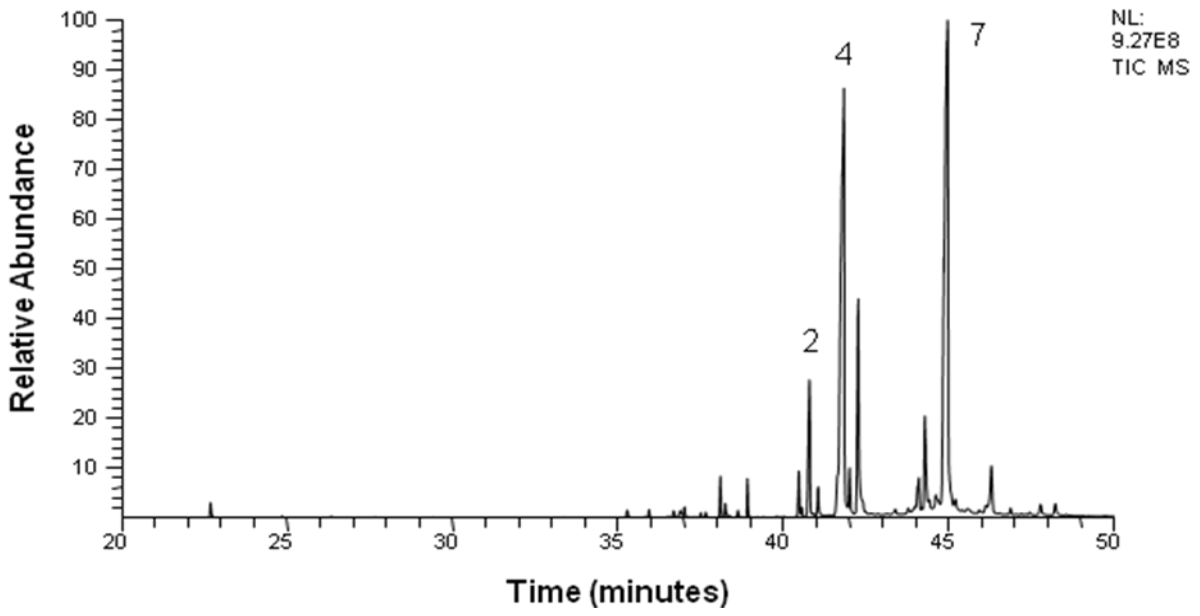



Figure 2-13. GC/MS total ion chromatogram of *Cx. quinquefasciatus* apical droplets collected by the micropipette sampling method and run on a capillary column with the adjusted temperature program

It is evident that better separation and peak shape is achieved with a capillary GC column (Figure 2-13) when compared to separation using a packed column (Figure 2-12). This FSOT column of Figure 2-13 is coupled to a mass spectrometer, which is a more sensitive detector and capable of producing a secondary amount of information, i.e. mass spectra, to aid in the identification of compounds.

## Conclusions

Sample dissolution resulted in poor peak separation. Thus, a solventless injection technique was pursued. The micropipette method introduced hydrocarbon contaminants from opening the injection port, which were also carried over into the following

experiment. These contaminants interfered with the interpretation of the chromatogram; thus, this collection method was not used further to obtain apical droplets for GC/MS analysis.

The SPME and glass fiber sampling techniques demonstrated less contamination carryover and were easily cleaned by baking out overnight in the injection port at 300 °C. The SPME and glass fibers were also more easily inserted and removed from the inlet, when compared to the micropipette method which involved opening up the inlet and introducing contamination. Additionally, the SPME and glass fiber techniques allowed for ramped heated and hot isothermal inlet temperature program, whereas the micropipette technique could only be ramped heated. The hollow glass fiber sampling technique had more carryover than the SPME technique, and may have collected fewer compounds from the apical droplets. Thus, the SPME fiber was the better choice of the three sampling methods.

The adjusted temperature program reduced the broadening of the later eluting peaks as well as the overall time of the experiment. Cryofocusing demonstrated destruction of stationary phase, so further cryofocusing analysis with the DB-5MS column was aborted. Finally, the DB-5MS capillary column gave better separation than the packed column.

# CHAPTER 3
# GC/MS STUDY OF APICAL DROPLETS FROM THE *CULEX QUINQUEFASCIATUS* MOSQUITO

## Introduction

*Cx. quinquefasciatus* is a significant vector for multiple arboviruses. These arboviruses that include, but are not limited to, St. Louis Encephalitis Virus, West Nile Virus, Rift Valley Fever Virus, Ross River Virus, Alfuy, Almpiwar, Corriparta, Sindbis, Japanese Encephalitis, and Reticuloendotheliosis [3,4,48,49]. One strategy to reduce disease transmission by these insects is to selectively target females that are ready to oviposit. This is the rationale behind the development of traps based on the attraction of adult female *Cx. quinquefasciatus* mosquitoes to semiochemicals that are cues that lead the mosquitoes to find sites for oviposition [8].

Semiochemicals are chemical substances that function to convey a message (such as attraction or avoidance) within a species or between different species. These chemical cues may originate from sources such as natural water bodies that contain breakdown products of bacterial origin or can originate from the mosquito itself. Both stimuli result in the oviposition in sites suitable for the survival of the larvae [50]. There are likely a number of undiscovered semiochemicals that contribute to the overall semiochemical-mediated egg-laying, and these may lead to an improved attractant bait that can be employed to direct females to oviposit in a location that is detrimental to their larvae.

In 1979, Bruno and Laurence performed experiments where *Cx. quinquefasciatus* females were offered a choice of oviposition sites [31]. A significant positive correlation was found between oviposition and egg raft densities. This research established that the attraction to the site resulted from the presence of the apical droplet material located

on top of each egg. Aharoni et al. determined the chemical composition of the *Cx. quinquefasciatus* apical droplet and found that the acidic hydrolysate of the droplet contained lauric acid (0.16%), β-OH lauric acid (13%), myristic acid (0.23%), β-OH myristic acid (72%), palmitic acid (9%), and stearic acid (5%) [37]. The remaining 0.6% consisted of unsaturated $C_{12}$ and $C_{14}$ and unknown material. The study involved the dissolution and reflux of the apical droplets in 0.1 N HCl in anhydrous methanol. This would have saponified any diglycerides and other fatty acid esters to free fatty acids, which are then methylated to form fatty acid methyl esters (FAMEs). The subsequent steps were extraction, concentration, and analysis of the methyl esters by gas-liquid chromatography using a packed column with a flame ionization detector (FID). Unfortunately, the chemical alteration of the natural compounds by saponification and methylization to form FAMEs for GC analysis provided limited information on the compounds in the droplets.

In 1982, GC/high resolution mass spectrometry (HRMS), chemical synthesis, and bioassays were employed to confirm that the droplet's main component was the ovipositional pheromone *erythro*-6-acetoxy-5-hexadecanolide [9]. This sample collection process involved washing the entire mosquito egg rafts in hexane and analysis by GC/HRMS using electron ionization (EI) and chemical ionization (CI) modes. The use of capillary GC and HRMS provided greater separation power and mass spectrometric information for elemental composition and structure identification. It was determined that the molecular weight of the active compound was 312.2009 u with major EI ions at *m/z* 142.062, 100.0474, and 99.0432. The ion at *m/z* 252.1992 likely indicated that it is acetylated and thus could be either a δ-lactone or a methyl γ-lactone. Based on the

HRMS results, the authors established that the *m/z* 142 fragment ion contained three oxygen atoms. It was also determined that *m/z* 142 "could only arise in an unusual shift of $CH_3CO$ from a neighboring group onto the lactone ring" [9]. The authors deduced that the molecular formula of this compound was $C_{18}H_{32}O_4$. Finally, it was concluded that "the compound showed no evidence of chain branching, the likely structure was 6-acetoxy-5-hexadecanolide" [9]. When compared, the *threo*-isomer gave more intense ions at *m/z* 269 and 252 than the *erythro*-isomer [9]. Thus, the authors were able to deduce that the pheromone was *erythro*-6-acetoxy-5-hexadecanolide, and that there was approximately 300 ng of this compound per egg raft.

More recent studies using *Aedes aegypti* eggs [51] showed that dodecanoic and (Z)-9-hexadecenoic acids produced significant positive ovipositional responses at different concentrations; whereas all the esters showed deterrent/repellent ovipositional effects, though this analysis was performed after sample collection by solvent wash of the entire raft. One consideration with a solvent wash is that the solution may contain compounds from the egg casings and not just the apical droplets. Additionally, the solvent may mask compounds that co-elute with the solvent, making a thorough investigation of the apical droplet compounds via GC/MS difficult.

The objective of this work is to identify additional compounds and confirm the presence of the known pheromone, *erythro*-6-acetocy-5-hexadecanolde, within the apical droplet through GC/MS using a solventless injection method.

## Experimental

### Mosquito Rearing

Mosquitoes were reared in insectaries in which light (10 hours of light per day), temperature (25 °C), and humidity (60% relative humidity) were controlled. The

mosquitoes were fed citrated avian blood once a week, defibrillated bovine blood three times a week, and there was a constant sugar source from cotton balls soaked in a 5% aqueous sucrose solution. The caged mosquitoes were offered an oviposition pan once a week overnight. The oviposition pan contained water with decaying organic matter from Bermuda grass. Egg rafts were collected after having access to the pan overnight.

**Sample Preparation**

Apical droplets were collected from a single raft to minimize sample bias. The apical droplets were obtained by touching a polydimethylsiloxane (PDMS) solid phase microextraction (SPME) fiber to apical droplets and adsorbing them onto the fiber.

**Gas Chromatograph**

Samples were introduced into the Trace GC Ultra (Thermo) by inserting SPME fiber into the GC inlet and leaving it there for the duration of the experiment. The injection port temperature was isothermal at 260 °C. A 30 m x 0.25 mm O.D. x 0.25 µm film thickness DB-5MS column was used for peak separation. The GC oven temperature program consisted of an initial hold at 35 °C for one min, then a ramp to 260 °C at 5 °C/min, and final hold at 260 °C for five min.

**Mass Spectrometer**

The mass spectrometer was a DSQ II (Thermo) single quadrupole system. The MS transfer line was set to 260 °C, and the ion source was set to 200 °C. Data acquisition started 0.25 s after injection. The system was operated in full scan mode with a scan rate of 2,000 u per s, over a mass range of 50 to 650 u. For EI, the detector gain was set at $3.00 \times 10^5$ with a multiplier voltage of 1,325 V. For CI, the reagent gas was either isobutane delivered at a rate of 1.0 mL/minute, or methane delivered at 2.5 mL/min.

## Compound Analysis

Multiple GC/MS analyses were conducted on the droplets. For some of the experiments, EI mode was used to obtain fragmentation information. Experiments were also conducted using identical parameters but with isobutane or methane as the reagent gas for CI to acquire molecular weight information.

# Results and Discussion

## Apical Droplet Profile

Figure 3-1 shows a chromatogram of ten *Cx. quinquefasciatus* apical droplets thermally desorbed from a PDMS SPME fiber. A small peak was observed around 20 minutes, but no analysis on this peak was performed because it was a trace level compound for which adequate fragmentation information could not be obtained. No peaks were observed after 48 minutes.

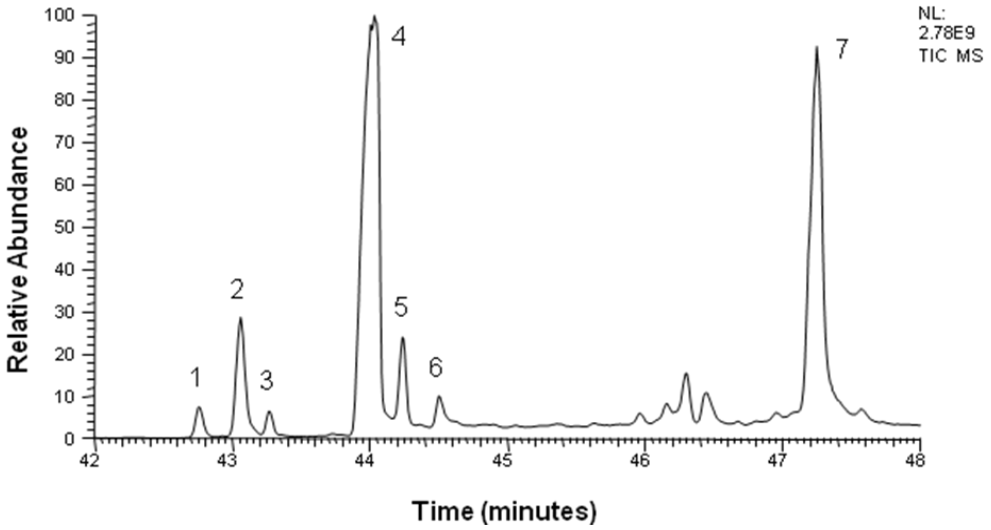



Figure 3-1. GC/(+)EI-MS total ion chromatogram (*m/z* 50-650) of *Cx. quinquefasciatus* apical droplets from 42 to 48 minutes

The peak numbers indicate those peaks for which compound identities have been confirmed or are known tentatively. In the chromatograms, there were produced as many as twelve detectable peaks.

**Mosquito Pheromone**

Laurence et al. identified the mosquito oviposition pheromone as *erythro*-6-acetoxy-5-hexadecanolide [9]. The major fragment ions they reported in the EI spectrum of this compound (MW 312.2009, $C_{18}H_{32}0_4$) are as follows:

Table 3-1. Major ions of *erythro*-6-acetoxy-5-hexadecanolide [9]

| *m/z* | Relative Abundance (%) | Molecular Formula |
|---|---|---|
| 312.2009 | 0.1 | $C_{18}H_{32}O_4$ |
| 252.1992 | 3.3 | $C_{16}H_{28}O_2$ |
| 142.0621 | 35.9 | $C_7H_{18}O_3$ |
| 100.0474 | 49.4 | $C_5H_8O_2$ |
| 99.0432 | 100 | $C_5H_7O_2$ |

The suggested fragmentation pathways to produce these ions are:

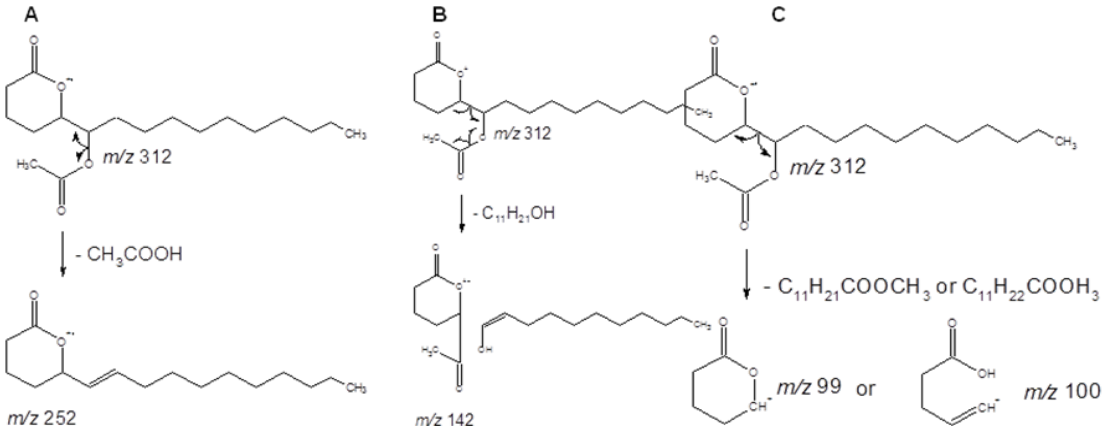



Figure 3-2. Suggested fragmentation [9] of the $M^+$ ion of *erythro*-6-acetoxyl-5-hexadecanolide to form (A) *m/z* 252, (B) *m/z* 142, and (C) *m/z* 100 or 99

The *m/z* 142 fragment may have been produced from cleavage of the carbonyl on the acetate group or the β-carbon. To verify this, the experiment would have to be repeated with compounds that are labeled with a different isotope, such as $O^{18}$ labeling.

The EI fragmentation pattern reported by Laurence et al.[9] closely matches that of Peak 4 (Figure 3-3A). The methane CI spectrum (Figure 3-3B) shows an abundant $[M+H]^+$ ion at *m/z* 313. The $[M+H]^+$ ion undergoes a neutral loss of acetic acid to form *m/z* 253 and sequential losses of water to produce fragment ions at *m/z* 235 and 217. The isobutane CI spectrum of Peak 4 (Figure 3-3C) also contains an abundant $[M+H]^+$ ion with low intensity fragment ions at *m/z* 235 and 99. Both the EI and the CI spectra of Peak 4 matched those of the commercially available mosquito pheromone (Bedoukian; Danbury, CT). These results were obtained using identical GC/MS parameters, and as a consequence, the retention times were nearly identical (Figure 3-4). Thus, Peak 4 is confirmed to be the previously identified mosquito oviposition pheromone, 6-acetoxy-5-hexadecanolide

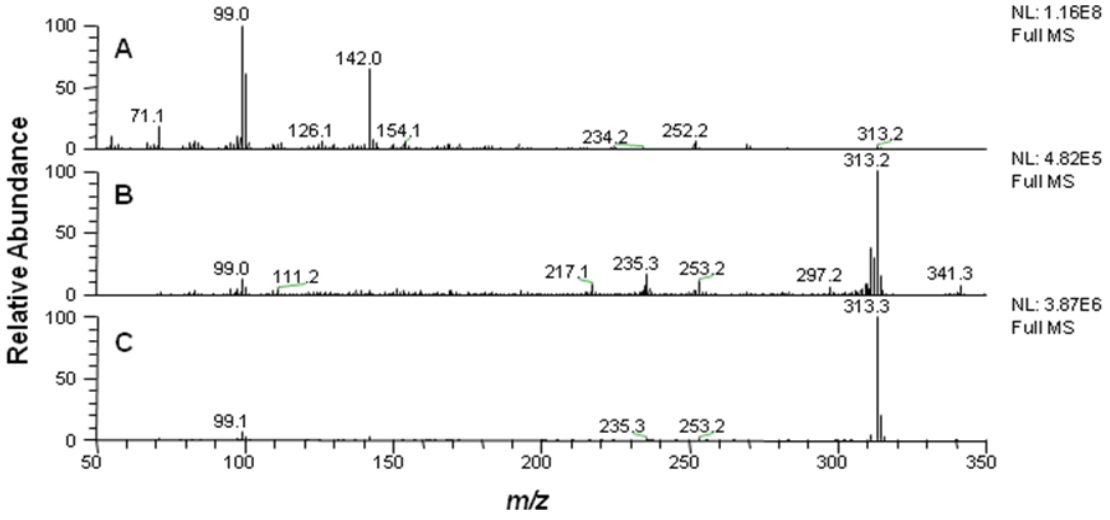



Figure 3-3. GC/MS mass spectra of Peak 4 in (A) GC/(+)EI-MS mode, (B) GC/(+)CI-MS (methane) mode, and (C) GC/(+)CI-MS (isobutane) mode

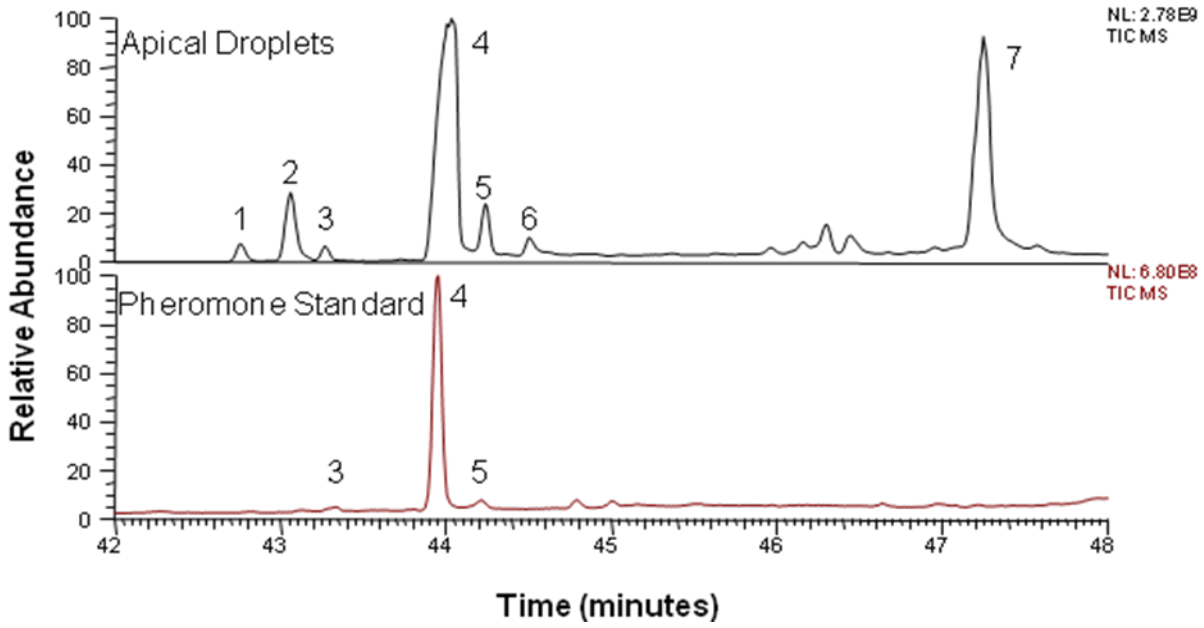



Figure 3-4. GC/(+)EI-MS total ion chromatograms of Peak 4 and standard mosquito ovipositioning pheromone, 6-acetoxy-5-hexadecanolide

Peaks 3 and 5 produce EI and CI spectra that are similar to those of Peak 4 (Figure 3-5).

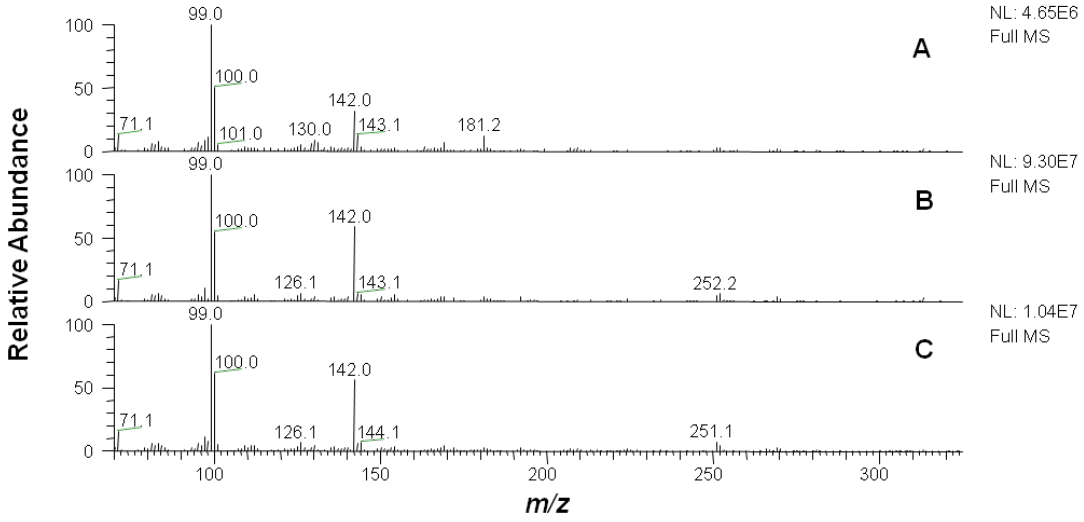



Figure 3-5. GC/(+)EI-MS mass spectra (A) Peak 3, (B) Peak 4, and (C) Peak 5; GC/(+)CI-MS (isobutane) mass spectra of (D) Peak 3, (E) Peak 4, and (F) Peak 5

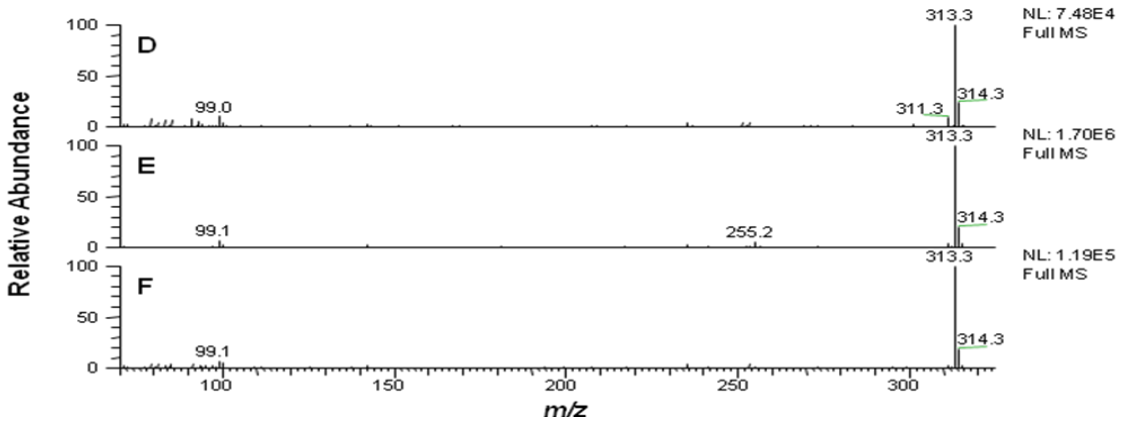



Figure 3-5. Continued

There are three possibilities as to why similar fragmentation occurs for different peaks. The first is that these compounds may be isomers of each other. The mass spectra of positional isomers are often difficult to distinguish because of their similar fragmentation patterns. Often, standards are needed to confirm the appropriate isomer. The second possibility is that the compounds may have different molecular weights but a common structural arrangement. The final possibility is that the compounds are fragments from higher molecular weight compounds. Higher molecular weight compounds may fragment easily and not produce a detectable molecular ion under EI conditions. This may happen from thermal decomposition in the hot ion source; i.e., a compound may lose a thermally-labile group prior to ionization. Isobutane CI generally results in less fragmentation of compounds than methane CI. When isobutane CI was performed, there were base peaks at *m/z* 313 for all three peaks (Peaks 3, 4, and 5), with no significant ions at higher *m/z*. This suggests that Peaks 3 and 5 are isomers of the more abundant Peak 4. Note that Peaks 3 and 5 are observed in the GC/MS spectrum of the pheromone standard at the same retention times as Peaks 3 and 5 (Figure 3-4) in the apical droplet sample, though their intensities in the standard are

lower. The EI spectra of Peaks 3 and 4 also match those of the apical droplets (Figure 3-4A). The *threo* isomer was determined by Laurence et al. to have a slightly longer retention time than the *erythro* isomer [9].

