ANALYSES OF ESSENTIAL AND EDIBLE OILS, AND CONSTITUENTS THEREIN, AS CANDIDATE REPELLENTS FOR THE YELLOW FEVER MOSQUITO *AEDES AEGYPTI* L. (DIPTERA: CULICIDAE)

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PEST MANAGEMENT

In the Department of Biological Sciences

SIMON FRASER UNIVERSITY

Fall 2009

APPROVAL

Name: **Cory James Campbell**

Degree: **Master of Pest Management**

Title of Thesis: **Analyses of Essential and Edible Oils, and Constituents Therein, as Candidate Repellents for the Yellow Fever Mosquito *Aedes aegypti* L. (Diptera: Culicidae)**

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ABSTRACT

Some plant essential and edible oils repel mosquitoes but often quantitatively minor repellent constituents therein remain unknown. In gas chromatographic-electroantennographic detection (GC-EAD) analyses of catnip, cinnamon, citronella, cumin, eucalyptus, geranium, ginger, melissa, peppermint, rosemary, and thyme essential oils, 43 constituents elicited responses from antennae of female yellow fever mosquitoes, *Aedes aegypti* L. (Diptera: Culicidae). GC-EAD analyses of soybean oil (active ingredient of BiteBlocker™) were inconclusive. In GC-EAD analyses of garlic oil, 10 constituents and 4 thermal degradation components stimulated antennae. Dual-port olfactometer and screened-cage experiments failed to reveal any repellent effect associated with soybean oil. Diallyl trisulfide and diallyl tetrasulfide as EAD-active constituents of garlic oil were significantly more repellent to female *Ae. aegypti* than control stimuli. I conclude that GC-EAD screening of plant essential oils and other sources is a viable technology to detect new quantitatively minor constituents that could be potent repellents when tested at an appropriate dose.

Keywords: Repellent; Mosquito; *Aedes aegypti*; Essential oils; Garlic; Soybean Oil; Gas Chromatographic-Electroantennographic Detection; GC-EAD

DEDICATION

To my mom… for everything. For being my educator, my guide, my friend.

To Christine De Pape, for standing by my side. For your unrelenting support and faith in me. For being my confidante, companion, and partner in crime.

ACKNOWLEDGEMENTS

I would like to thank my senior supervisor, Dr. Gerhard Gries, for his invaluable guidance, enthusiasm, and support throughout the course of this work. Thank you to Mrs. Regine Gries for her wealth of experience and knowledge, for all her technical and moral support, and for truly being the lab mum.

I thank Dr. Grigori Khaskin for his chemistry expertise and my Gries Laboratory colleagues for advice. I would also like to thank Dr. Carl Schwarz for his assistance with statistical analyses, and Robert Birtch and Stevo DeMuth for their contributions to graphical illustrations.

I thank my supervisory committee member Dr. Carl Lowenberger for review of my thesis and constructive feedback through the course of this research, as well as providing the initial *Aedes aegypti* colony necessary to complete this work. I also thank my external examiner Dr. Sheila Fitzpatrick for her valuable suggestions that have led to improvements of this thesis.

Special thanks go to Thomas Cowan, Christine De Pape and Eric Siljander for assistance with feeding, and to Pilar Cepeda with assistance in maintaining the colony.

I would also like to thank Thomas Cowan, Zaid Jumean, Kevin Lam, Eloise Rowland, Eric Siljander, and Nathan Woodbury for their very good friendship. Along with advice and guidance on this work, they also provided many pub nights, movie

nights, games nights, camping trips, and ski weekends that were integral to the research experience.

This research has been made possible with significant financial support. I thank the Dr. John Yorston Memorial Graduate Scholarship in Pest Management, a Graduate Fellowship from Simon Fraser University, Natural Sciences and Engineering Research Council of Canada (NSERC)-Industrial Research Chair to G.G. with SC Johnson Canada, Contech Enterprises Inc. and Global Forest Science (GF-18-2007-226 and GF-18-2007-227) as the industrial sponsors.

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GLOSSARY

Anthropophilic	The preference of a parasite for the human host as a source of blood or tissues over an animal host.
Attraction inhibitor	An insect fails to proceed to a previously attractant stimulus; involves combination of at least 2 chemicals.
Cosmotropical	Found globally within tropical regions.
Deterrent	Something that prevents feeding or oviposition when present in a place where insects would, in its absence, feed or oviposit; something that protects against bodily harm.
Emulsion	Any colloidal suspension of a liquid in another liquid.
Essential oil	Terpenes and other volatile compounds from plants obtained by steam or water distillation or pressing; they are hydrophobic and mostly aromatic.
Excipient	An inert substance used as a diluent or vehicle for a drug.
Excitorepellent	The ability of an insecticide, such as DDT and some pyrethroids, to irritate insects sufficiently that they fly away before knockdown, even from sublethal exposure.
Formulation	Defined chemical product mixture, usually meaning the commercialized version of a special formula.
Repellent	Something that causes insects to make oriented movements away from its source.
Synanthropism	Ecologically associated with humans; habit of an organism of living in or around human dwellings.
Vector	An organism that transmits a disease agent (pathogen) from one host to another.