Peaks 1 and 2 produce EI fragmentation patterns similar to that of Peak 4 (the known pheromone) (Figure 3-6).

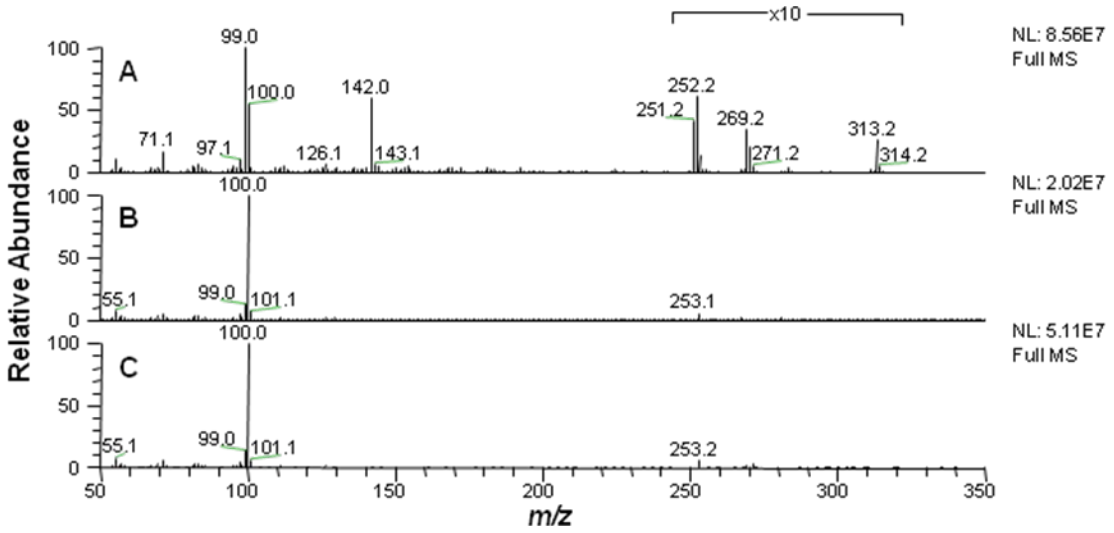



Figure 3-6. GC/(+)EI-MS mass spectra of (A) Peak 4, (B) Peak1, and (C) Peak 2

However, there are noticeable absences of some previously detected fragment ions. The absence of *m/z* 142 in Peaks 1 and 2 indicates that these two compounds are structurally dissimilar from the pheromone (Peak 4). Though these three compounds have similar fragmentation in the lower *m/z* region, the intensity of the *m/z* 99 and 100 in Peaks 1 and 2 differs; thus, confirming that these are unique compounds from the pheromone that is found in Peak 4.

The difference was also observable in the isobutane CI mass spectra (Figure 3-7).

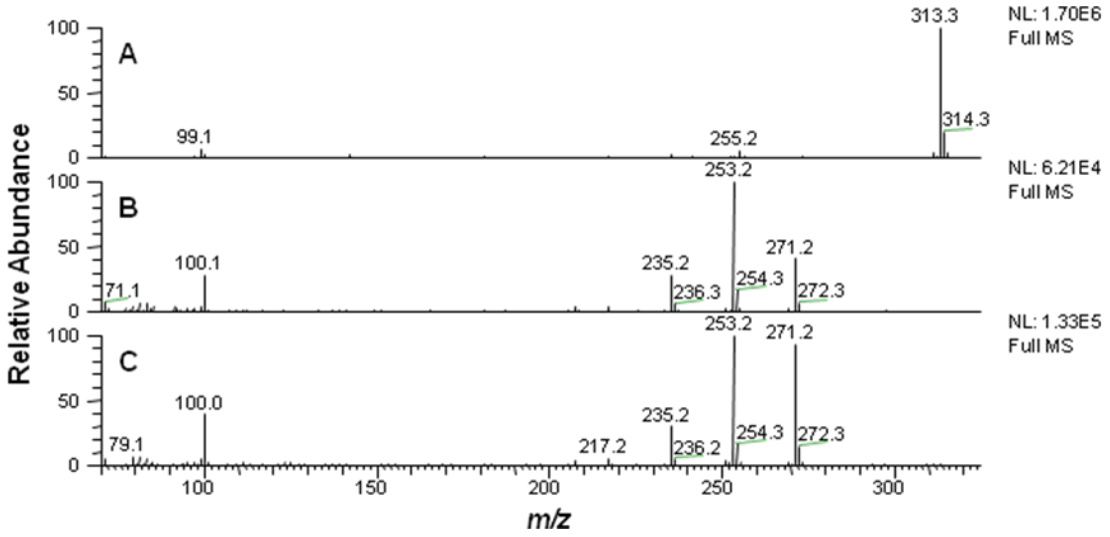



Figure 3-7. GC/(+)CI-MS (isobutane) mass spectra of (A) Peak 4, (B) Peak 1, and (C) Peak 2

During isobutane CI, alcohols may not produce an $[M+H]^+$ ion; instead an $[M+H-OH]^+$ ion will be observed due to OH abstraction by $C_4H_9^+$ [52]. This would mean that the molecular weight of the compounds that produced both Peaks 1 and 2 is 270 u. Two neutral losses of $H_2O$ from the $[M+H]^+$ ion (*m/z* 271 to *m/z* 253 and *m/z* 253 to *m/z* 235) imply that there are two hydroxyl groups in this compound. The *m/z* 100 ion leads to the conclusion that the base structure of this compound is similar to the pheromone. Laurence et al. synthesized a MW 270 compound (Figure 3-8) by hydrolysis of the droplet extract, which had a retention time earlier than that of the pheromone and an EI mass spectrum similar to that of Peaks 1 and 2. It is believed that the compound is formed by the conversion of the acetoxy group to a hydroxyl group (loss of 42 u).

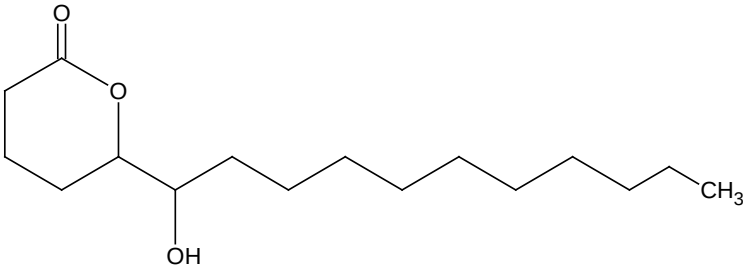



Figure 3-8. Suggested structure of Peaks 1 and 2 compound

Figure 3-8 is thought to be the general structure of Peaks 1 and 2. Various isomers are possible, and the lactone ring could “open” with the hydroxyl group connected to a keto group (Figure3-9).

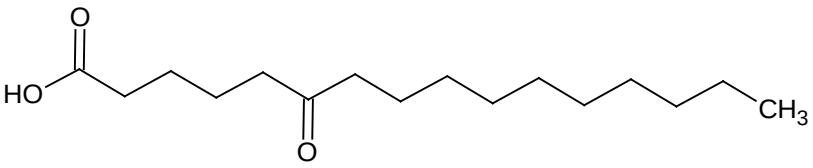



Figure 3-9. Suggested isomer of Peaks 1 and 2 compound

**Peaks 6 and 7**

The EI mass spectra of Peaks 6 and 7 contain fragment ions similar to each other (Figure 3-10).

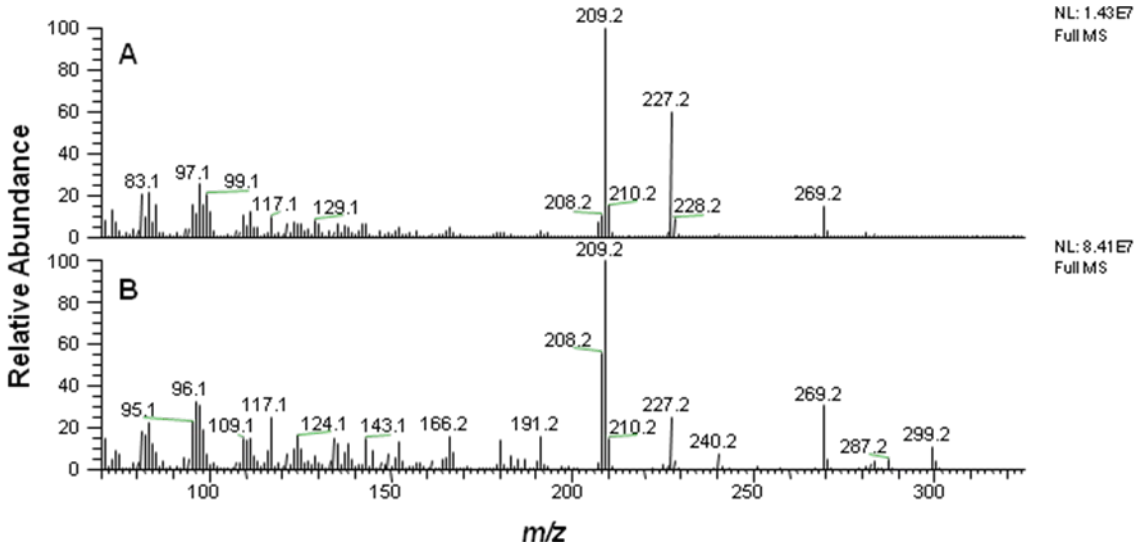



Figure 3-10. GC/(+)EI-MS mass spectra of (A) Peak 6 and (B) Peak 7

Peaks 6 and 7 also have CI isobutane spectra similar to each other, though ion intensities vary and Peak 7 has an *m/z* 343 which Peak 6 does not (Figure 3-11).

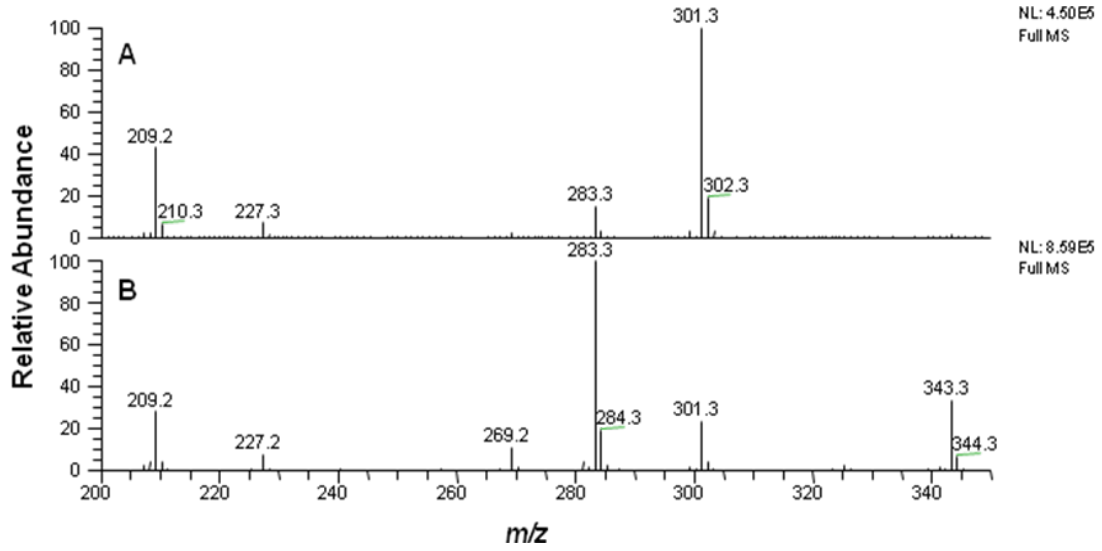



Figure 3-11. GC/(+)CI-MS (isobutane) mass spectra of (A) Peak 6 and (B) Peak 7

Based on the CI mass spectrum of Peak 6, the molecular weight is thought to be 300 u. The $[M+H]^+$ ion at *m/z* 301 loses water to form an ion at *m/z* 283. Peak 7 has a possible molecular weight of 342 u; the $[M+H]^+$ ion at *m/z* 343 loses 60 u ($CH_3COOH$), and therefore is probably an acetylated version of the compound that is found in Peak 6. The *m/z* 343 and 283 ions are discussed later in Chapter 4.

**Fatty Acids**

Aahroni et al. reported the presence of fatty acids, specifically lauric acid, β-OH lauric acid, myristic acid, β-OH mystic acid, palmitic acid, stearic acid in the acidic hydrolysate of the apical droplets [37]. They dissolved and refluxed the apical droplets in O.1 N HCl in anhydrous methanol. This would have saponified, hydrolyzed, and methylated any diglycerides and other fatty acid esters into FAMEs. It would have also methylated any free fatty acids naturally occurring in the apical droplet.

To determine if the apical droplet contained any free fatty acids, *Cx. quinquefasciatus* apical droplets were collected and derivatized on a PDMS SPME fiber

using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [53, 54]. However, derivatization and subsequent analysis did not provide evidence of free fatty acids being present in the apical droplet.

Previous studies revealed that the apical droplet's active fraction contained1,3-diglycerides that are composed of mono- and dihydroxy fatty acids [35]. The major monohydroxy acids were 3-hydroxytetradecanoic acid, 3-hydroxyhexadecanoic acid, and 3-hydroxyoctadec-*cis*-II-enoic acid. The major dihydroxy- acid was *erythro*-5,6-dihydroxyhexadecanoic acid. The acid hydrolysis performed by Aahroni et al. would have caused the diglyceride bonds to cleave and the resulting fatty acids to become FAMEs. Chapter 4 proposes structures for the 1,3-diglycerides, and if these structures had undergone same reactions of the method of Aahroni et al., the resulting compounds would have produced lauric acid, β-OH lauric acid, myristic acid, β-OH mystic acid, and palmitic acid, as they observed.

## Conclusions

This GC/MS study has resulted in the tentative identification of six new compounds from the *Cx. quinquefasciatus* apical droplet. It has also confirmed the presence of the known pheromone, *erythro*-6-acetoxy-5-hexadecanolide. The avoidance of solvent masking and interference coupled with better sensitivity, has likely allowed for the discovery previously unreported, similarly structured compounds. Table 3-1 lists the characteristics of Peaks 1-7 found in *Cx. quinquefasciatus* apical droplets via GC/MS analysis.

Table 3-1. Established characteristics of Peaks 1-7

| Characteristic | Peak 1 | Peak 2 | Peak 3 | Peak 4 | Peak 5 | Peak 6 | Peak 7 |
|---|---|---|---|---|---|---|---|
| Retention time (minutes) | 42.67-42.85 | 42.98-43.20 | 43.22-43.33 | 43.85-44.14 | 44.17-44.29 | 44.45-44.57 | 47.13-47.52 |
| Molecular Weight | 252 | 252 | 312 | 312 | 312 | 300 | 342 |
| Elemental Composition | $C_{16}H_{28}O_2$ | $C_{16}H_{28}O_2$ | $C_{18}H_{32}O_4$ | $C_{18}H_{32}O_4$ | $C_{18}H_{32}O_4$ | TBD | TBD |

Peak 4 was confirmed to be the known pheromone through standard comparison. Peaks 3 and 5 displayed EI and CI fragmentation patterns similar to the pheromone. These are believed to be isomers of the pheromone. Peaks 1 and 2 produced similar EI and CI spectra, and presumably are deacetylated versions of Peaks 3, 4, and 5. Peaks 6 and 7 had similar EI spectra, but their isobutane CI spectra implicated Compound 7 as the acetylated version of Compound 6, which is believed to be hydroxylated. These new compounds may aid in exploiting control of this species and entomological monitoring the vector populations.

# CHAPTER 4
# LC/MS$^N$ STUDY OF APICAL DROPLETS FROM THE *CULEX QUINQUEFASCIATUS* MOSQUITO

## Introduction

The *Culex pipiens quinquefasciatus* (*Cx. quinquefasciatus)* mosquito is one of the most common and widespread members of the *Culex pipiens* (*Cx. pipiens*) complex [1]. Worldwide, *Cx. quinquefasciatus* is an important vector of many prominent arboviruses. Pathogen infection requires the taking of at least one blood meal by a female mosquito vector for egg production. Thus, "disease transmission typically necessitates the completion of at least one egg-laying (oviposition) cycle before pathogen transfer can occur with a successive blood meal" [7]. Oviposition is, therefore, an important aspect of mosquito behavior that can be used to manage mosquito-borne diseases.

In 1979, Bruno et al. studied *Cx. pipiens fatigans* (*Cx. quinquefasciatus*) females which were offered a choice of oviposition sites [31]. The authors observed a large number of egg-carrying females oviposition in water that contained *Cx. quinquefasciatus* egg rafts at various densities. This response was hypothesized to result from the presence of the apical droplet on top of each egg. In understanding the chemical make-up of the apical droplet, one may be able to manipulate a mosquito's oviposition behavior and control disease transmission.

One method for analyzing the *Cx. quinquefasciatus* apical droplet involves LC/MS. Although this technique is a powerful method for organic analysis, very little has been reported on its use in the study of mosquitoes. In 2010, various LC/MS experiments were performed on egg casings of *Aedes aegypti* mosquitoes [55-57]. The authors found 44 proteins in the eggshell along with seven odorant binding proteins. Glucosamine was also identified within the *Aedes aegypti* eggshells, eggs, and ovaries. As for *Cx.*

*quinquefasciatus*, LC/MS analysis has been performed only on its odorant-binding protein [4], with no reported work on the *Cx. quinquefasciatus* apical droplet using LC/MS.

The purpose of this work is to provide greater insight into the chemical compounds within the *Cx. quinquefasciatus* apical droplet using LC/MS. The neat apical droplets from freshly laid *Cx. quinquefasciatus* egg rafts were analyzed using LC/ESI/MS. Neat collection allows for direct analysis of only the apical droplet and not the egg casing; LC separates and ESI ionizes the major organic compounds within the apical droplet. Discovery of new biologically active oviposition compounds may lead to methods for improved surveillance and control.

## Experimental

### Sample Acquisition

In order to lay eggs, *Cx*. *quinquefasciatus* female mosquitoes must acquire blood [7]. In this experiment, mosquitoes were bloodfed once a week and offered an egg-laying pan five days after being fed. Eggs are laid in rafts consisting of eggs bound together, with a typical raft consisting of 200 eggs. The egg rafts were collected twelve hours after being laid. The apical droplets were accumulated from each raft into a single micro pipette to give a typical volume of 5 µL. All apical droplets used in a single LC/MS analysis were collected from a single raft. A micropipette collection method was used to remove the apical droplets from the egg rafts. This method involved preparing tapered capillary collection tubes by heating and pulling the tubes (World Precision Instruments, Sarasota, FL) using a PUL-1 (World Precision Instruments) micropipette puller. The final dimensions of the micropipettes were 66 mm long with a tip being 50 µm O.D., and 7.5 µm I.D. The laying pan was placed under a microscope, and the apical droplets

removed through capillary action by touching the tip of the tapered capillary collection tube to the droplets.

**Sample Preparation**

The micropipette tips containing the apical droplets were broken off and sealed in glass GC 2 mL vials (Supelco, 29084-U). The vials were stored at -20 °C prior to analysis, at which time the vials were removed from the freezer 30 minutes prior to analysis. Fifteen minutes prior to analysis, 20 µL of water or isopropanol (LC-grade, Fisher) and 20 µL of methanol (LC-grade, Honeywell Burdick & Jackson, Muskegon, MI) were added to the vials containing the micropipette tips. The vials were swirled and finger vortexed.

**LC/MS**

The vials containing apical droplet samples were analyzed by LC/MS. The LC used for compound separation was an Agilent Technologies (Santa Clara, CA) 1100 series G1312A binary pump with a ThermoFisher Scientific (Waltham, MA) Hypurity C8 column (5µm particle diameter; 2.1 x 100 mm long + guard column). The mobile phase consisted of Solvent A (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in water (LC-grade, Honeywell Burdick & Jackson)), and Solvent B (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in isopropanol (LC-grade; Honeywell Burdick & Jackson)). The flow rate was 0.18 mL/min; the gradient varied. The UV/Vis detector was an Agilent 1100 G1314A UV/Vis detector set to 205 - 220 nm.

The mass spectrometer was a ThermoFinnigan (San Jose, CA) LCQ quadrupole ion trap mass spectrometer with electrospray ionization (ESI). The ESI sheath gas and auxiliary gas were $N_2$ at instrument settings of 65 and 5 (no units), respectively. The heated capillary temperature was 250 °C. The positive ESI [(+)ESI] spray voltage was

+3.3 kV, a heated capillary voltage of +12.5 V. The negative ESI [(-)ESI] spray voltage was -3.2 kV, a heated capillary voltage of -10 V.

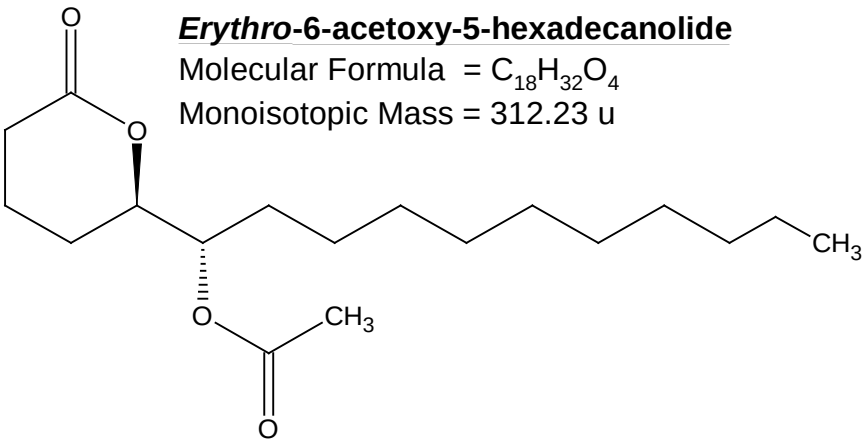
Initial LC/$MS^n$ analyses were performed in (+)ESI and (-)ESI modes; 120 minutes per run. Data-dependent $MS^n$ scans (5-6 u) were centered on the *m/z* of the precursor ion. Collision-induced dissociation (CID) was performed at 37.5% max CID energy with a qCID of 0.25 for 30 ms.

**LC/HRMS**

Samples were separated by LC using the same column as described above. The mobile phase consisted of Solvent A (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in water (LC-grade, Honeywell Burdick & Jackson, Muskegon, MI)), and Solvent B (2.0 mM ammonium acetate in methanol, (LC-grade; Honeywell Burdick & Jackson, Muskegon, MI)). The flow rate was 0.18 mL/min; the gradient was [A:B(min)]: 95:5(0):35:65(20)>15:85(65)>5:95(100-120). After LC separation, samples were introduced into a Agilent 6210 TOF-MS by ESI. Experiments were performed by Yaoling Long.

## Results and Discussion

With (+)ESI-MS and in the presence of ammonium acetate, most ester-containing compounds form $[M+NH_4]^+$ ions in addition to the commonly observed $[M+Na]^+$ ions (due to $Na^+$ ions in solution). The mass difference between these two ions is 5 u (atomic mass units), and can aid in determining the compound's molecular weight (the molecular weight being $[M + NH_4]^+$ - 18 or $[M+Na]^+$ - 23). Figure 4-1 illustrates the structure of the published *Cx. quinquefasciatus* active oviposition pheromone [9], *erythro*-6-acetoxy-5-hexadecanolide.




***Erythro*-6-acetoxy-5-hexadecanolide**
Molecular Formula = $C_{18}H_{32}O_4$
Monoisotopic Mass = 312.23 u

Figure 4-1. *Erythro*-6-acetoxy-5-hexadecanolide

*Erythro*-6-acetoxy-5-hexadecanolide contains two ester groups and will most likely display an $[M+NH_4]^+$ ion at *m/z* 330 and/or an $[M+Na]^+$ ion at *m/z* 335 rather than an $[M+H]^+$ ion at m/z 313 since it lacks any basic functional groups that would be protonated in solution. MS/MS spectra of *m/z* 335 resulted in loss of 60 u. Once the "characteristic" ionization and MS/MS or $MS^n$ dissociation are established based upon a known standard, one can use these to assist in the determination of unknowns.

Normal (+)ESI mass spectra were obtained over four different mass ranges in order to increase sensitivity for the ions in each range and the number of data-dependent $MS^n$ scans: *m/z* 85 to 200, *m/z* 190 to 700, *m/z* 690 to 1,000, and *m/z* 980 to 2,000. There were no significant apical droplet-related compounds detected with the two extreme mass ranges (*m/z* 85-200 and *m/z* 980-2000). However, numerous droplet-related compounds were detected in the other two *m/z* ranges.

Figure 4-2 illustrates that the chemical profiles from *m/z* 190 to 700 collected from multiple rafts under the same conditions were similar.

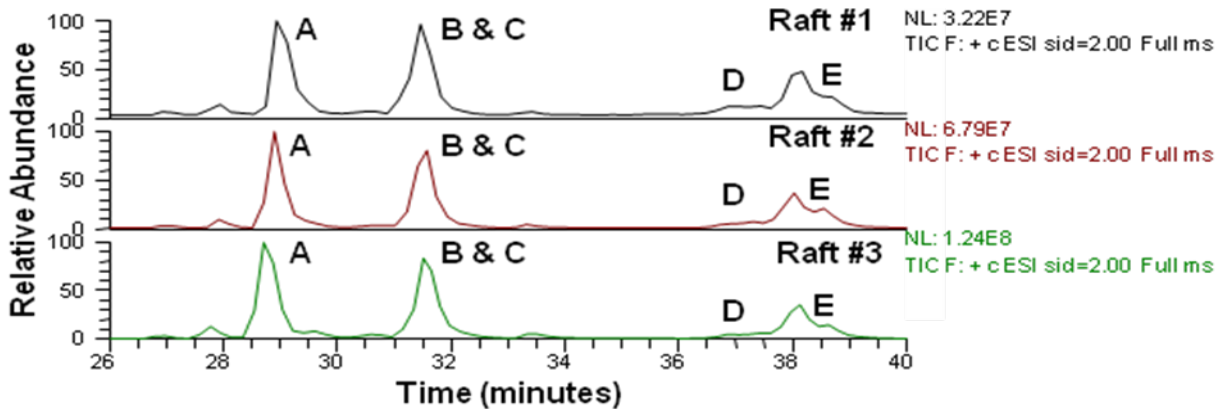



Figure 4-2. LC/(+)ESI-MS (*m/z* 190-700) base peak chromatograms for Rafts #1, 2, and 3

However, the effect(s) of various parameters (temperature, age, season, etc.) still needs to be explored.

**Peak A**

As illustrated in Figure 4-2, there were five major peaks, with Peaks B and C co-eluting, within the retention time 26-40 minute window of the (+)ESI-MS (*m/z* 190-700) base peak chromatograms. The first peak, Peak A, corresponded to a compound which produced *m/z* 390 $[M+NH_4]^+$ and *m/z* 395 $[M+Na]^+$ ions, indicative of a 372 u molecular weight (Figure 4-3). HRMS of these ions resulted in a monoisotopic mass of 372.2512, which best matched a molecular formula of $C_{20}H_{36}O_6$; an increase of 60 u and $C_2H_4O_2$ over the published pheromone (Figure 4-1).

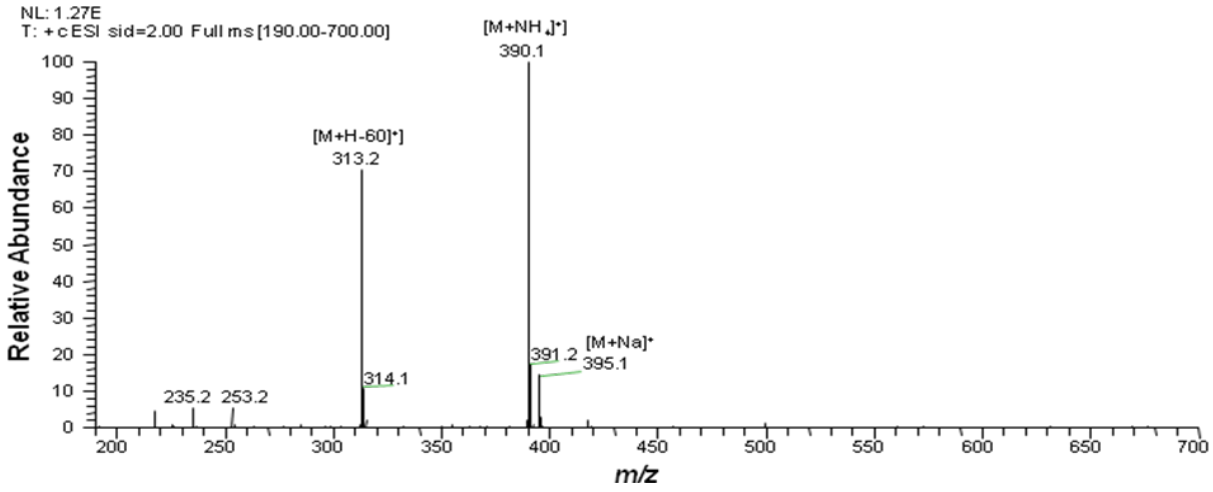



Figure 4-3. (+)ESI ion chromatogram of low mass spectrum of Peak A (see Figure 4-2)

Peak A, with MW 372, produced only very low abundances of the *m/z* 373 $[M+H]^+$ ion; this was likely due to the low proton affinity of the compound in solution or due to the ready loss of 60 u from any formed $[M+H]^+$ ion to form the *m/z* 313 fragment ion. The $MS^2$ collision-induced dissociation (CID) of the *m/z* 390 $[M+NH_4]^+$ ion resulted in an *m/z* 313 product ion via loss of $NH_3$ and 60 u. Further CID of the *m/z* 313 primary product ion produced *m/z* 253 (a second loss of 60 u), 235, and 217 product ions (Figure 4-4A). The presence of these same ions in the normal mass spectrum indicates that these are likely fragment ions of this compound and not some other compound. The (+)ESI-$MS^2$ CID of *m/z* 313 $[M+H]^+$ ion of the mosquito pheromone standard gave rise to very similar product ions (Figure 4-4B).

.

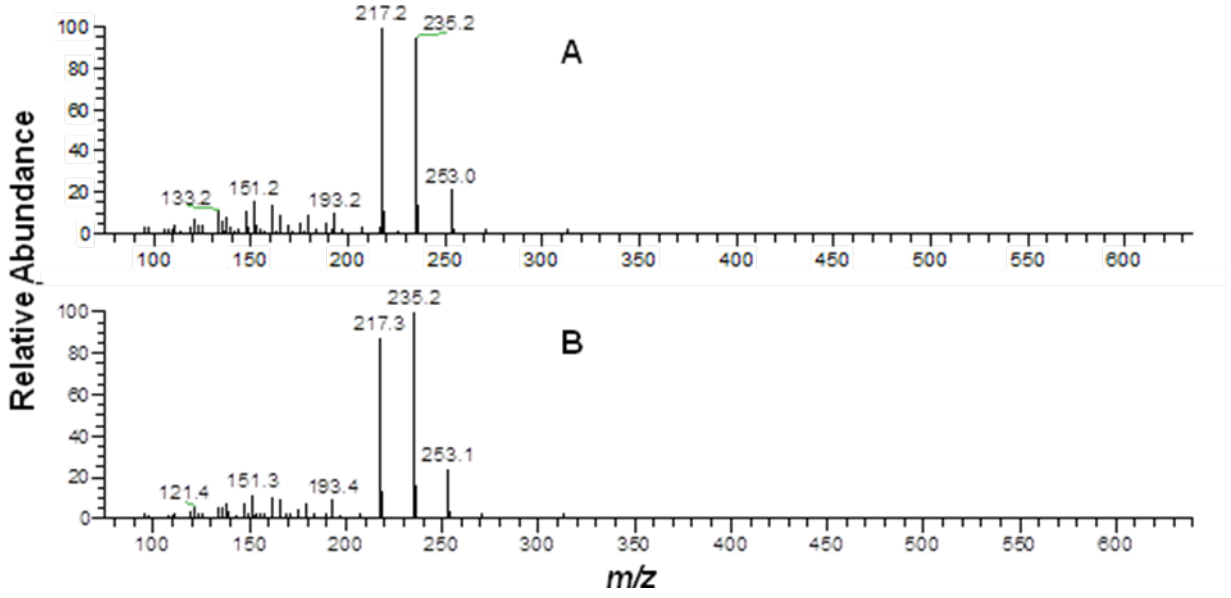



Figure 4-4. (+)ESI-MS$^3$ product ion spectrum of the *m/z* 313 product ion of *m/z* 390 (A) and (+)ESI-MS$^2$ product ion spectrum of *m/z* 313 $[M+H]^+$ of 6-acetoxy-5-hexadecanolide (B)

The similarities between Figure 4-4A and 4B suggest that the *m/z* 313 product ion of 390 (Figure 4-3) has a similar structure to that of the *m/z* 313 $[M+H]^+$ ion of the mosquito pheromone, 6-acetoxy-5-hexadecanolide.

The HRMS data indicated the *m/z* 313 fragment and product ions associated with Peak A were due to loss of $C_2H_4O_2$ (60 u) from the *m/z* 373 $[M+H]^+$ ion and from the loss of 77 u from the *m/z* 390 $[M+NH_4]^+$ via a combined loss of ammonia and $C_2H_4O_2$. Due to the similarity of the product spectra of Figure 4-4, the $C_2H_4O_2$ is likely due to loss of acetic acid ($CH_3COOH$) from an acetoxy group. The loss of another 60 u from *m/z* 313 to form *m/z* 253 was also due to loss of $C_2H_4O_2$, indicating a second acetoxy group. The HRMS spectrum for Peak A also had ions at 217.1943, 235.2055, and 253.2164 with their corresponding elemental compositions being $C_{16}H_{25}$, $C_{16}H_{27}O$, and $C_{16}H_{29}O_2$, respectively. Below is a table of observed HRMS ions, their experimental and accurate

masses, mass errors, and their suggested elemental composition for Peak A (Table 4-1).

Table 4-1. Peak A's HRMS major ions

| Observed Ions (m/z) | Experimental Mass (u) | Accurate Mass (u) | Mass Error (ppm) | Elemental Composition |
|---|---|---|---|---|
| $[M+Na]^+$ | 395.2411 | 395.2404 | +2 | $C_{20}H_{36}NaO_6$ |
| $[M+NH_4]^+$ | 390.2850 | 390.2863 | -3 | $C_{20}H_{40}NO_6$ |
| $[M+H-CH_3COOH]^+$ | 313.2378 | 313.2373 | +2 | $C_{18}H_{33}O_4$ |
| $[M+H-2CH_3COOH]^+$ | 253.2164 | 253.2162 | +1 | $C_{16}H_{29}O_2$ |
| $[M+H-CH_3COOH-H_2O]^+$ | 235.2055 | 235.2056 | 0 | $C_{16}H_{27}O$ |

Peak A also produced an *m/z* 417 $[(M-H+Na)+Na]^+$ ion in (+)ESI-MS and a strong *m/z* 371 $[M-H]^-$ ion in (-)ESI-MS. This indicates the presence of an exchangeable proton. These data suggest that the lactone moiety of Peak A, 6-acetoxy-5-hexadecaonolide, "opened" to form a free carboxylic acid, and the resulting hydroxyl group was acetylated. (Figure 4-5).

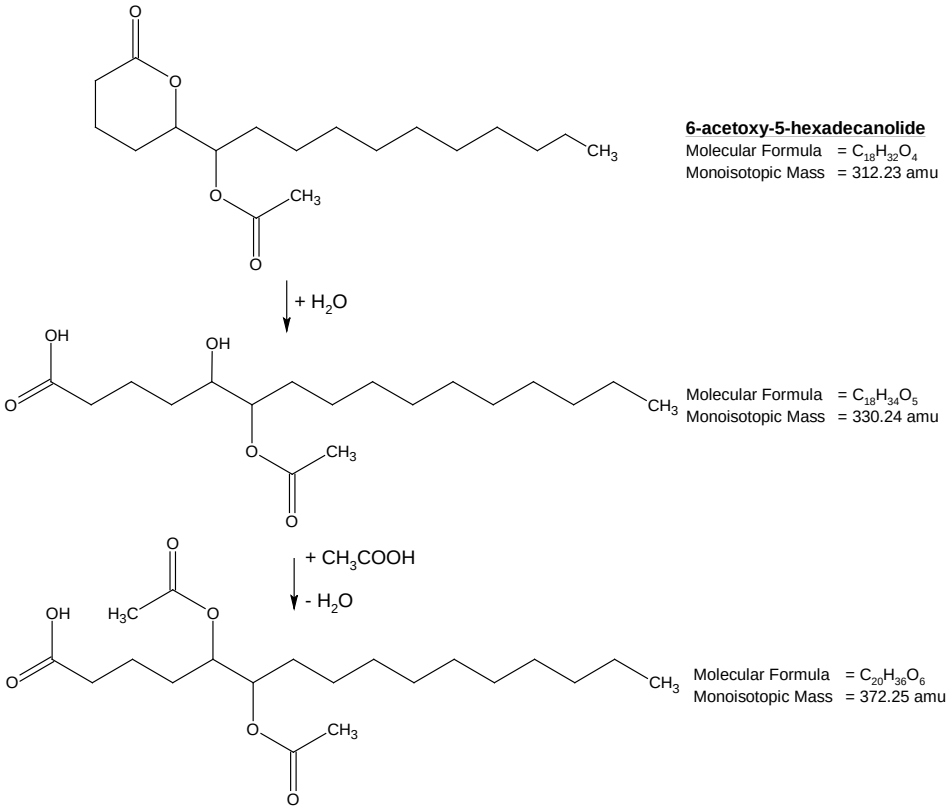



Figure 4-5. Suggested formation of Peak A

With two acetoxy groups on the compound within Peak A, there are two possible losses of acetic acid. Figure 4-6 illustrates how Peak A may have fragmented.

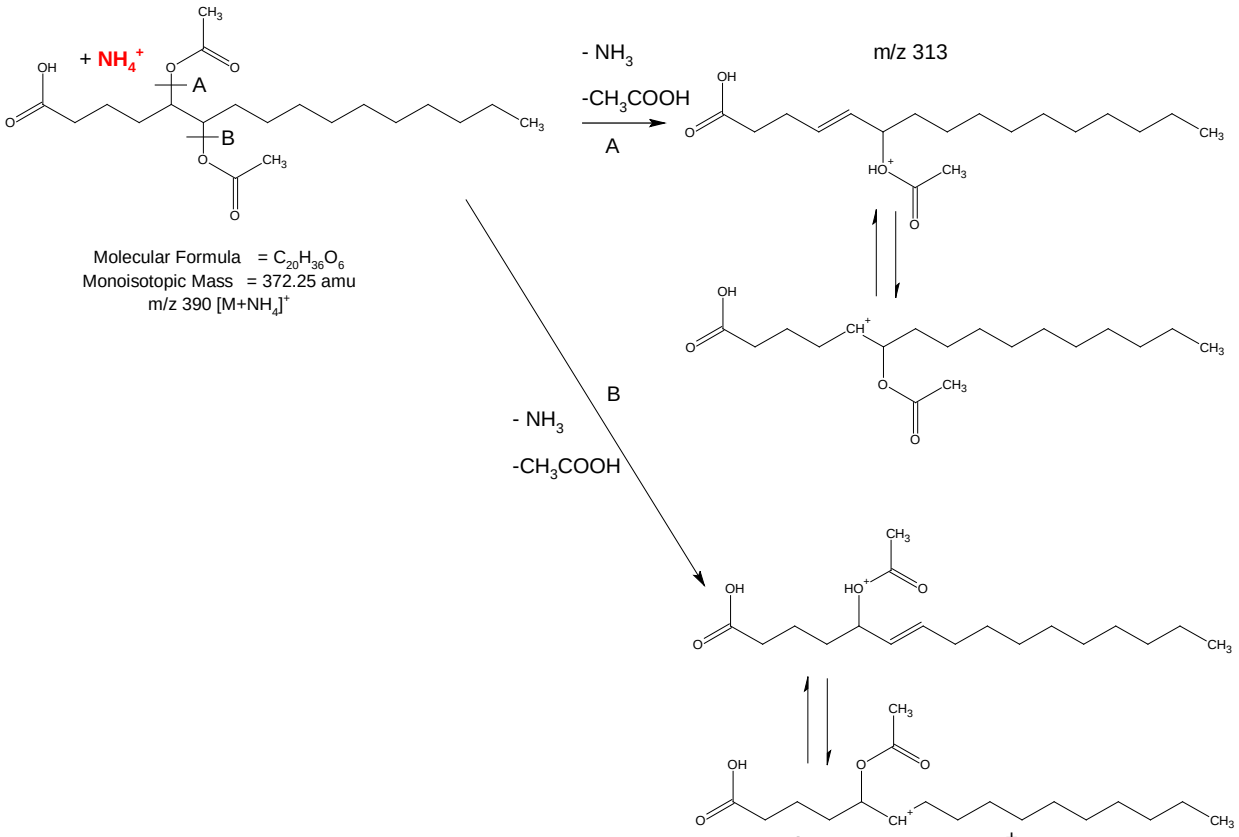



Figure 4-6. Suggested (+)ESI-MS dissociation of the *m/z* 390 $[M+NH_4]^+$ ion of Peak A to form *m/z* 313 product ions

The (+)ESI of Peak A also formed *m/z* 789 $[2M-H+2Na]^+$ ions and *m/z* 811 $[2M-H+3Na]^+$ ions (Figure 4-7). (+)ESI-$MS^2$ CID of *m/z* 789 and *m/z* 811 produced *m/z* 417 $[M-H+2Na]^+$ product ions. This indicates that Peak A is capable of forming dimers when ionized under +ESI conditions.

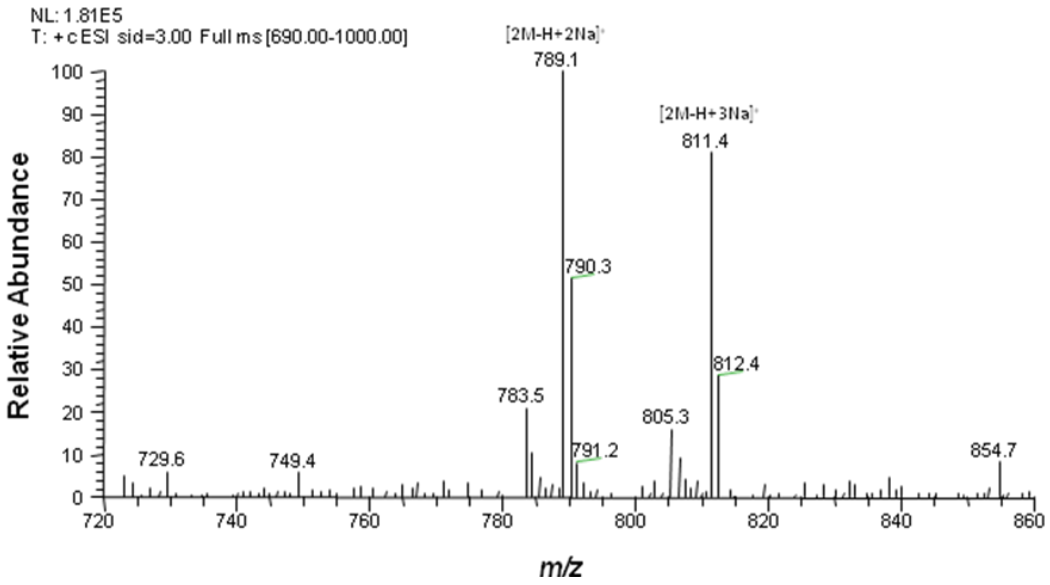



Figure 4-7. (+)ESI high mass spectrum of Peak A

**Peaks B and C**

The mass chromatograms of the major ions of Peaks B and C show that there are two compounds present (B and C) (Figure 4-2).

The earlier eluting compound (Peak B) produced *m/z* 313 $[M+H]^+$ and *m/z* 330 $[M+NH_4]^+$ ions (Figure 4-8); thus, Peak B has a molecular weight of 312 u. These ions and their relative abundances and retention time match those of the standard, 6-acetoxy-5-hexadecanolide (Figure 4-1). Thus, B is most likely the mosquito oviposition pheromone.

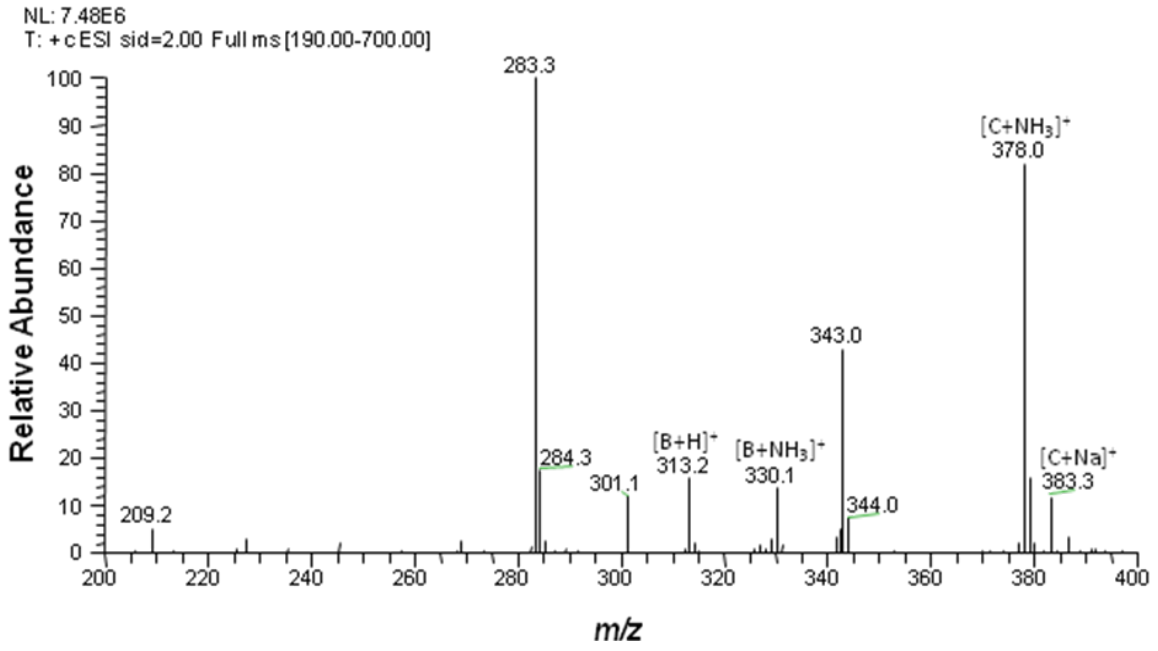



Figure 4-8. (+)ESI mass spectrum of Peaks B and C

The second peak (Peak C), co-eluting with Peak B, had *m/z* 378 $[M+NH_4]^+$ and *m/z* 383 $[M+Na]^+$ ions (Figure 4-8). HRMS of these ions indicated a molecular weight of 360.2512 and a molecular formula of $C_{19}H_{36}O_6$. Peaks B and C were incompletely separated chromatographically, as seen in their ion chromatograms (Figure 4-9)

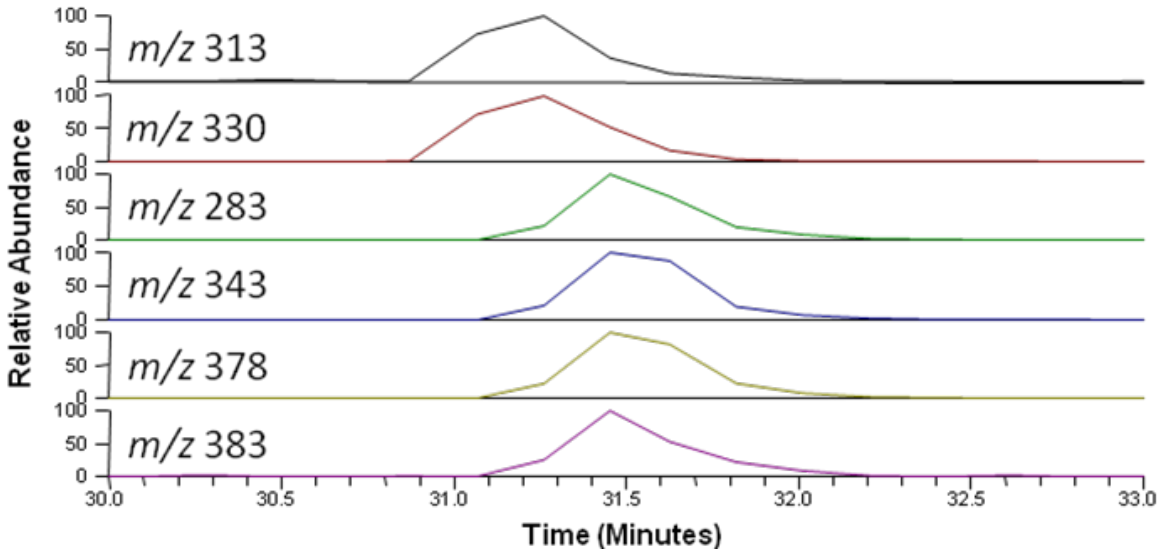



Figure 4-9. (+)ESI ion chromatograms of *m/z*, 313 330 (both from Peak B) and 283, 343, 378, and 383 (from Peak C)

The *m/z* 343 ion of Figure 4-8 is probably the $[M+H-H_2O]^+$ fragment ion of Peak C. The HRMS spectrum had an *m/z* 343.2484 ion with a composition of $C_{19}H_{35}O_5$, which is consistent with the suggested fragmentation. CID of the *m/z* 378 $[M+NH_4]^+$ ion formed *m/z* 343, via loss of $NH_3$ and $H_2O$. That this molecule readily loses water indicates an available OH group on the compound. The CID of the *m/z* 343 fragment ion lost an acetoxy group to form *m/z* 283.

No major ion peaks were observed in B and C's high mass scan.

Below is a table of observed HRMS ions, their experimental and accurate masses, mass errors, and their suggested elemental composition for Peak C (Table 4-1)

Table 4-2. Peak C's HRMS major ions

| Observed Ions (m/z) | Experimental Mass (u) | Accurate Mass (u) | Mass Error (ppm) | Elemental Composition |
|---|---|---|---|---|
| $[M+Na]^+$ | 383.2413 | 383.2404 | +2 | $C_{19}H_{36}NaO_6$ |
| $[M+NH_4]^+$ | 378.2856 | 387.2850 | +2 | $C_{19}H_{40}NO_6$ |
| $[M+H-H_2O]^+$ | 343.2482 | 343.2479 | +1 | $C_{19}H_{34}O_5$ |
| $[M+H-H_2O-CH_3COOH]^+$ | 283.2269 | 283.2268 | 0 | $C_{17}H_{30}O_3$ |

**Peaks D and E**

The third and fourth major peaks in this mass range, Peaks D and E, were better separated utilizing a mobile phase with methanol rather than isopropanol (Figure 4-10):

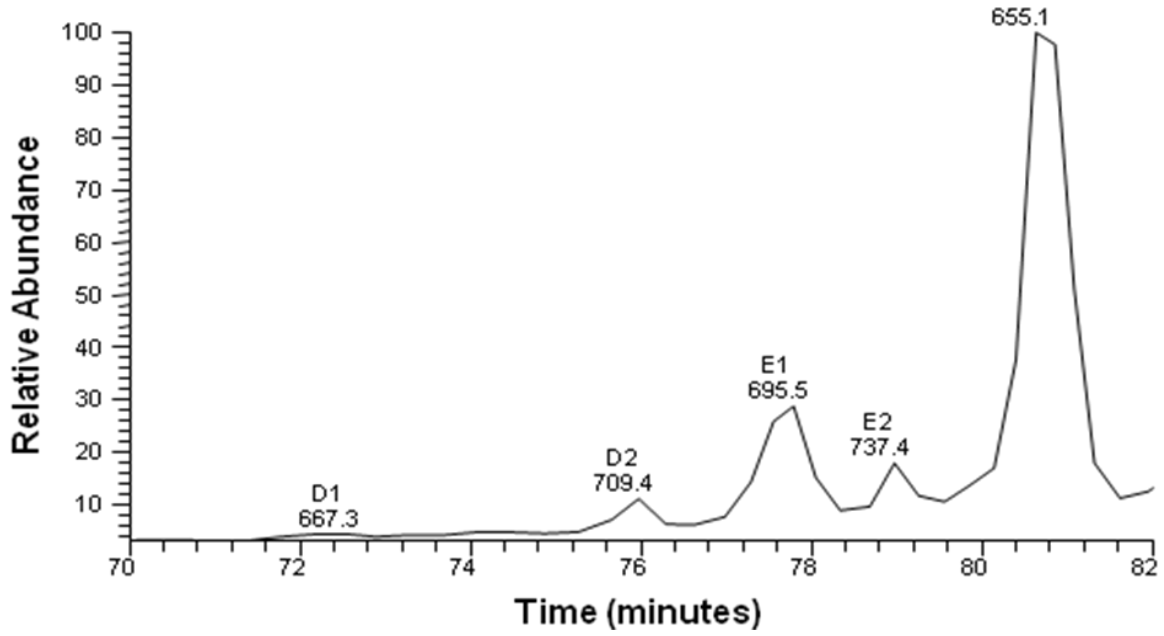



Figure 4-10. LC/(+)ESI-MS (*m/z* 667,709, 695, 737, and 655) base peak chromatogram for the higher molecular weight compounds of Peaks D and E of Figure 4-2 separated using methanol instead of isopropanol

The ion chromatograms of the major ions of the peaks in Figure 4-10 indicate the presence of at least four compounds with different molecular weights, *m/z* 627 being a common ion in one pair (D1 and D2) and *m/z* 655 being a common ion in another pair (E1 and E2) (Figure 4-11).

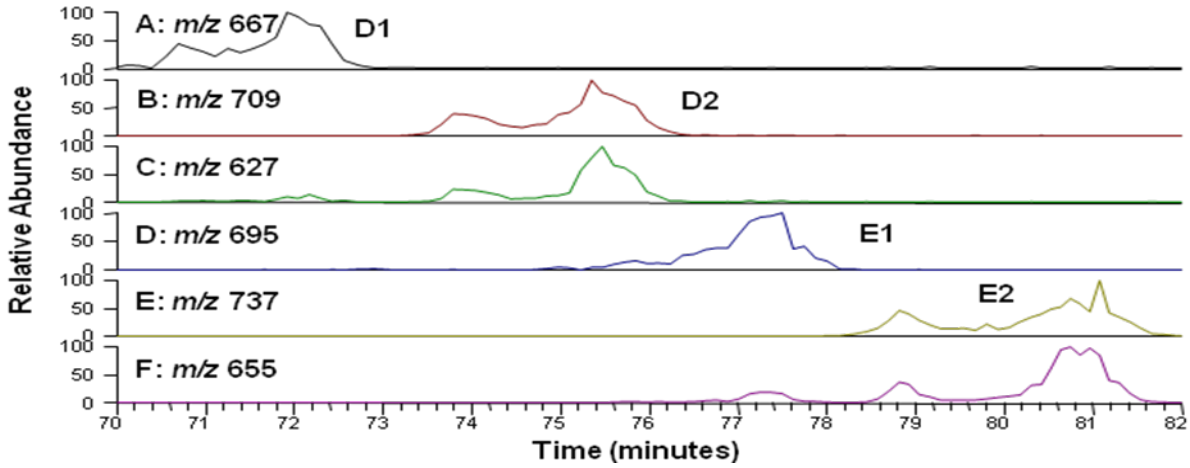



Figure 4-11. Ion chromatograms of base peaks *m/z* 667, 709, 627, 695, 737, and 655

Figure 4-12 shows that Peak D1 at $t_r$ of 70.34 to 72.82 minutes produced *m/z* 667 $[M+Na]^+$, *m/z* 662 $[M+NH_4]^+$, and *m/z* 627 $[M+H-H_2O]^+$ ions.

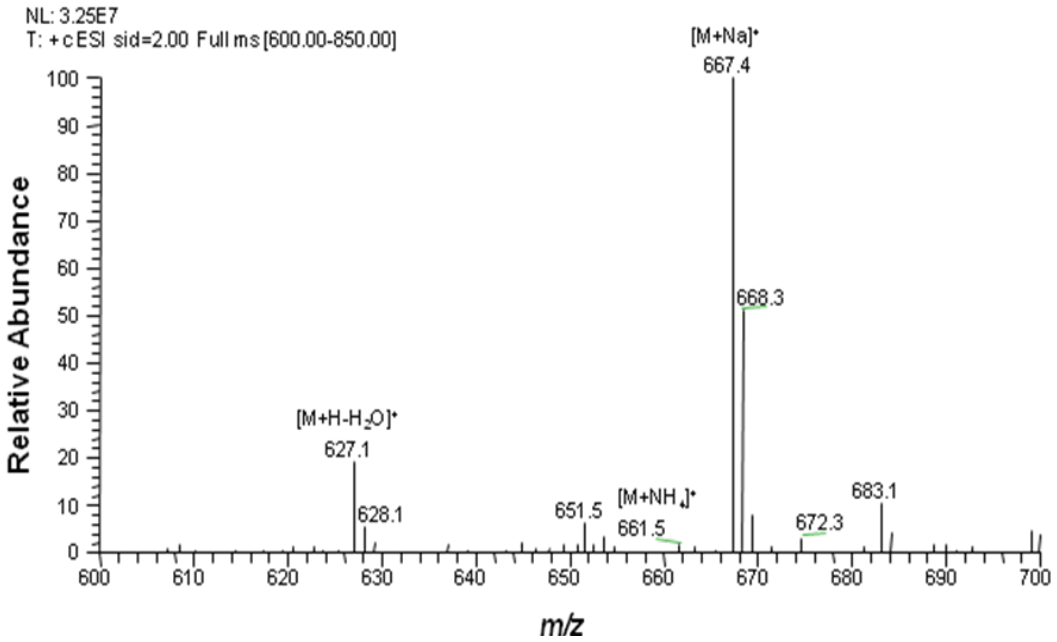



Figure 4-12. (+)ESI mass spectrum of Peak D1 of Figure 4-11

The *m/z* 667 $[M+Na]^+$ ion underwent CID to produce *m/z* 627 $[M+H-18]^+$, *m/z* 607 (loss of 60 u), and *m/z* 547 (a second loss of 60 u). Again, the loss of water indicates an OH group on this compound.

HRMS data of Peak D1 indicated a monoisotopic mass of 644.4499 u and elemental composition of $C_{35}H_{64}O_{10}$. Below is a table of observed HRMS ions, their experimental and accurate masses, mass errors, and their suggested elemental composition for Peak D1.

Table 4-3. Peak D1's HRMS major ions

| Observed Ions (*m/z*) | Experimental Mass (u) | Accurate Mass (u) | Mass Error (ppm) | Elemental Composition |
|---|---|---|---|---|
| $[M+Na]^+$ | 667.4405 | 667.4392 | +2 | $C_{35}H_{64}NaO_{10}$ |
| $[M+NH_4]^+$ | 662.4835 | 662.4838 | 0 | $C_{35}H_{68}NO_{10}$ |
| $[M+H-H_2O]^+$ | 627.4468 | 627.4467 | 0 | $C_{35}H_{63}O_9$ |
| $[M+H-H_2O-CH_3COOH]^+$ | 567.4279 | 567.4255 | +4 | $C_{33}H_{59}O_7$ |
| 343 | 343.2484 | 343.2479 | +1 | $C_{19}H_{35}O_5$ |
| 255 | 255.1953 | 255.1955 | -1 | $C_{15}H_{27}O_3$ |

+ESI of Peak D2 ($t_r$ from 73.61 to 76.19 minutes) produced *m/z* 709 $[M+Na]^+$, m/z 704 $[M+NH_4]^+$, and *m/z* 627 $[M+H-60]^+$ fragment ion (Figure 4-13).

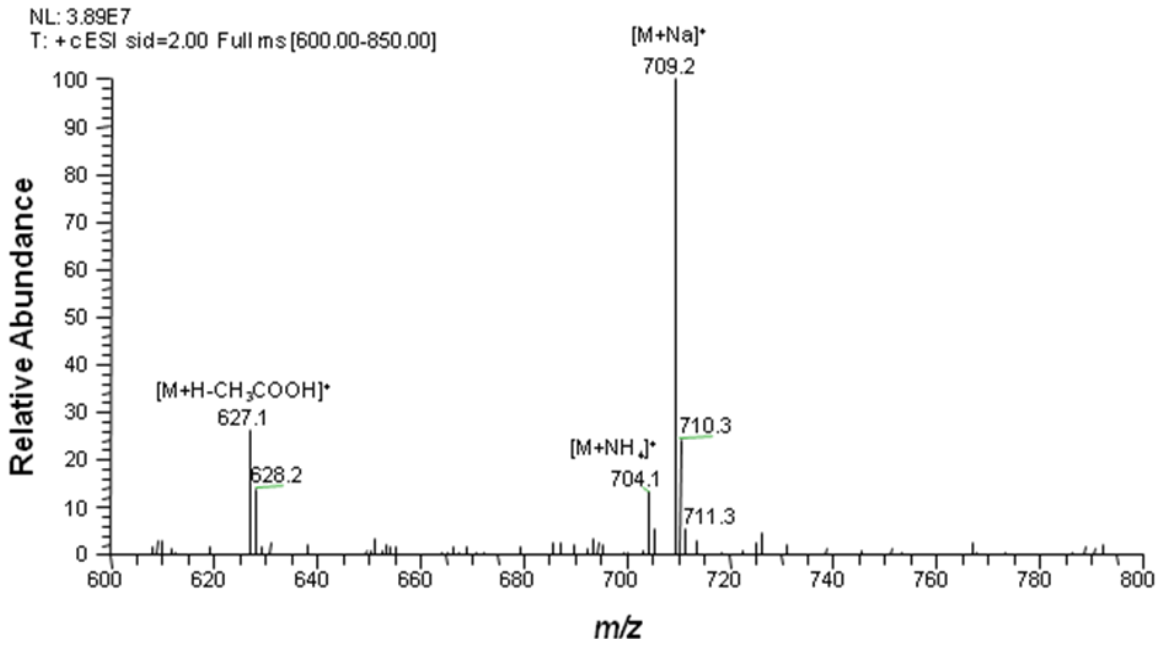



Figure 4-13. (+)ESI mass spectrum of Peak D2 from Figure 4-11

The *m/z* 709 $[M+Na]^+$ underwent CID in $MS^2$ to produce *m/z* 649 and 589 via consecutive losses of 60 u. The *m/z* 649 was further dissociated in $MS^3$ and $MS^4$ to *m/z* 589 and 529 via additional losses of 60 u, indicating that at least three acetoxy groups were present (Figure 4-14).

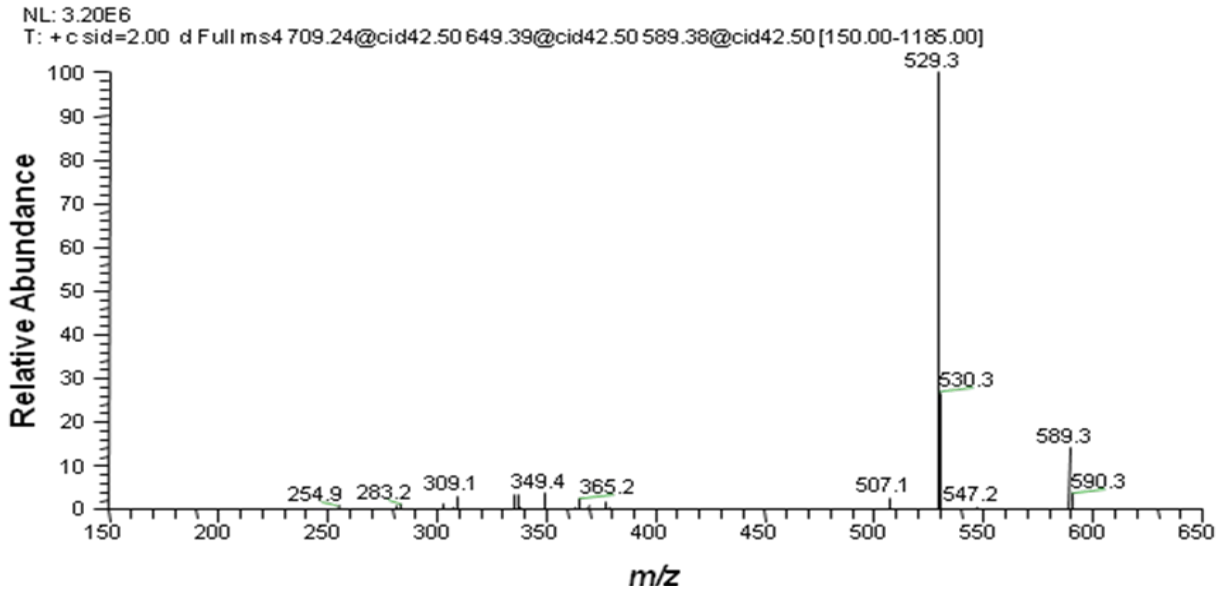



Figure 4-14. (+)ESI $MS^4$ mass spectrum of *m/z* 709 → *m/z* 649 → *m/z* 589 → *m/z* 529

Below is a table of observed HRMS ions, their experimental and accurate masses, mass errors, and their suggested elemental composition for Peak D2 (Table 4-4).

Table 4-4. Peak D2's HRMS major ions

| Observed Ions (*m/z*) | Experimental Mass (u) | Accurate Mass (u) | Mass Error (ppm) | Elemental Composition |
|---|---|---|---|---|
| $[M+Na]^+$ | 709.4513 | 709.4497 | +2 | $C_{37}H_{66}NaO_{11}$ |
| $[M+NH_4]^+$ | 704.4959 | 704.4943 | +2 | $C_{37}H_{70}NO_{11}$ |
| $[M+H-CH_3COOH]^+$ | 627.4464 | 627.4467 | 0 | $C_{35}H_{63}O_9$ |
| 343 | 343.2468 | 343.2479 | -3 | $C_{19}H_{35}O_5$ |
| 315 | 315.2182 | 315.2166 | +5 | $C_{17}H_{31}0_5$ |
| 283 | 283.2265 | 283.2268 | -1 | $C_{17}H_{31}O_3$ |
| 255 | 255.1962 | 255.1955 | +3 | $C_{15}H_{27}O_3$ |

With Peaks D1 and D2 having *m/z* 627 in common, a possible structure can be established. The *m/z* 627 ion loses one acetoxy group to form *m/z* 567 and an additional acetoxy and hydroxyl group to form *m/z* 489 (Figure 4-15).

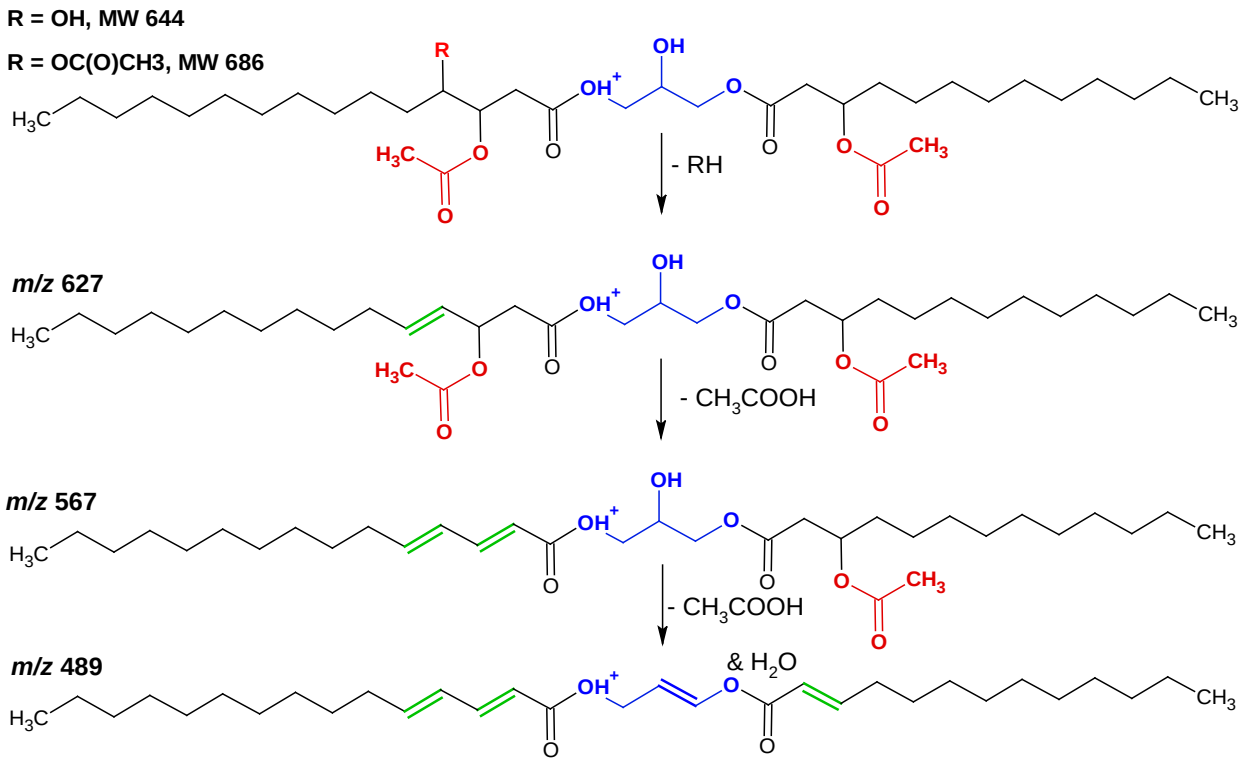



Figure 4-15. Suggested fragmentation of *m/z* 627

The next major ions are *m/z* 343, 315, 283, and 255. There is a difference of 60 u between *m/z* 343 and 283 as well as *m/z* 315 and 255. The difference between *m/z* 343 and 315 is 28 u. This would indicate that these ions come from *m/z* 627. In 1973, Starratt and Osgood [36] found that the *Cx. quinquefasciatus* mosquito's apical droplet active fraction contained 1,3-diglycerides. Assuming that Peaks D1 and D2 both correspond to 1,3-diglycerides, the loss of *m/z* 343 and 283 could be from one side of the molecule while *m/z* 315 and 255 come from the other (Figure 4-16) .

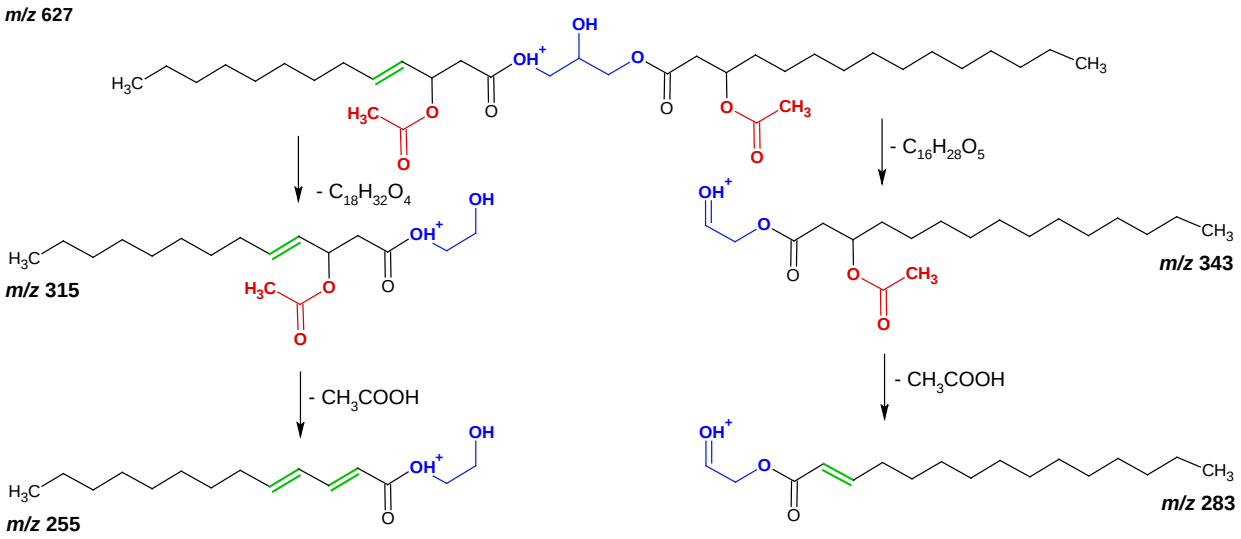



Figure 4-16. Suggested formation of *m/z* 343, 315, 283, and 255 from *m/z* 627

Peak E1, $t_r$ 75.48 to 78.18 minutes, (Figure 4-11) produced *m/z* 695 $[M+Na]^+$ and *m/z* 690 $[M+NH_4]^+$ ions, as well as $[M+H-H_2O]^+$ at *m/z* 655 (Figure 4-17).

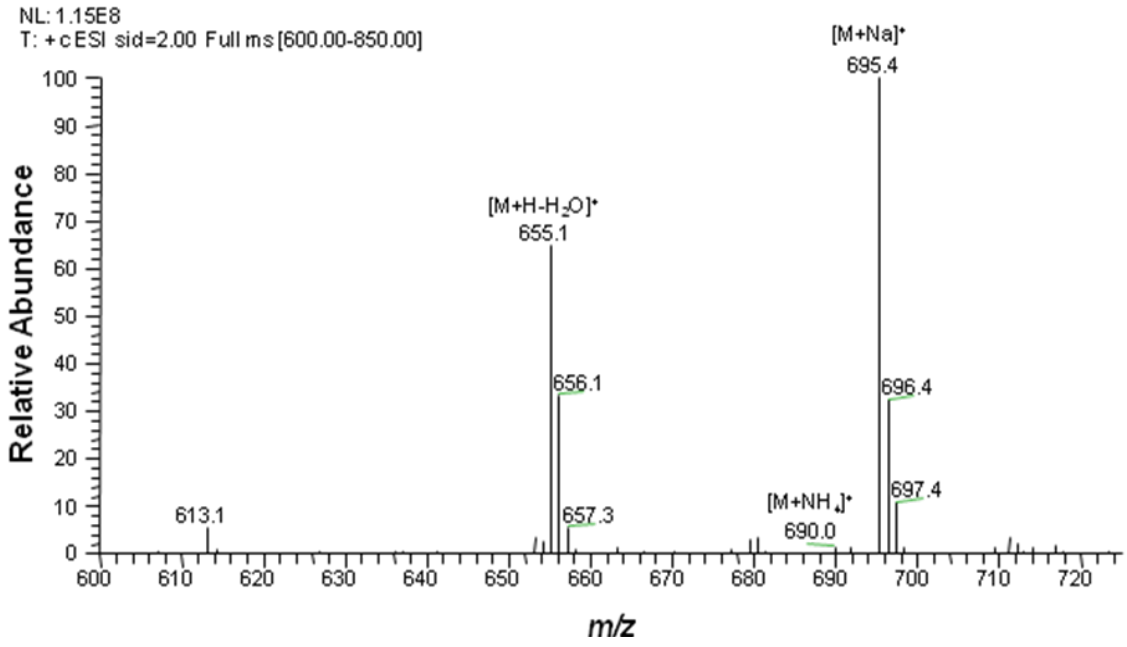



Figure 4-17. (+)ESI mass spectrum of Peak E1

$MS^2$ of m/z 695 gave m/z 635 (-60 u) and 575 (-60 u) due to successive loses of $CH_3COOH$. The next lowest mass ion is at *m/z* 343. Often such a large difference in

mass between the parent and daughter ions suggests that the daughter ion results from a fragmentation at a labile bond between two major “pieces” of a molecule.

Below is a table of observed HRMS ions, their experimental and accurate masses, mass errors, and their suggested elemental composition for Peak E1 (Table 4-5).

Table 4-5. Peak E1’s HRMS major ions

| Observed Ions (*m/z*) | Experimental Mass (u) | Accurate Mass (u) | Mass Error (ppm) | Elemental Composition |
|---|---|---|---|---|
| $[M+Na]^+$ | 695.4718 | 695.4705 | +2 | $C_{37}H_{68}NaO_{10}$ |
| $[M+NH_4]^+$ | 690.5164 | 690.5151 | +2 | $C_{37}H_{72}NO_{10}$ |
| $[M+H-H_2O]^+$ | 655.4791 | 655.4780 | +2 | $C_{37}H_{67}O_9$ |
| $[M+H-H_2O-CH_eCOOH]^+$ | 595.4565 | 595.4568 | -1 | $C_{35}H_{63}O_7$ |
| 343 | 343.2475 | 343.2479 | -1 | $C_{19}H_{35}O_5$ |
| 283 | 283.2266 | 283.2268 | -1 | $C_{17}H_{31}O_3$ |

+ESI of Peak E2, $t_r$ 78.34 to 81.63 minutes, produced *m/z* 732 $[M+NH_4]^+$ and *m/z* 737 $[M+Na]^+$ ions, as well as $[M+H-CH_3COOH]^+$ at *m/z* 655 (Figure 4-18). HRMS of these ions indicated a molecular weight of 714.4918 and a molecular formula of $C_{39}H_{70}O_{11}$.

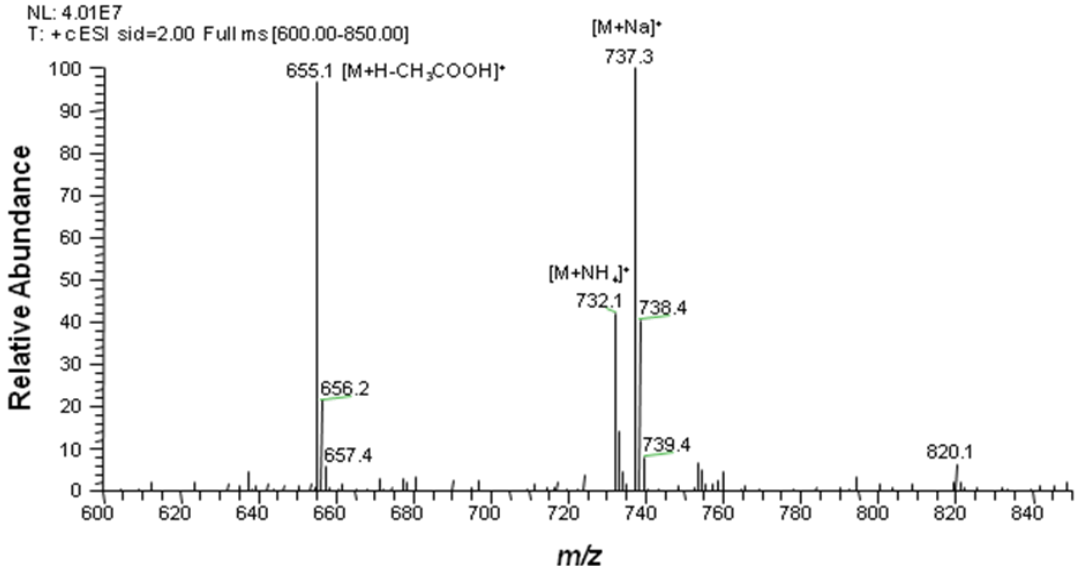



Figure 4-18. (+)ESI mass spectrum of Peak E2

$MS^3$ of *m/z* 737 produced *m/z* 677 (-60 u), 617 (-60 u), and 577 (-60 u).

Below is a table of observed HRMS ions, their experimental and accurate masses, mass errors, and their suggested elemental composition for Peak E2 (Table 4-6).

Table 4-6. Peak E2's HRMS major ions

| Observed Ions (*m/z*) | Experimental Mass (u) | Accurate Mass (u) | Mass Error (ppm) | Elemental Composition |
|---|---|---|---|---|
| $[M+Na]^+$ | 737.4826 | 737.4810 | +2 | $C_{39}H_{70}NaO_{11}$ |
| $[M+NH_4]^+$ | 732.5273 | 732.5256 | +2 | $C_{39}H_{74}NO_{11}$ |
| $[M+H-CH_3COOH]^+$ | 655.4782 | 655.4780 | 0 | $C_{37}H_{67}O_9$ |
| $[M+H-2CH_3COOH]^+$ | 595.4563 | 595.4568 | +1 | $C_{35}H_{63}O_7$ |
| 343 | 343.2474 | 343.2479 | -2 | $C_{19}H_{35}O_5$ |
| 283 | 283.2262 | 283.2268 | -2 | $C_{17}H_{31}O_3$ |

With E1 and E2 having *m/z* 655 in common, a possible structure can be established for these peaks as well. This ion loses an acetoxy group and then an acetoxy group with a hydroxyl group to form *m/z* 595 and 517 (Figure 4-19).

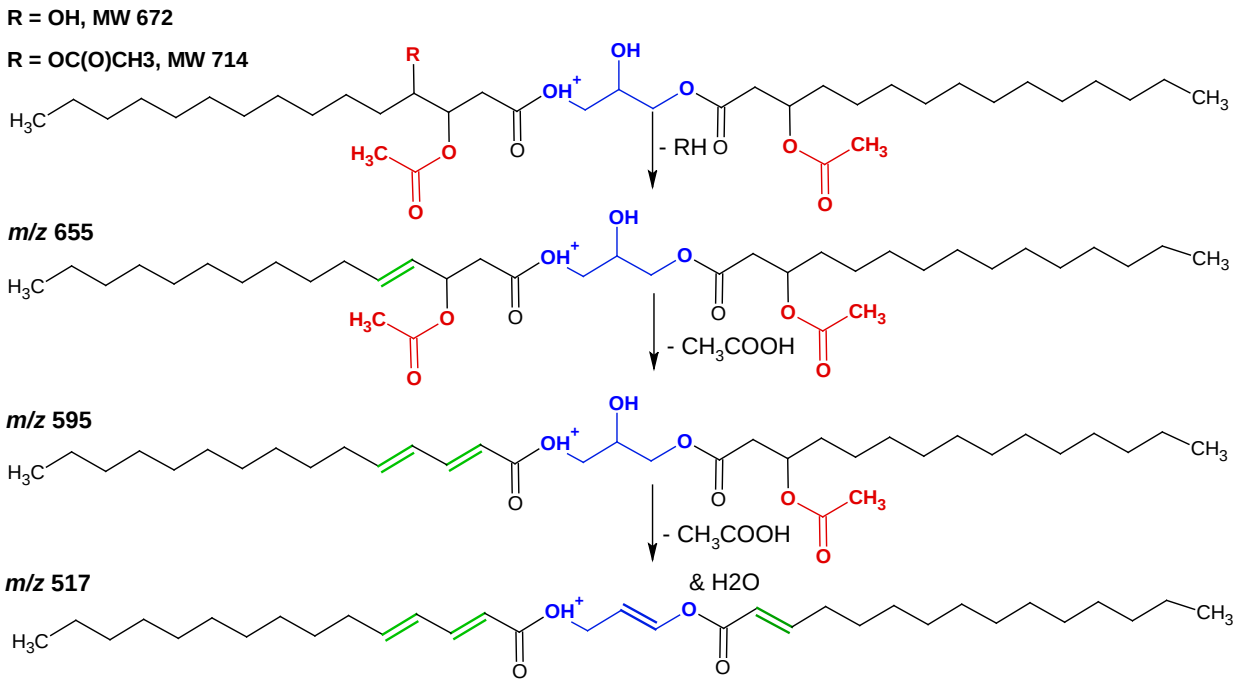



Figure 4-19. Suggested fragmentation of *m/z* 655

The next two major ions are *m/z* 343 (-312 u) and 283 (-372 u). The difference between these two losses is 60 u from an acetoxy group. Based on HRMS and LC/$MS^n$ data, this ion has only two acetoxy groups, which means that *m/z* 343 and 283 come

from *m/z* 655. If *m/z* 655 fragments similarly to *m/z* 627, then two pairs of ions should be observed with 28 u difference between the pairs and 60 u difference within the pairs. However, only one pair, *m/z* 343 and 283, is observed. It should be noted that *m/z* 655 is 28 u greater than *m/z* 627. Based on HRMS data of these two ions, *m/z* 655 is $C_2H_4$ longer than *m/z* 627. Therefore, if one side of *m/z* 627 increases by $C_2H_4$, then *m/z* 655 is obtained with similar fragmentation on both sides of the 1,3-diglyceride (Figure 4-20).

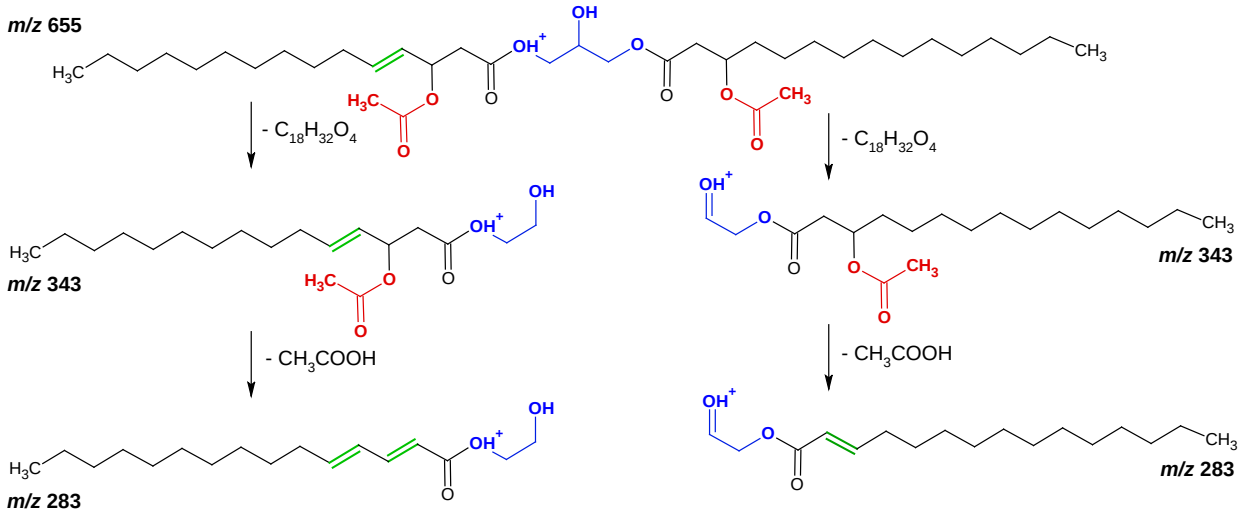



Figure 4-20. Suggested formation of *m/z* 343 and 283 from *m/z* 655

**Comparison to previous findings**

In 1972, Starratt and Osgood [35] found that the 1,3-diglycerides in *Cx. tarsalis* mosquito's apical droplet active fraction consisted of mono- and dihydroxy fatty acids. The major monohydroxy acids were 3-hydroxytetradecanoic acid, 3-hydroxyhexadecanoic acid, and 3-hydroxyoctadec-cis-II-enoic acid. The major dihydroxy acid was *erythro*-5,6-dihydroxyhexadecanoic acid. The authors concluded that the fatty acid hydroxyl groups were acetylated in the diglycerides. They also proposed that the monohydroxy acid groups were esterified at one of the α-positions of

the glycerol moiety, and the dihydroxy acid residues with the other. This would give the following structures (Figure 4-21):

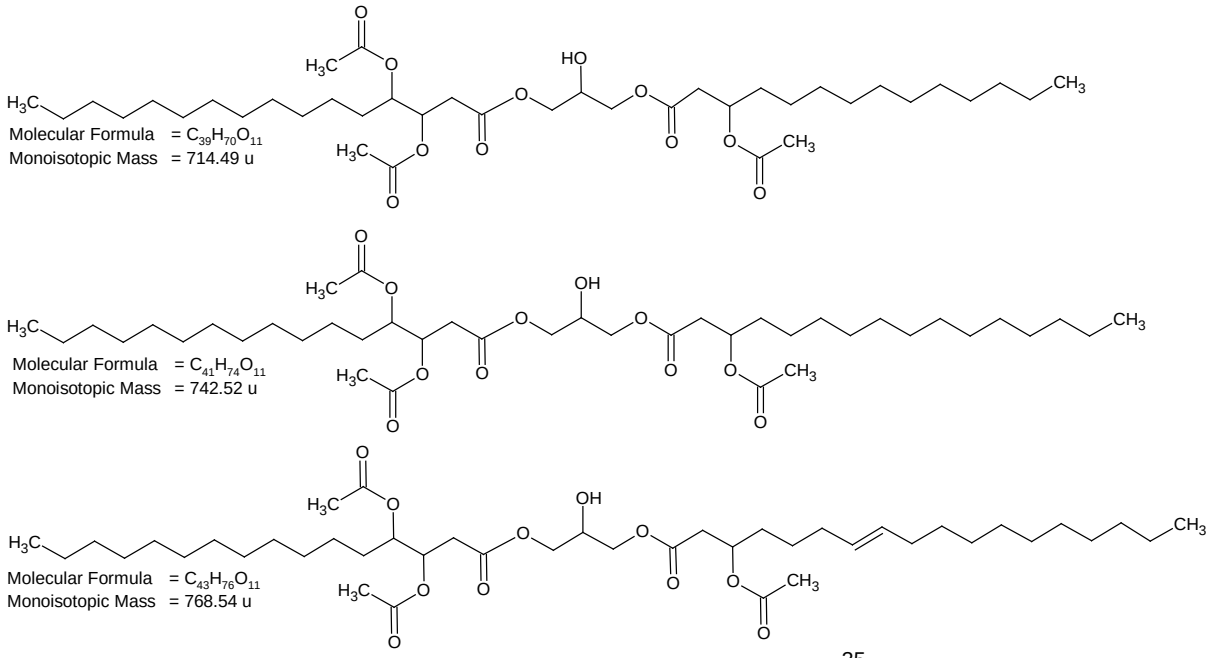



Figure 4-21. Structures suggested by Starratt and Osgood [35]

An study in 1973 showed that ether washings of the *Cx. tarsalis* and the *Cx. quinquefasciatus* egg rafts had very similar 1,3-diglyceride compositions [36].

Not all the structures hypothesized by Starratt and Osgood in Figure 4-21 agree with the data shown here. The first two (MW 714 and MW 742) were detected, with MW 742 being in very low abundance. However, the LC/$MS^n$ data show the presence of hydroxyl groups, which is supported by HRMS data. Peaks D1 and D2 have a similar base structure, *m/z* 627, but differ in that Peak D1 is hydroxylated and Peak D2 is acetylated. Peaks E1 and E2 also have a similar base structure, *m/z* 655, with Peak E1 being hydroxylated and Peak E2 being acetylated. Thus, the following structures are suggested:

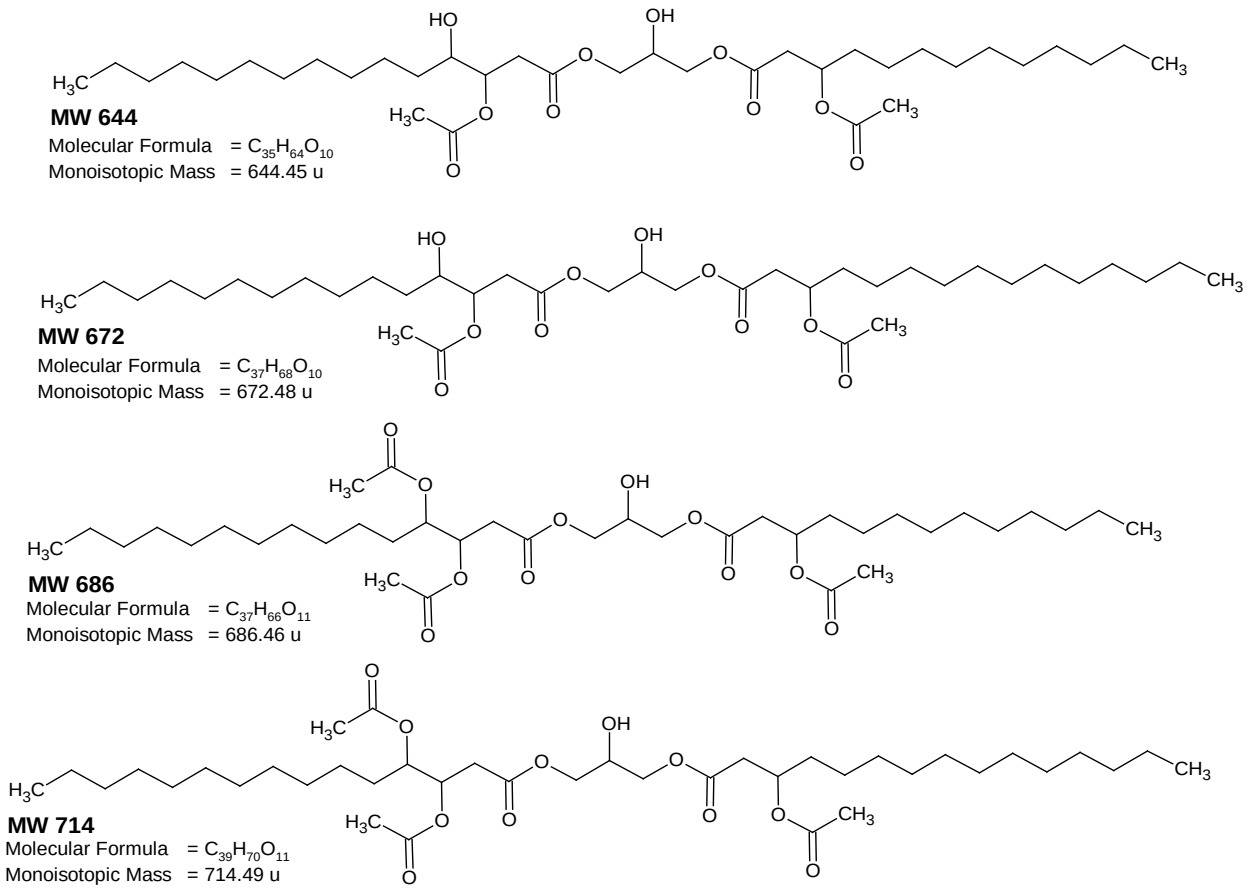



Figure 4-22. Suggested structures for peaks D1, D2, E1, and E2 in *Cx. quinquefasciatus* apical droplets

## Conclusion

Seven major peaks were separated and analyzed from female *Cx. quinquefasciatus*' apical droplets via LC/ESI/MS and HRMS. One of these peaks, Peak B, was the known mosquito pheromone, 6-acetoxy-5-hexadecanolide. The remaining peaks have the following molecular weights and formulae:

Table 4-7. $LC/MS^n$ and HRMS Data

| Trait | Peak A | Peak C | Peak D1 | Peak D2 | Peak E1 | Peak E2 |
|---|---|---|---|---|---|---|
| $[M+Na]^+$ (m/z) | $C_{20}H_{36}NaO_6$ | $C_{19}H_{36}NaO_6$ | $C_{35}H_{64}NaO_{10}$ | $C_{37}H_{66}NaO_{11}$ | $C_{37}H_{68}NaO_{10}$ | $C_{39}H_{70}NaO_{11}$ |
| $[M+Na]^+$ (*m/z*) | 395.2411 | 383.2413 | 667.4405 | 709.4513 | 695.4718 | 737.4826 |
| Acetoxy Groups | 2 | 1 | 2 | 3 | 2 | 3 |
| Hydroxyl Groups | 0 | 1 | 1 | 0 | 1 | 0 |

$MS^n$ scans illustrated that Peak E1 and E2 have a common ion of *m/z* 655, and D1 and D2 have a common ion of *m/z* 627. With a difference of 42 u between E1 and E2, as well as for D1 and D2, E1 and E2 may have a similar base structure with E1 being hydroxylated and E2 being acetylated. The same is true for D1 and D2, with D1 being hydroxylated and D2 being acetylated. Both *m/z* 627 and 655 fragment similarly, with *m/z* 283 and 343 (the acetylated version of *m/z* 283) being present. It should be noted that the *m/z* 655 ion is $C_2H_4$ longer than the *m/z* 627 ion. This accounts for *m/z* 255 and 315 (the acetylated version of *m/z* 255) observed in Peak D1 and D2. Thus, one side of *m/z* 655 is $C_2H_4$ longer than *m/z* 627. Suggested structures are shown in Figure 4-22. The hydroxylated versions of *m/z* 627 and 655 contradict the findings of Starratt and Osgood [36], who proposed that all compounds were only acetylated.

# CHAPTER 5
# NMR STUDY OF APICAL DROPLETS FROM THE *CULEX QUINQUEFASCIATUS* MOSQUITO

## Introduction

*Culex quinquefasciatus* (*Cx. quinquefasciatus*) is a vector of many pathogens of humans and animals. Due to development of resistance in vectors to traditional insecticides, it is necessary to explore the development of new chemical agents for vector control.

Gravid (egg-laying) mosquitoes use a combination of sensory cues to find and determine the suitability of water-containing sites for egg deposition. Habitat-related odors and a pheromone associated with the egg rafts can influence the inclination of gravid female *Culex* mosquitoes to oviposit [58]. Bruno et al. observed that a large number of female mosquitoes oviposit in water containing *Cx. quinquefasciatus* egg rafts at various densities [31]. This response resulted from the pheromone *erthyo*-6-acetoxy-5-hexadecanolide within the apical droplet on each egg [9]. Studies show that mosquitoes react only to the *erythro* enantiomer of the pheromone, and not to the *threo* enantiomer [10, 59]. Therefore, an understanding of the chemical structure and stereochemistry is necessary to fully exploit the use of these compounds to manipulate mosquitoes' oviposition behavior and control disease transmission.

In addition to the previously identified mosquito pheromone, *erythro*-6-acetoxy-5-hexadecanolide, several other compounds have been discovered in the *Cx. quinquefasciatus* apical droplet through GC/MS and LC/$MS^n$ analysis in this study Chapters 3 and 4). However, the chemical structure of these compounds has yet to be determined.

To be able to accurately determine each compound's structure, the compounds should be analyzed by NMR. Although on-line LC/NMR has been applied successfully to characterize many compounds, the S/N ratios in the NMR spectra are low because of the poor sensitivity of NMR and the relatively low sample concentrations in the LC eluate; this makes the spectra difficult to interpret. To provide more concentrated samples, the compounds' retention times were determined by LC/MS, seven fractions were manually collected from twelve injections, the mobile phases were evaporated off from each fraction, and the fractions were then reconstituted in deuterated chloroform.

The purpose of this work is to provide greater insight into the chemical structure of the *Cx. quinquefasciatus* apical droplet compounds using NMR. Discovery of new biologically active oviposition compounds may lead to methods for improved mosquito surveillance and control.

## Experimental

### Mosquito Rearing

The *Cx. quinquefasciatus* mosquitoes were reared in insectaries at 25 °C and 60% relative humidity, following procedures outlined in Gerberg et al. [60] Fluorescent and incandescent lights were controlled by an adjustable timer to simulate natural daylight for ten hours. For egg-raft production, female mosquitoes were bloodfed on bovine blood, and then held for five days for the eggs to develop. For oviposition, gravid females were provided a pan containing water with decaying organic matter (Bermuda grass) in the evening of the fifth day. Egg rafts were collected about twelve hours after being laid.

**Sample Preparation**

Apical droplets were acquired from egg rafts from *Cx. quinquefasciatus* mosquitoes. Sampling of apical droplets was done with tapered capillary collection tubes prepared by heating and pulling the glass capillary tubes (World Precision Instruments, Sarasota, FL, 1.5 mm outer diameter, 1.12 mm ID, 100 mm length) using a PUL-1 (World Precision Instruments) micropipette puller. The pan containing egg rafts was placed under a microscope at 90x, and the apical droplets were removed by touching them with the tip of the micropipette.

The apical droplets in the vial were diluted with a 0.5 mL solution of methanol and isopropanol at a 2:1 ratio. The LC used for compound separation was an Agilent Technologies (Santa Clara, CA) 1100 series G1312A binary pump with a ThermoFisher Scientific (Waltham, MA) Hypurity C8 column (5µm particle diameter; 2.1 x 100 mm long+ guard column). The mobile phase consisted of Solvent A (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in water (LC-grade, Honeywell Burdick & Jackson)), and Solvent B (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in isopropanol (LC-grade; Honeywell Burdick & Jackson)). The gradient was [A:B(min)]: 95:5(0):35:65(20)>15:85(65)>5:95(100-120) with a flow rate of 0.2 mL/minute. Approximately 20 µL of apical droplet solution was injected each run, and twelve injections were performed for this experiment. Once the retention time of each compound was determined by mass spectrometry, the flow tube to the mass spectrometer was disconnected and seven fractions were manually collected based on time. The fractions were collected in 10 mL glass vials, and pooled from all twelve injections. The fractions were then dried down with nitrogen to evaporate the mobile phase.

**NMR**

Thirty µL of d-chloroform (Sigma-Aldrich) were added to the vial. The vial was swirled and finger vortexed. The solution was removed with a microsyringe and injected into a 1-mm NMR tube.

NMR analysis occurred that the Advanced Magnetic Resonance Imaging and Spectroscopy (AMRIS) facility at the University of Florida. The instruments used were an Agilent VNMRS-600 at 600 MHz spectrometers. All NMR experiments were performed by Jim Rocca.

A newly developed NMR probe that utilizes high temperature superconducting (HTS) coils coupled with cryogenic cooling and small volumes were used for fraction analysis. The HTS material provides very high sensitivity detection, the cryogenic cooling reduces the noise, and the small volume improves the overall efficiency by approximately 1/d where d is the diameter of the probe. The probe was optimized for $^{1}H$ sensitivity. It utilized a 1-mm sample tube and has a $^{1}H$ mass sensitivity about 20x greater than a conventional 5-mm probe at room temperature.

## Results and Discussion

Figure 5-1 illustrates the structure of the *Cx. quinquefasciatus* oviposition pheromone, *erythro*-6-acetoxy-5-hexadecanolide

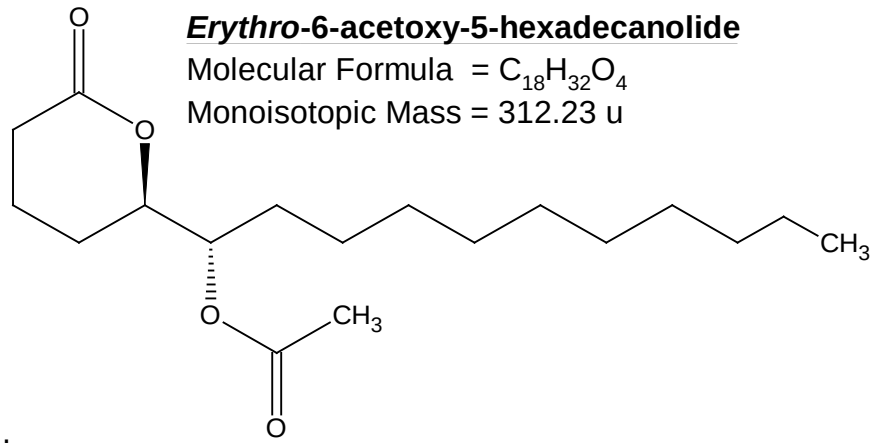



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Figure 5-1. *Erythro*-6-acetoxy-5-hexadecanolide

Figure 5-2 illustrates the separation of the components from *Cx. quinquefasciatus* apical droplets. Peak C was identified as the established pheromone, e*rythro*-6-acetoxy-5-hexadecanolide. Seven fractions (shown by the bands on the figure) were collected for NMR analysis.

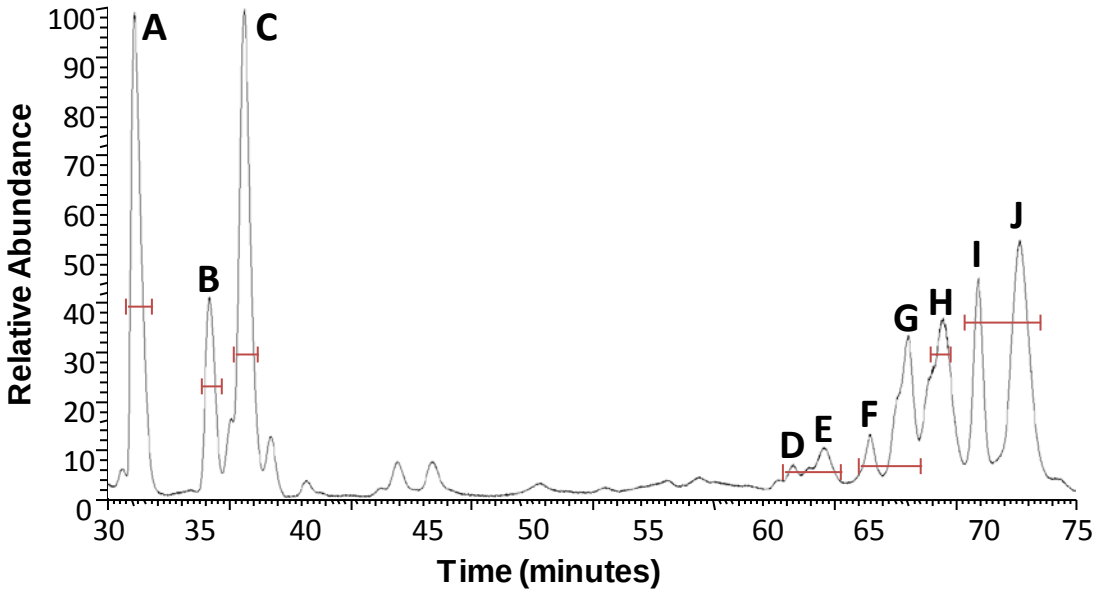



Figure 5-2. Total ion chromatogram of LC/(+)ESI-MS peaks from *Cx. quinquefasciatus* apical droplets showing each fraction's collection time window

LC-MS$^n$ studies (Chapter 4) showed that there are three sets of isomers (Peak D and E, F and G, and I and J). During collection, these sets of isomers were collected together in a single fraction. Table 5-1 displays each peak's molecular weight and corresponding molecular formula, as determined by HRMS, and the calculated degree of unsaturation (rings and double bonds).

Table 5-1. Molecular weights and formulae for previously identified compounds

| | Peak A | Peak B | Peak C | Peak D, E | Peak F, G | Peak H | Peak I, J |
|---|---|---|---|---|---|---|---|
| MW | 372.2512 | 312.2512 | 360.2512 | 644.4499 | 686.4605 | 672.4812 | 714.4918 |
| MF | $C_{20}H_{36}O_6$ | $C_{18}H_{32}O_4$ | $C_{19}H_{36}O_6$ | $C_{35}H_{64}O_{10}$ | $C_{37}H_{66}O_{11}$ | $C_{37}H_{68}O_{10}$ | $C_{39}H_{74}O_{11}$ |
| Unsaturation | 3 | 3 | 2 | 4 | 5 | 4 | 5 |

All proton NMR spectra showed methanol and water contamination due to not all of the mobile phase evaporating off. The methanol was identified by its $CH_3$ proton peak at δ3.49, while the water proton peak was observed at δ1.31.

$^1$H, Correlation spectroscopy (COSY) was performed on Peak A, C, D, E, F, G, H, I, and J. Peak B was collected but used only for bioassays (Chapter 6).

**Peak A**

Figure 5-3 is a suggested structure for Peak A.

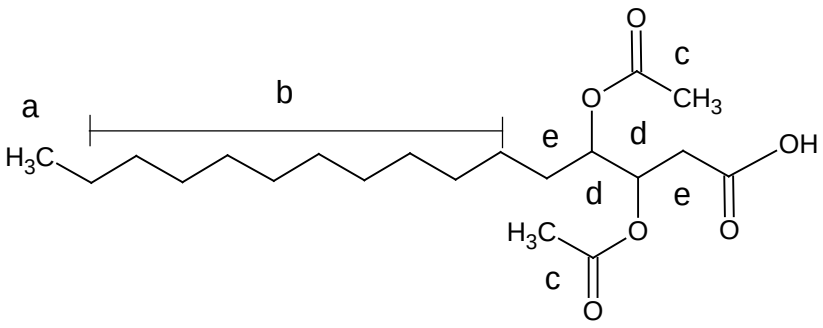



Figure 5-3. Suggested structure for Peak A

The $^1$H NMR of the fraction containing Peak A (MW 372) has a single methyl triplet (a) at δ0.88, which is COSY to the $CH_2$ singlet (b) at δ1.24 (Figure5-4). This

corresponds to an alkyl chain. The methyl protons on acetoxy groups (c) are observed at δ2.06, along with their adjacent $CH_2$ protons (e) at δ2.34.

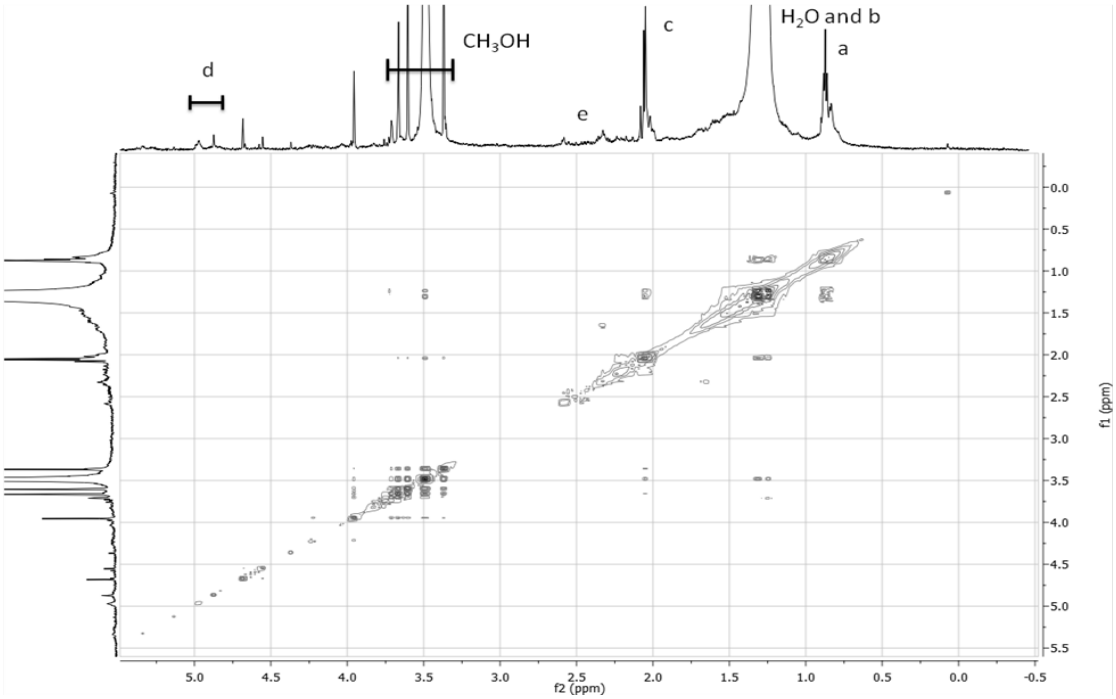



Figure 5-4. $^1$H NMR COSY spectrum of Peak A

LC/MS$^n$ analysis (Chapter 4, Table 4-1) showed that this compound contains two acetoxy groups; the CHs (d) α to these acetoxy groups are the multiplets at δ4.98 and δ4.87. With two acetoxy groups and an unsaturation number of 3, Peak A must contain an additional double bond.

LC/MS$^n$ data (Chapter 4, Figure 4-4) show that the base structure for the known pheromone, Peak B, is very similar to the base structure of Peak A. The only difference between the two molecules is that Peak B loses a hydroxyl whereas Peak A loses an additional acetoxy group. Figure 5-3 agrees with these findings, though the location of the second acetoxy group cannot be determined at this time.

**Peak C**

Figure 5-5 is a suggested structure of Peak C.

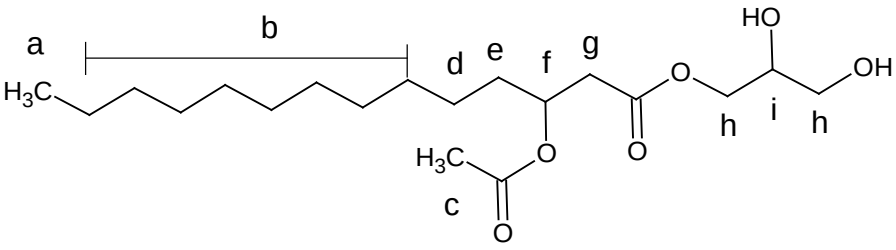



Figure 5-5. Suggested structure of Peak C

The $^{1}H$ spectrum for Peak C (MW 360) in Figure 5-6 shows a methyl triplet (a) at δ0.87. This is followed by a $CH_2$ singlet at δ1.25 (b) and two multiplets (d) at δ1.59 and δ1.61. The singlet is similar to $CH_2$ found on fatty acid chains. The multiplets correspond to hydrogens near an electron withdrawing group. The acetoxy $CH_3$ peaks (c) at δ2.05 integrates at a 1:1 ratio with the methyl peak at δ0.87 (Figure 5-6). Starratt and Osgood noted a single acetoxy $CH_3$ peak also at δ2.06 [35] in their apical droplet active fraction (Figure 5-7).

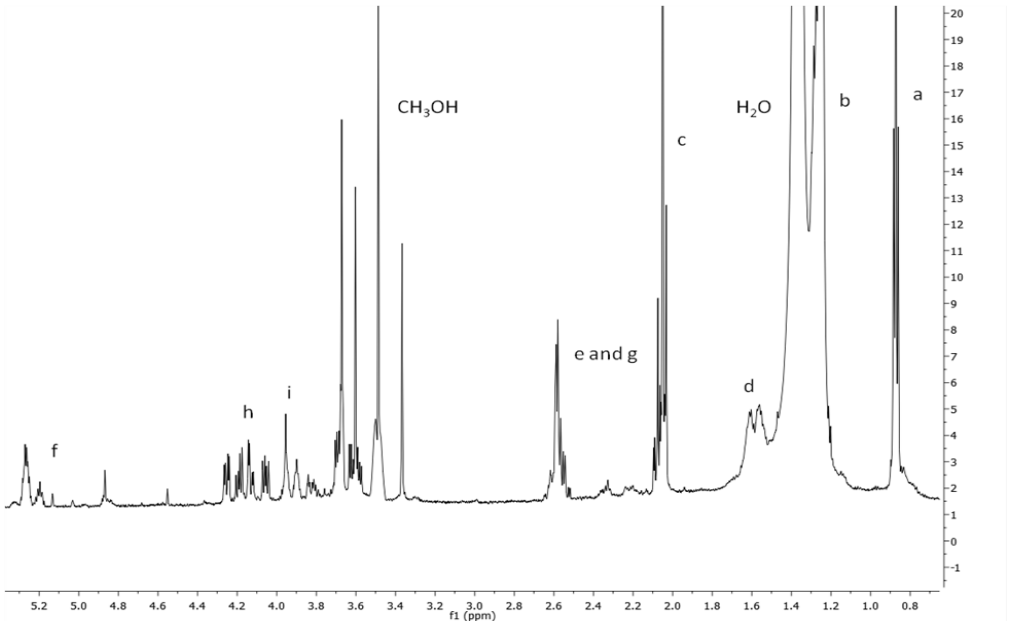



Figure 5-6. $^{1}H$ NMR spectrum of Peak C

Additional $CH_2$ are at δ2.34 (e) and δ2.58 (g). These can be attributed to hydrogens closer than the δ1.59 and δ1.61 multiplets (d), possibly β, to an electron

withdrawing group and are COSY to CH peaks (f) around δ5.2 and δ5.3. The multiple CH peaks are from the presence of isomers. This is confirmed by the multiple acetoxy $CH_3$ peaks seen at δ2.05. According to Starratt and Osgood, the CH multiplets around δ5.2 and δ5.3 come from hydrogens on a carbon α to acetoxy group or to olefinic protons. While the multiplets are observed, there is no evidence of alkenes. However, pervious literature findings suggest the presence of 1,3-digycerides consisting of acetylated fatty acids [35], which indicate the presence of a carbonyl group. The hydrogens β (f) to this carbonyl group would be the multiplets at δ2.58. Starratt et al. observed a doublet at δ2.53 (Figure 5-7), which they "attributed to methylenic protons α to a carbomethoxy grouping appearing as a doublet at δ2.54 in the spectrum of methyl 3-acetoxypalmitate, which enabled the assignment of a doublet at δ2.60 in their spectrum to protons in a similar environment" [35]. Therefore, Starratt and Osgood suggest that there is a methoxy group (most likely part of the glycerol) which is β to a C=O, which is γ to an acetoxy group. Our data agree with this.

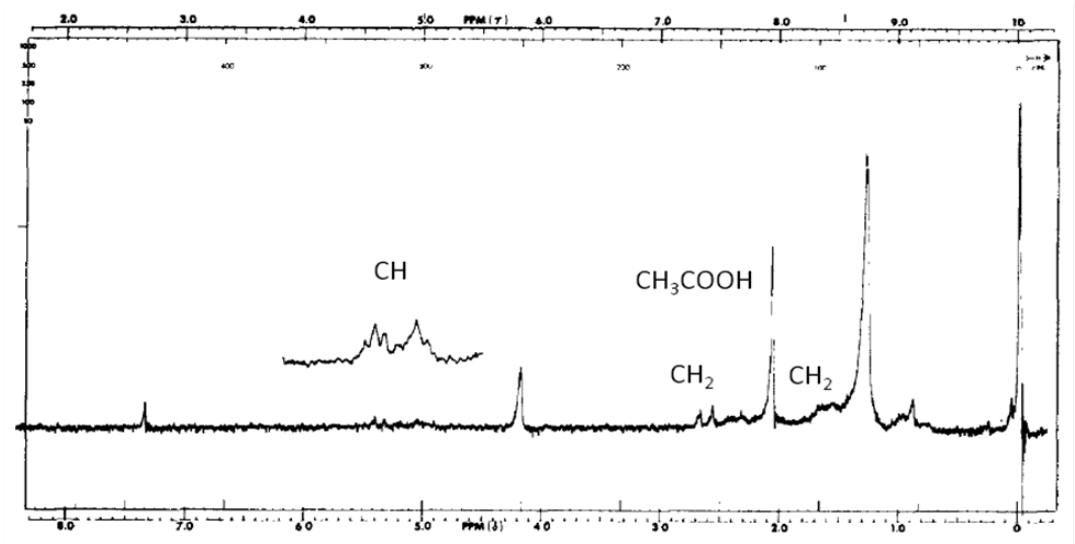



Figure 5-7. [1]HNMR spectrum of Starratt's oviposition attractants from *Cx. tarsalis* eggs [Reprinted by permission from Starratt, A., Osgood, C. An Oviposition Pheromone of the Mosquito Culex tarsalis: Diglyceride Composition of the Active Fraction. *Biochim. Biophys Acta.* **1972***, 208*, 187-193]

There are two sets of doublets of doublets seen between δ4.03 and δ4.35 (Figure 5-8). A single set of doublet of doublets is from the vicinal and geminal hydrogens on a glycerol. That there are two sets of doublets of doublets shows that there are at least two isomers present. The first set doublet of doublets at δ4.25 and δ4.05 are COSY to the second set of doublet of doublets between δ4.13 and δ4.19. The corresponding multiplet is seen at δ3.84. The remaining doublet of doublets between δ4.1 and δ4.2 are COSY to each other as well as the multiplet at δ3.96 (Figure 5-9). It should be noted that the two doublets of doublets shown in Figure 5-8 were also seen in Peaks D, E, F, G, H, I, and J.

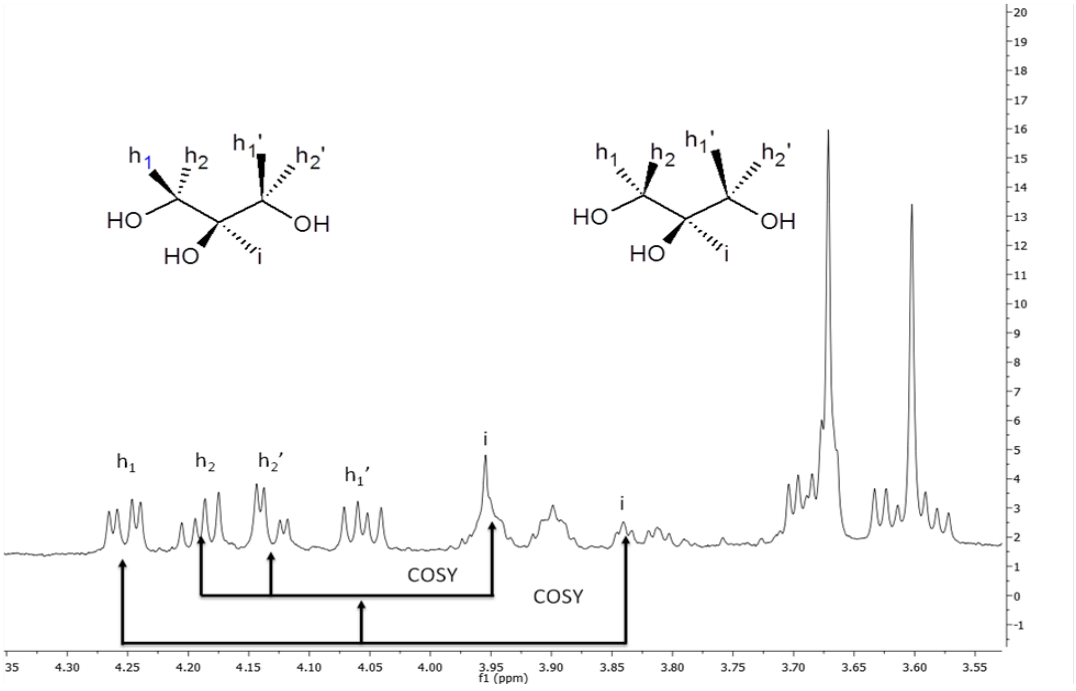



Figure 5-8. Expansion of the 1H NMR spectrum of Peak C between 3.55 and 4.35 showing two doublets of doublets: the first at δ4.25 and δ4.05 and the second at δ4.13 and δ4.19

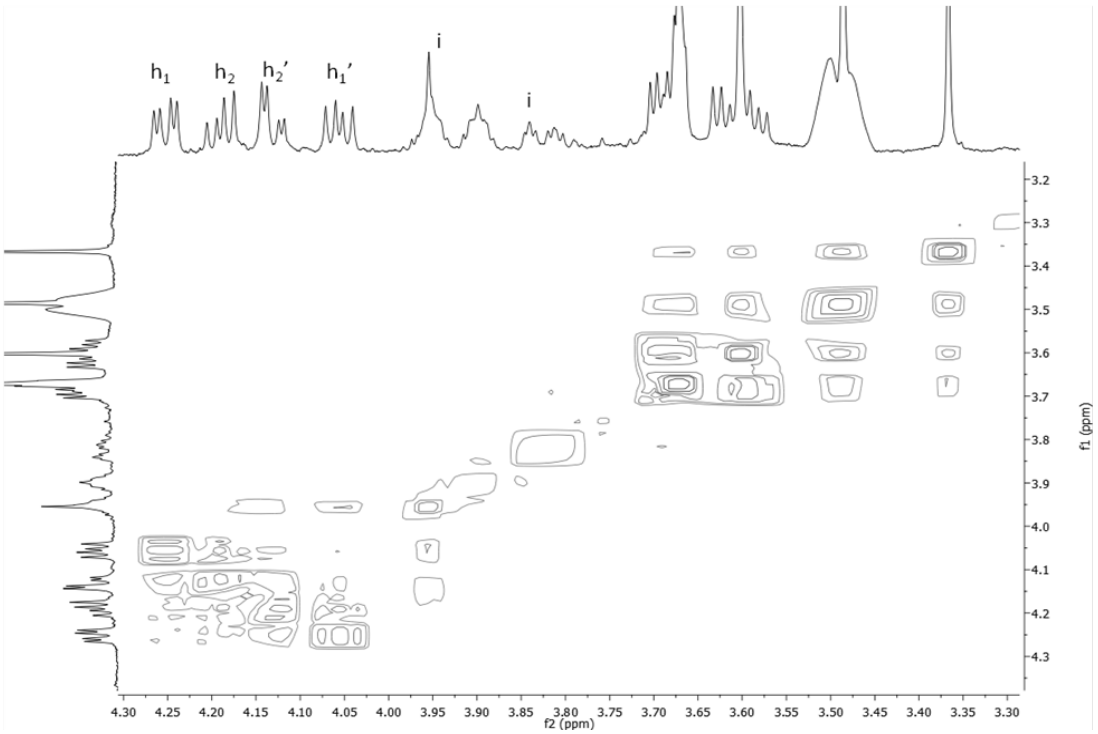



Figure 5-9. $^{1}$H COSY spectrum of doublets of doublets in Peak C

Overall, this data is consistent with the structure suggested in Figure 5-5.

**Peaks D and E**

A suggested structure for Peaks D and E is shown in Figure 5-10.

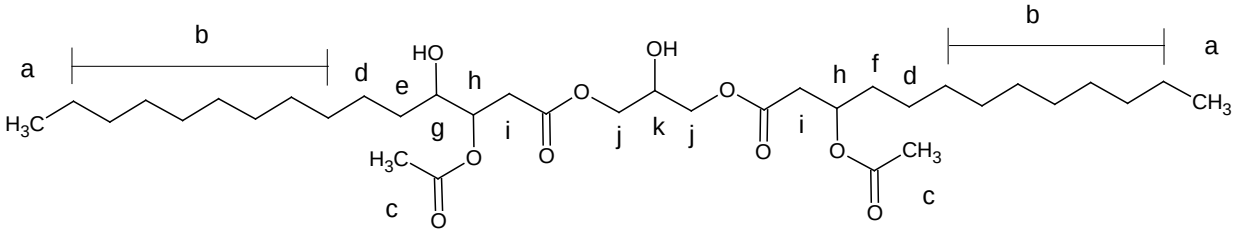



Figure 5-10. Suggested structure for Peaks D and E

The $^{1}$H spectrum of Peaks D and E is very similar to that of Peak C. The both spectra have $CH_2$ hydrocarbons (b) at δ1.24, methyl groups (a) at δ0.87, and an acetoxy methyl (c) at δ2.05; the methyl and acetate methyls integrate 1:1. LC/MS$^{n}$ fragmentation suggests that there are two acetoxy groups; thus, there must also be two

methyl groups. With four degrees of unsaturation, there are two other double bonds besides the two found in the two acetoxy groups. As with Peak C, the hydrogens β (d) to the carbonyl group would be the multiplet at δ1.62. The multiplet at δ2.59 corresponds to the $CH_2$ α (i) to the acetoxy group and the glycerol group. The multiplet at δ2.24 would be the α $CH_2$ (f) on the other side of the acetoxy group, while the $CH_2$ at δ2.36 may be hydrogens α (e) to an electron withdrawing group other than the acetoxy group. The singlet at δ4.87 may be a hydroxyl group (g); LC/$MS^n$ confirms the presence of a hydroxyl group. Finally, the proton peak at δ5.29 would correspond to the CH (h) attached to the acetoxy group.

LC/$MS^n$ fragmentation for Peaks D and E shows that the hydrocarbon chain (b) on one side of the glycerol is longer by $C_2H_4$, and that a hydroxyl group is on the shorter hydrocarbon chain .Although NMR analysis suggests the location of the acetoxy groups, the presence of multiple isomers makes the location of the hydroxyl group difficult.

**Peaks F and G**

LC/$MS^n$ data suggests (Chapter 4, Table 4-4) that Peaks F and G have the following structure (Figure 5-11).

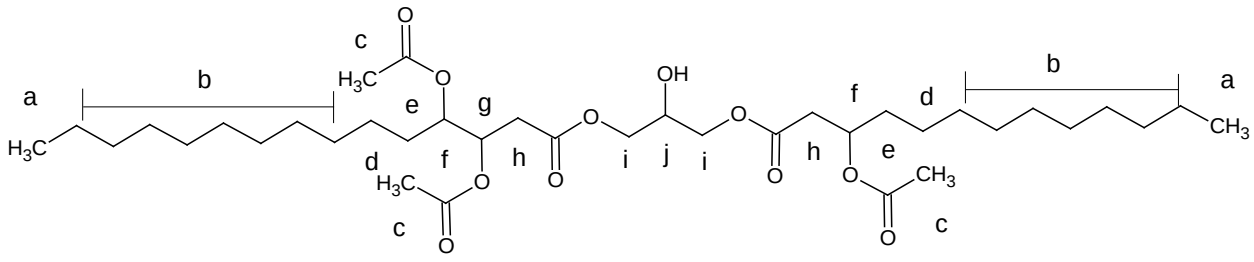



Figure 5-11. Suggested structure for Peaks F and G

The $^1H$ NMR spectra of Peaks F and G (MW 686) also have methyl groups (a) at δ0.87 which are COSY to the $CH_2$s (b) at δ1.25. The multiplets at δ1.61 are $CH_2$ (d),

most likely β to an electron withdrawing group. Acetoxy methyl protons (c) are again observed at δ2.05 with possibly six different peaks, suggesting the presence of isomers; LC/$MS^n$ indicated that this compound has three acetoxy groups (Chapter 4, Figure 4-14). With an unsaturation number of five, this compound would have two additional double bonds. The multiplet (e) at δ2.37 would be α to the acetoxy group; the $CH_2$ protons (g) at δ2.59 would be between the acetoxy group and the carbonyl, as suggested by Peak C. A multiplet at δ5.27 would be the CH (f) next to an acetoxy group.

Several CH singlets at δ5.14, δ4.87, and δ4.56 are also near electron withdrawing groups. Since the location of the third acetoxy group is not determined, these singlets may be from different isomers.

**Peak H**

The $^1H$ spectrum of Peak H (MW 672) suggests the following structure, based on its comparison to Peak C (Figure 5-12).

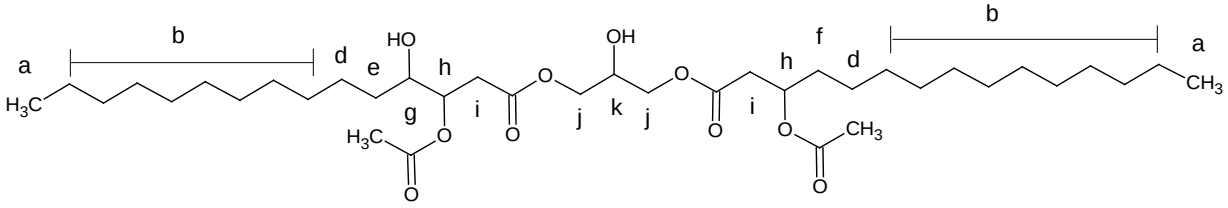



Figure 5-12. Suggested structure of Peak H

Both Peaks C and H show methyl groups (a) at δ0.87 that integrate at a 1:1 ratio with the acetate methyl protons (c) at δ2.05. Hydrocarbon $CH_2$ (b) are observed at δ1.27. LC/$MS^n$ (Chapter 4, Figure 4-17) shows that there are two acetoxy groups, indicating that there are also two methyl groups. The acetoxy methyl protons are COSY to the CH protons (h) at δ5.27 and to the $CH_2$ (i) multiplet found at δ2.58, corresponding

to hydrogens near a carbonyl group. As with Peak C, the hydrogens β to the carbonyl group would be the doublet at δ2.58. The doublet would indicate that the δ2.58 hydrogens are near a single hydrogen, possibly from the acetoxy group. The $CH_2$ (f) multiplet at δ2.24 may also be hydrogens β to an electron withdrawing group different from the acetoxy. The multiplet at δ2.36 corresponds to the $CH_2$ protons (e) β to the acetoxy group, but not near the carbonyl. With four degrees of unsaturation and two acetoxy groups, there must be two carbonyl groups. The two sets of doublets of doublets (j) again suggest the presence of a glycerol group. The doublet of doublets at δ4.25 is COSY to the doublet of doublets at δ4.05; the doublet of doublets at δ4.19 is COSY to the doublet of doublets at δ4.13, which is also COSY to a multiplet at δ3.96 (k). Finally, the multiplet at δ5.27 may be a hydroxyl group (g); LC/$MS^n$ confirms the presence of a hydroxyl group.

**Peaks I and J**

A suggested structure for Peaks I and J is shown in Figure 5-13.

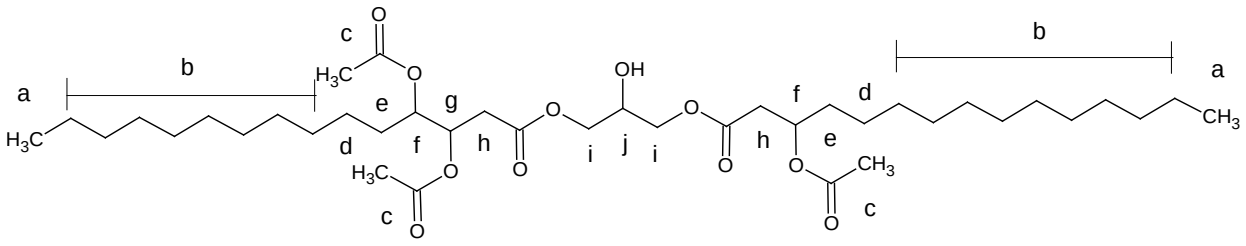



Figure 5-13. Suggested structure for Peaks I and J

Peaks I and J's (MW 714) $^1H$ spectra are also similar to Peak C and show methyl groups (a) at δ0.87, which integrate at a 3:4 ratio with the acetoxy methyl groups (c) at δ2.05. The hydrocarbon $CH_2$s (b) were observed at δ1.27. LC/$MS^n$ analysis shows that there are three acetoxy groups in this compound, which would indicate the presence of two equivalent methyl groups. With five degrees of unsaturation, Peaks I and J have

two additional double bonds besides those in the three acetoxy groups. The acetoxy groups are COSY to the $CH_2$ (d) multiplet at δ1.52 and the δ2.37 $CH_2$ (e) doublet, which is also COSY to the CH (f and g) multiplets at δ5.26 and δ5.20. The $CH_2$ at δ1.27 is COSY to the $CH_3$, which reiterates the hypothesis of a $CH_2$ chain. The $CH_2$ (g) at δ2.60 is most likely a carbonyl similar to those seen in Peaks C and H. Two carbonyl groups would account for the remaining two double bonds in this molecule.

LC/MS data indicated that the structure of Peaks H, I, and J have a common ion at *m/z* 655, and differ only in that one is hydroxylated (Peak H) and one is acetylated (Peaks I and J). Therefore, Peaks I and J should also have a glycerol group. Based on the fragmentation pattern of *m/z* 655 (loss of acetic acid from the $[M+H]^+$ ion at *m/z* 715), both sides of the glycerol group are the same after the loss of one acetic acid. This structure is consistent with the NMR data in that the acetoxy groups are COSY to the carbonyl groups and the hydrocarbon chains on either side. The location of the third acetoxy group may be anywhere on either side of the compound.

## Conclusion

Six major components from *Cx. quinquefasciatus* females' apical droplets were separated by LC/ESI and analyzed by NMR. Table 5-1 gives a list of each peak's known characteristics. Peak B was determined to be the known pheromone, *erytho*-6-acetoxy-5-hexadecanolide, through sample spiking. Structures were suggested for Peaks A, C, D, E, F, G, H, I, and J (Chapter 6, Figure 6-2). The NMR data supports the concept that there are hydroxylated and acetylated versions of the same compound, as well as glycerol groups present in Peaks C, D, E, F, G, H, I and J. Stereochemistry was not determined due to the presence of isomers. Future experiments should include NMR of fractions containing individual isomers.

# CHAPTER 6
# EVALUATION OF OVIPOSITIONAL BEHAVIOR IN THE PRESENSE OF COMPOUNDS IDENTIFIED IN *CULEX QUINQUEFASCIATUS* APICAL DROPLETS

## Introduction

*Erythro*-6-acetoxy-5-hexadecanolide is a known mosquito oviposition pheromone found on each egg. The eggs of *Cx. quinquefasciatus* are laid as floating rafts, and each egg has an apical droplet containing the oviposition aggregation pheromone [9]. Gravid (egg-laying) *Cx. quinquefasciatus* females orient towards *erythro*-6-acetoxy-5-hexadecanolide [31]. While fatty acids have also been found within the acidic hydrolysate of the apical droplets [37], the attraction to these compounds is moderate in the field; thus, they are not considered effective for surveillance.

LC/MS studies (Chapter 4) have demonstrated that the apical droplet contains at least six additional compounds besides the known pheromone. However, the ovipositional activity of these compounds has yet to be determined. Therefore, the objective of this study was to compare the effect of these six additional compounds in LC fractions to *erythro*-6-acetoxy-5-hexadecanolide.

## Experimental

### Mosquito Rearing

*Culex quinquefasciatus* mosquitoes were reared in an insectary at 25 °C and 60% relative humidity. Fluorescent and incandescent lights were controlled by an adjustable timer to simulate natural daylight for ten hours. Two-week old mosquitoes were fed avian blood once a week (UF IACUC protocol D469) and defibrillated bovine blood three times a week; there was a constant sugar source from cotton balls saturated in a 5% sucrose solution. Once a week for colony maintenance, bloodfed mosquitoes were provided a pan containing water with decaying organic matter from

Bermuda grass as an oviposition site. To produce eggs rafts, laboratory raised female mosquitoes were bloodfed with bovine blood and held for five days for completion of ovarian development. On the evening of the fifth day, a pan with water containing organic matter was placed in the cage, and mosquitoes were allowed to oviposit at night. Egg rafts were collected about twelve hours after being laid.

**Sample Collection**

Apical droplets were acquired from egg rafts from *Cx. quinquefasciatus* mosquitoes. Sampling of apical droplets was done with tapered capillary collection tubes previously prepared by heating and pulling the capillary tubes (World Precision Instruments, Sarasota, FL, 1.5 mm outer diameter, 1.12 mm ID, 100 mm length) using a PUL-1 (World Precision Instruments) micropipette puller. The pan containing egg rafts was placed under a microscope at 90x, and the apical droplets were collected by touching them with the tip of the micropipette.

The micropipette tips containing the apical droplets were broken off and sealed in glass GC 2 mL vials (Supelco, 29084-U). Droplets were collected twice a week over a five month period, and stored at -20 °C.

**LC Separation**

Prior to LC separation, the vial was removed from the freezer and the apical droplets were diluted with a 0.5 mL solution of methanol (LC-grade, Honeywell Burdick & Jackson, Muskegon, MI) and isopropanol (LC-grade, Fisher) at a 2:1 ratio. The LC used for compound separation was an Agilent Technologies (Santa Clara, CA) 1100 series G1312A binary pump with a ThermoFisher Scientific (Waltham, MA) Hypurity C8 column (5µm particle diameter; 2.1 x 100 mm long+ guard column). The mobile phase consisted of Solvent A (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in

water (LC-grade, Honeywell Burdick & Jackson)), and Solvent B (2.0 mM ammonium acetate (Fisher Scientific, LC-grade) in isopropanol (LC-grade; Honeywell Burdick & Jackson)). The gradient was [A:B(min)]: 95:5(0):35:65(20)>15:85(65)>5:95(100-120) with a flow rate of 0.2 mL/minute. Approximately 20 µL of apical droplet solution was injected each run, and twelve injections were performed for this experiment. Once the retention time of each fraction was determined by mass spectrometry, the flow tube to the mass spectrometer was disconnected and the seven fractions were manually collected based on retention time. The fractions were collected in 10 mL glass vials. The fractions were then blown down with nitrogen to evaporate the mobile phase, and reconstituted in 1 mL of 100% methanol (Sigma-Aldrich, M3641).

**Bioassays**

Bioassays were conducted using the following design. Two 3 oz, black, plastic oviposition cups, one test cup containing 25 µL of one of the reconstituted compounds in 50 mL of well water and one control cup containing 50 mL of only well water, were placed in opposite corners of a Plexiglas and mesh 12” x 12” x 12” cage containing twenty bloodfed, gravid mosquitoes. A 3 oz white, plastic cup containing cotton balls saturated with a 5% sucrose solution was placed in the middle of the cage.

Attraction was assessed by the number of egg rafts from each cage laid in each oviposition cup overnight. The oviposition activity is expressed as oviposition activity index (OAI) calculated as follows [1]:

$$OAI = \frac{NT - NC}{NT + NC} \quad (6\text{-}1)$$

where *NT* denotes the number of egg rafts in a treated cup and *NC* denotes the number of egg rafts in the control cup. A positive OAI represents a positive ovipositional response, i.e. attraction, whereas a negative OAI represents negative ovipositional response, i.e. repellency.

Each fraction was tested eight times in a ventilated environmental chamber at 25 °C and 60% relative humidity with twenty gravid mosquitoes for each test. Fluorescent and incandescent lights were controlled by an adjustable timer to simulate natural daylight for ten hours. Tests were run overnight because *Culex* are nocturnal mosquitoes; each test ran for approximately sixteen hours. A blank consisting of only water was tested in both the control and test cup. To determine the influence methanol had on *Cx. quinquefasciatus* oviposition, 25 µL of only methanol in 25 mL of water was tested against the control cup containing only water.

**Statistical Analysis**

A student's t-test was performed to compare the different fractions' influence on *Cx. quinquefasciatus* oviposition. A student's t-test determines if two means at a $P < 0.05$ level are considered significantly different by ascertaining if the difference between the means is on account by the error or variation of the samples or due to the treatment.

## Results and Discussion

The separation of each fraction is shown in Figure 6-1. The volume of each fraction tested was determined by comparing the reconstituted pheromone fraction to that of 0.05% v/v of the pheromone standard in methanol.

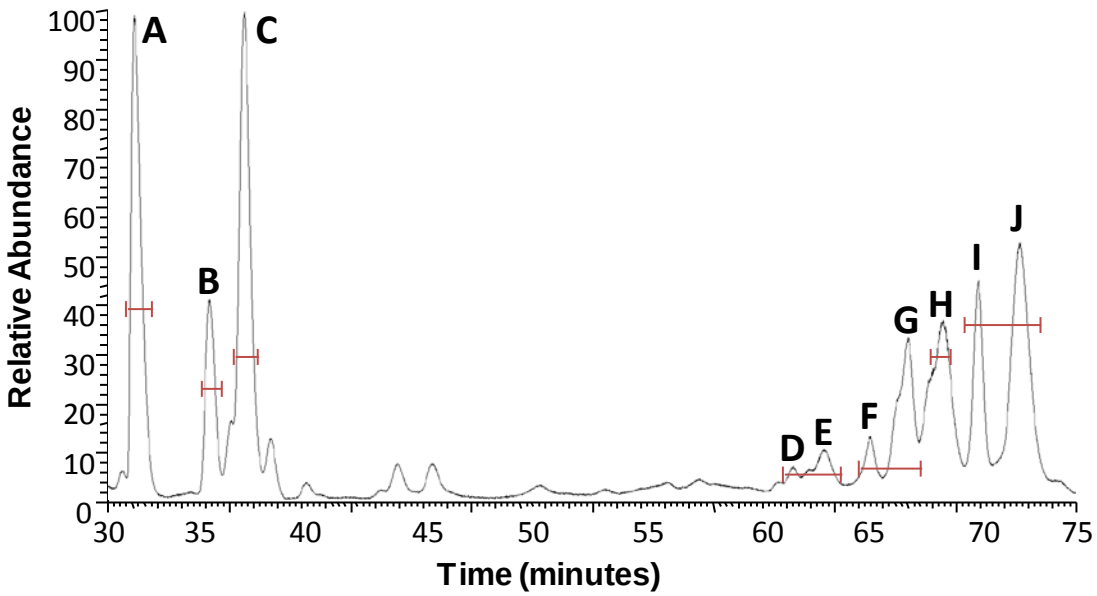



Figure 6-1. Total ion chromatogram of LC/(+)ESI-MS peaks from *Cx. quinquefasciatus* apical droplets showing each fraction's collection time window

Approximately 25 µL of Fraction B (pheromone) solution provided the same ovipositional response as 12.5 µL of the pheromone standard solution in methanol. This corresponded to 0.05% v/v of reconstituted fraction in water. No response was observed with lower doses of either solution. While the concentration of the fractions was unknown, the volumes of the material in the fractions were present in the same ratio as found in *Cx. quinquefasciatus* apical droplets. Thus, if a fraction did not demonstrate any influence on oviposition behavior in the bioassays, it could be assumed that the same fraction would not influence *Cx. quinquefasciatus* oviposition behavior in the wild.

The oviposition activity of the seven fractions, water, and methanol (solvent) were compared to water alone (control) (Figure 6-2). This is the first time these fractions were separated from the apical droplets and compared for relative attraction.

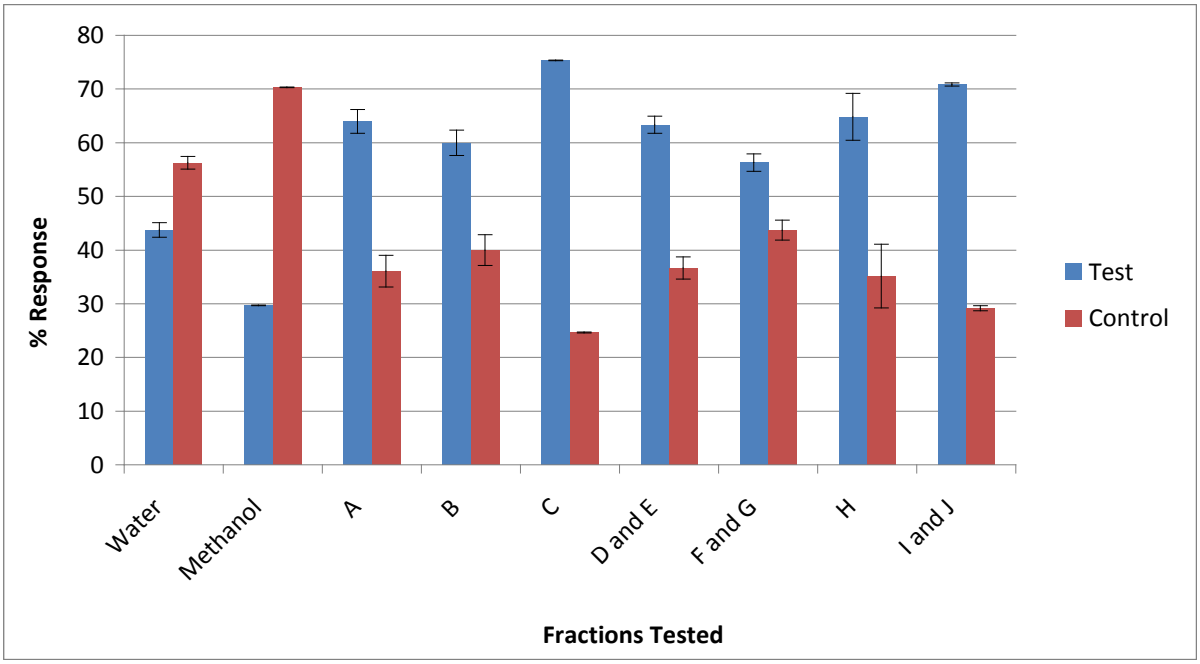



Figure 6-2. Percentage of total of eight replicates of *Cx. quinquefasciatus* egg rafts laid in test cups containing 50 mL of water and 25 µL of reconstituted fraction and control cups containing 50 mL of water

The 12% difference between the two water cups indicates that additional replicates need to be performed to demonstrate that there is little to no preference between cups containing the same material. However, 25 µL of methanol in 50 mL of water had a negative influence on *Cx. quinquefasciatus* oviposition. All tested fractions and the pheromone, Fraction B, in 25 µL of methanol overcame the methanol's negative influence and produced a positive effect on *Cx. quinquefasciatus* oviposition.

Fractions F and G had the least influence on where *Cx. quinquefasciatus* chose to lay their eggs. However, the fraction's influence by itself needs to be established to determine the influence that only the fraction has on oviposition, rather than the fractions in methanol. Fractions A, D, and E have a similar influence when compared to the known pheromone. This is confirmed when each fraction's OAI was calculated (Table 6-1):

Table 6-1. Oviposition activity index and student t-test values of tested fractions

| Fraction | OAI | T-Test |
|---|---|---|
| Methanol | -0.41 | -1.96 |
| Water | -0.13 | -0.53 |
| F and G | 0.13 | 0.92 |
| B | 0.20 | 1.88 |
| D and E | 0.27 | 1.14 |
| A | 0.28 | 1.18 |
| H | 0.30 | 2.28 |
| I and J | 0.42 | 2.45 |
| C | 0.51 | 2.30 |

Table 6-1 shows that all fractions have a positive influence on *Cx. quinquefasciatus* oviposition. The student's t-test showed that Fractions C, H, I, and J had a 95% confidence interval of having a positive influence on *Cx. quinquefasciatus* oviposition; Fraction B, the pheromone, had only a 90% confidence interval. Methanol had a 90% confidence interval of having a negative influence on oviposition. All other fractions tested did not demonstrate a statistically significant difference between them and the water control.

Suggested structures of the fractions tested are shown in Figure 6-3

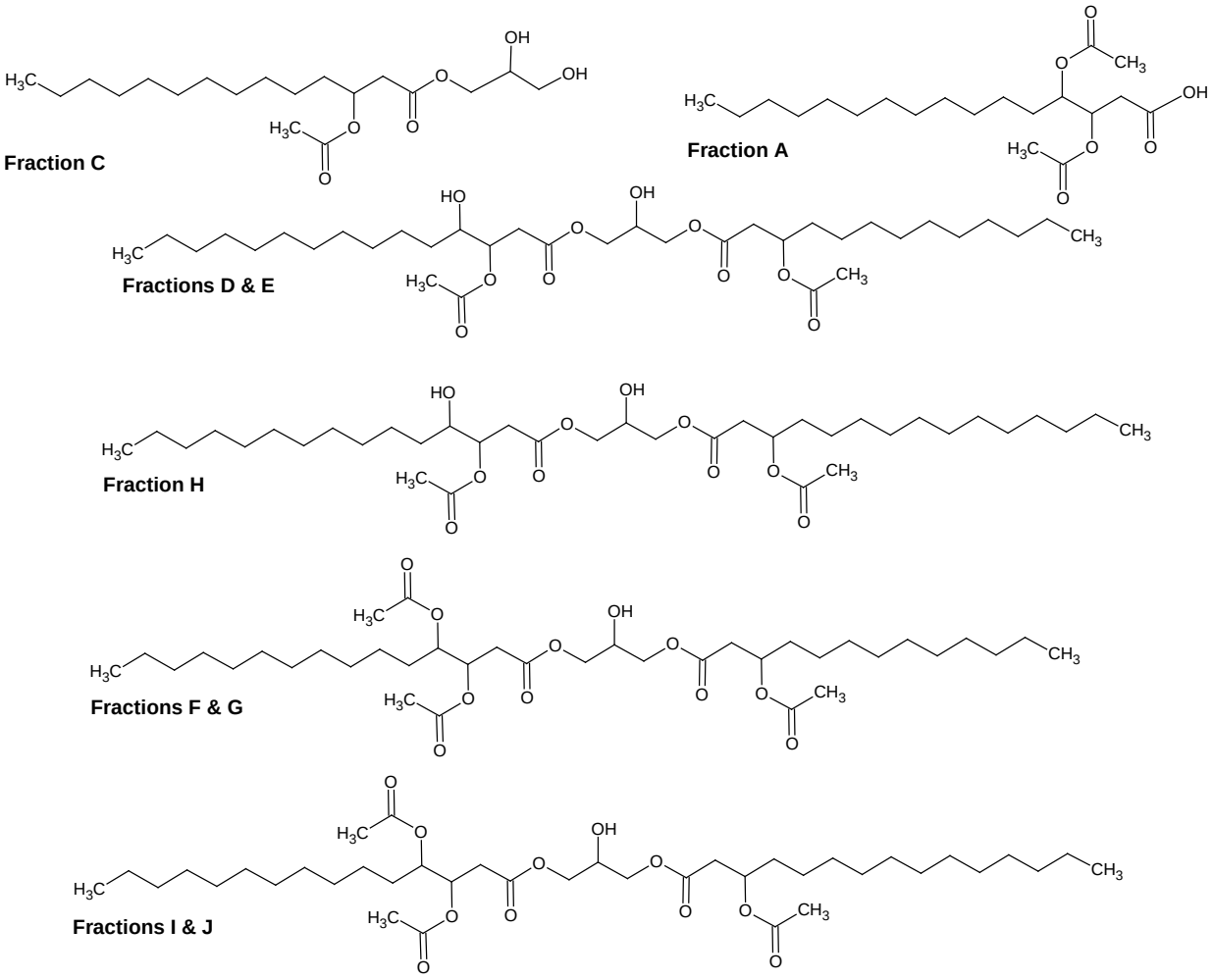



Figure 6-3. Suggested structures for *Cx. quinquefasciatus* apical droplet fractions

Fraction A differs from the other fractions in that it is not a mono or diglyceride. It is possible that fractions with a statistically significant positive influence on *Cx. quinquefasciatus* oviposition must contain a glycerol group. However, this would imply that Fractions D, E. F, and G should have a positive influence, which they did not. Therefore, another factor must come into play, possibly the hydrocarbon chain length.

Previous research indicated that *Cx. quinquefasciatus* are repelled by straight-chain fatty acids containing $C_5$ – $C_{13}$ [21]. In fractions D, E, F and G, the shorter hydrocarbon chain is a $C_{15}$. It is possible that the straight-chain fatty acid repellent range might extend beyond $C_{13}$ to $C_{15}$.

Overall, five of the fractions had a positive influence on *Cx. quinquefasciatus* oviposition, with Fractions B, H, I, and J having a more positive influence than the known pheromone. This observation would make these three fractions possible candidates for improved mosquito control. Additional research would involve comparing these fractions and their compounds against each other.

## Conclusions

Because of mosquitoes' ability to develop resistance to insecticides, the evolution and spread of resistance genes among mosquitoes across the continents is a topic of considerable scientific interest [6]. Repellents, which in contrast to pesticides do not kill the mosquitoes, reduce the likelihood of disease transmission. Many repellents feel greasy and unpleasant on the skin, making people less inclined to use them. Pheromone-based mosquito control could provide a beneficial alternative method to reduce the use of pesticides and repellents.

In this research, mosquitoes tended to avoid cups containing methanol, but chose to oviposit in cups containing all seven fractions of the apical droplet. All fractions had a positive influence on mosquito oviposition. Three of these fractions, Fractions B, H, I, and J, more positively influenced oviposition by gravid *Cx. quinquefasciatus* than the known pheromone, *erythro*-6-acetoxy-5-hexadecanolide. Methanol had a statistically significant negative influence on *Cx. quinquefasciatus* oviposition.

Future testing should involve chirally separating each fractions' isomers, determining each isomer's stereochemistry using NMR, and performing bioassay with each isomer in a solvent that does not influence mosquito oviposition in order to avoid unintentional influences, such as that observed with methanol.

# CHAPTER 7
# CONCLUSIONS AND FUTURE WORK

## Conclusions

The nuisance created and the diseases transmitted by mosquitoes still exist in areas where significant control strategies are in use. Therefore, additional approaches to mosquito control are needed to complement existing strategies. The use of oviposition pheromones in combination with lethal traps remains an under-explored area of research.

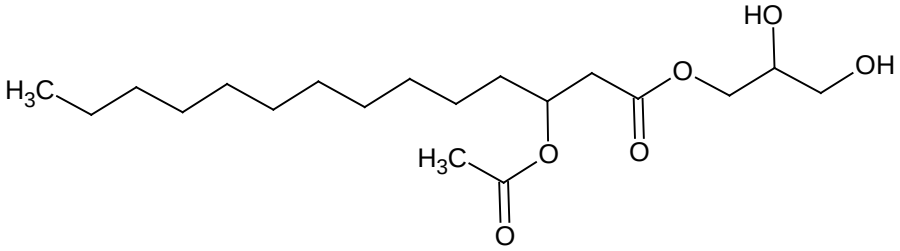
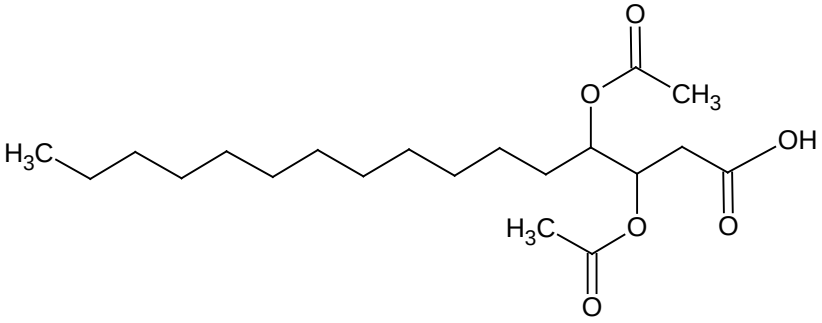
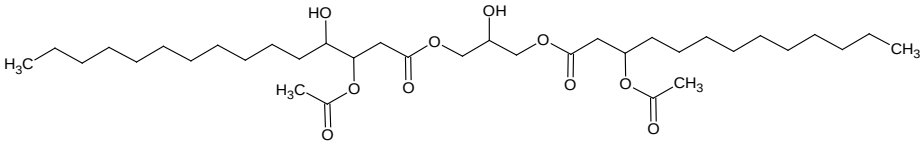
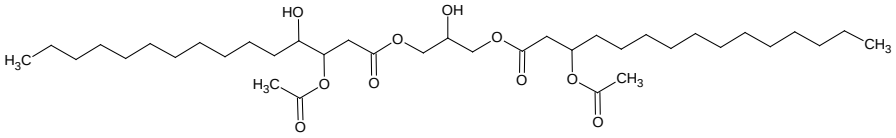
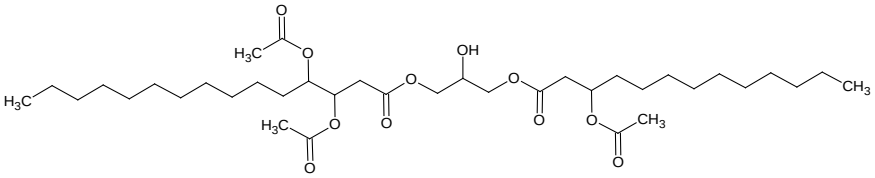
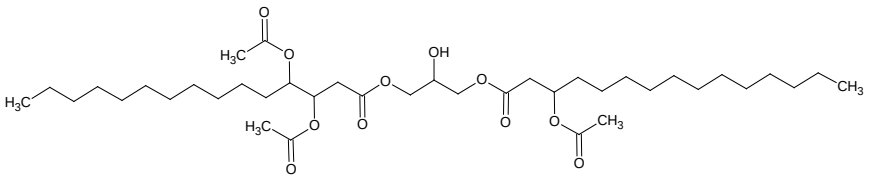
Pheromones play an important part in nature by eliciting a social response in members of the same species. Pheromones can act as alarms or aggregates, mark territories or trails, or pass on information such as the availability of the female for breeding. Through a better understanding of the pheromones of insects such as mosquitoes, one may be able to manipulate behavior of the species for surveillance or management purposes [30].

For analysis of egg pheromone extracts, the initial concern involved optimizing the GC/MS parameters, injection port temperature, oven temperature program, column selection, and sampling methods, in order to obtain good resolution of the components in the apical droplet. An injection port isothermal temperature of 260 °C with an oven temperature program of a 1 minute hold at 35 °C followed by a 5 °C/minute ramp to 260 °C, concluding with a twenty minute hold on a DB-5MS column demonstrated good resolution for compounds with a wide range of boiling points. A PDMS SPME fiber was used for sample collection from the eggs because of its low sample carryover and ability to be easily cleaned and reused.

Preliminary GC/MS findings indicated the presence of the known *Culex* oviposition pheromone, *erythro*-6-acetoxy-5-hexadecanolide, as well as several novel compounds. Additionally, possible isomers of the pheromone were observed. Because of the novel compounds' high proton affinity, isobutane CI was employed; however, molecular weights of the novel compounds could not be determined by this method due to a lack of $[M+H]^+$ ions. However, novel compounds included hydroxylated and acetylated versions of the same compounds.

Data from the $LC/MS^n$ and HRMS spectra provided evidence of seven major compounds within the *Cx. quinquefasciatus* apical droplet, one of which is the known pheromone, *erythro*-6-acetoxy-6-hexadecanolide. NMR aided in establishing possible structures for the remaining six compounds. The six novel compounds' molecular formulae, molecular weights, and suggested structures are shown below (Table 7-1). One is a monoglyceride (MW 360); four are diglycerides (MW 644, 672, 686, and 714), and several are present as isomers, based on LC separation, and the sixth (MW 372) is a diacetylated fatty acid diol.

Table 7-1. Molecular formulae, molecular weights, and suggested structures of compounds found within the *Cx. quinquefasciatus* apical droplet

| Molecular Formula | Molecular Weight (g/mol) | Suggested Structure |
|---|---|---|
| $C_{19}H_{36}O_6$ | 360.2512 |  |
| $C_{20}H_{36}O_6$ | 372.2512 |  |
| $C_{35}H_{68}O_{10}$ | 644.4499 |  |
| $C_{37}H_{68}O_{10}$ | 672.4812 |  |
| $C_{37}H_{66}O_{11}$ | 686.4605 |  |
| $C_{39}H_{74}O_{11}$ | 714.4918 |  |

Of these compounds, MW 360, 672, and 714 demonstrated greater biological activity to positively influence the oviposition of *Culex quinquefasciatus* mosquito than the pheromone, *erythro*-6-acetoxy-5-hexadecanolide, alone.

## Future Work

Due to their positive influence on *Cx. quinquefasciatus* oviposition, the MW 360, 672, 714 compounds may be good candidates to control the spread of certain arboviral diseases. However, certain aspects must be determined:

The bioassays performed within this work have shown that methanol has a negative influence on *Cx. quinquefasciatus* oviposition. With this in mind, the influence of the fractions presented in methanol may be stronger than observed. Therefore, experiments should be repeated using material diluted in a solvent that does not influence mosquito oviposition.

Stereochemistry can play a role in biological activity. When the bioassays were performed, the fractions were tested as mixtures of isomers. Additional studies need to be performed with each novel fractions' individual isomers. Such separation can be performed on an LC chiral column. This separation will also allow for NMR experiments with purer fractions. Once the stereochemistry of the biologically active fractions has been established, synthetic compounds may be developed and tested.

Concentration and synergy also plays an important part in oviposition; too much of one compound or the lack of another and can result in a negative influence. Therefore, the concentration of each novel compound in relation to *erythro*-5,6-hexadecanolide should to established. Additional studies with identified compounds examining mixtures for synergy and optimal concentration of each compound should be conducted to maximize influence over *Cx. quinquefasciatus* oviposition.

LC/MS$^n$ also established the presence of other novel compounds not discussed in this work. Due to the low abundance of these other compounds, their influence on oviposition was not determined. Future studies should investigate these compounds to determine their molecular formulae, molecular weights, structures, stereochemistries, and biological activities.

Finally, previous studies have demonstrated that other *Culex* species are attracted to *erythro*-6-acetoxy-5-hexadecanolide [59]. However, other publications have indicated different species within the same genus are not necessarily attracted to each others' egg rafts [61]. Therefore, additional studies should be conducted to determine the major compounds within other *Culex* species' apical droplets as well as the compounds' concentrations.

In conclusion, by performing these future experiments, one will be able to ascertain the compounds, their isomer structures, and their biologically active concentrations to more positively influence mosquito oviposition beyond the currently known pheromone alone. These novel compounds could provide the basis for management strategies that could encourage female mosquitoes to lay their eggs in an environment detrimental to their larvae, which could result in control of mosquitoes and reduction of vector-borne diseases.

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## BIOGRAPHICAL SKETCH

Hillary Lathrop is from outside the Boston area in Massachusetts. She received her B.A. in Chemistry from Smith College in 2004 and her M.S. in Analytical Chemistry in 2009 from UMASS Lowell. She was employed full-time while she pursued her M.S. degree. She has held R&D positions at adhesive companies (Ivex Novacel and DSM Neoresins), engineering companies (InfoSciTex), and manufacturing companies (Bionostics). She began her graduate career at UF in the spring of 2010 and graduated in the summer of 2013.

It was through her employment that she was first involved in mosquito-related chemical research in response to a U.S. government solicitation for an effective solution for repelling mosquitoes and sand flies from soldiers fighting abroad. The procedure involved synthesizing European Elm Beetle pheromone. Looking at the two together, she formulated a plan of synthesizing mosquito and sand fly oviposition pheromones to encourage female arthropods to lay their eggs where they could easily be destroyed. While the initial proposal was not a success, she continued with her research, and two proposals later she was granted research funding. More importantly, she found that she had a true passion for this research.

During her proposal writing, she came across Dr. Yost's co-authored paper titled "Analysis of Human Skin Emanations by Gas Chromatography/Mass Spectrometry. 2. Identification of Volatile Compounds That Are Candidate Attractants for the Yellow Fever Mosquito (*Aedes aegypti*)". Upon her arrival at UF, Dr. Yost put in her in contact with Dr. Bernier at the USDA, and she has been working on the analysis of the apical droplet from *Cx. quinquefasciatus* egg ever since. In understanding the chemical composition of the *Culex* apical droplet, she hopes to have a better understanding of

this mosquito and manipulate the insect in order to suit our needs (e.g., influence *Culex* mosquitoes to oviposit at a site which is detrimental to the larvae).