1 PRINCIPLES OF INSECT REPELLENTS AND MOSQUITO BIOLOGY

1.1 Repellent Terminology

The term "repellent" is often used with different meanings. The traditional definition describes a repellent as a thing that causes oriented movement away from a source, essentially the opposite of an attractant, which is a thing that causes oriented movement towards a source (Dethier *et al.* 1960). With respect to arthropod communication, repellents or attractants may have visual, bioacoustic, or chemical, characteristics. Chemicals mediating interactions between organisms are termed semiochemicals (message bearing chemicals) (Nordlund and Lewis 1976). Colloquially the term repellent has a broad, generic meaning describing commercial products rather than any technical properties of a semiochemical. It is important to discern between chemicals that cause specific oriented movement away from a source (repellent chemical), and chemicals that physiologically prevent an insect from responding to an otherwise attractive stimulus. The latter chemicals are termed attraction inhibitors (Dogan and Rossignol 1999).

Repellent chemicals express themselves through two modes of action based, in part, on the chemical's physical state. Contact repellents have to be contacted by the arthropod to elicit a repellent response, whereas a vapour or volatile repellent is detected in air (White 2006). Repellent chemicals applied to skin and clothes for protection from haematophagous arthropods are termed topical repellents and are capable of functioning

through either mode of action. A topical repellent often requires the use of a carrier agent and/or specific formulation for (better) delivery of the repellent chemical. A spatial repellent is a volatile repellent used to protect an area of space. Here, I will describe the history and development of repellents and the significance of their use for protection from haematophagous arthropods, particularly with respect to the yellow fever mosquito, *Aedes aegypti*.

1.2 Biology and Ecology of Mosquitoes

The taxonomic family Culicidae (mosquitoes) belongs to the order of the true flies (Diptera) that include 41 genera in the subfamilies Anophelinae and Culicinae. Adult mosquitoes have one pair of scale-covered wings and one pair of halteres, long legs and a long slender abdomen. The females' mouthparts are modified for piercing and sucking (solenophagy) with the proboscis consisting of the six piercing stylets labrum-epipharynx, a pair of mandibles, a pair of maxillae, and the hypopharynx, all ensheathed in the elongated labium. Adult females of almost all genera are ectoparasitic on vertebrates. Except for Antarctica, mosquitoes are present on every continent of the globe and adapted to almost every habitat. According to larval habitat species are grouped into container/tree-hole breeding and pool breeding (Chase and Knight 2003; Julian 2009). The former include most *Aedes* spp., which deposit eggs singly along the inside wall of a container at the waterline. The eggs of these species typically undergo a period of desiccation and hatch when immersed in water. Pool breeding species further divide into three specific groups (Chase and Knight 2003; Julian 2009): 1) Permanent pool species such as *Anopheles* spp., *Culex* spp., and *Mansonia* spp. that require a stagnant body of nutrient rich water, with females ovipositing directly on the water

surface; 2) Transient pool water species, such as *Culiseta* spp., that also oviposit is directly on the water surface, often associated with culverts, canals, or ditches; 3) Floodwater species, such as many *Aedes* spp., that deposit eggs singly on damp soil in areas prone to flooding, with eggs remaining dormant and awaiting an influx of water for hatching.

The overall fitness and survival of mosquitoes results from mostly genetically determined behaviour in response to various internal and external stimuli, the latter of which are principally olfactory. Olfactory receptors on the antennae and maxillary palps detect semiochemicals for sugar feeding, host-seeking, oviposition, and to a lesser extent mate finding. Species-specific contact pheromones have been described, but their functional roles are not completely understood. Mating behaviour, which normally occurs 3- to 5-d post-emergence, is believed to be mediated primarily by acoustic signals. Conspecific males recognize the slightly lower wing-beat frequency of females (Göpfert *et al.* 1999).

Nectar is the sole food of male mosquitoes. Females of many species imbibe nectar prior to host seeking, presumably as a source of energy for the host-seeking flights. Mosquitoes locate host plants by way of volatile semiochemicals. For example, volatiles of the oxeye daisy, *Leucanthemum vulgare*, are attractive to *Ae. aegypti* (Jepson and Healy 1988). The nectar sugars supplement energy reserves thus enabling females to fly for extended distances in search of a blood host, a behaviour that is mediated heavily by olfaction. Host-seeking mosquitoes detect host-derived chemical cues by the two major sensory organs. The maxillary palps detect and assess CO_2 levels which activate and sensitize mosquitoes to other host-related odors that are received primarily by the

olfactory receptors of the antennae and some receptors on the palps (Zwiebel and Takken 2004; Dekker *et al.* 2005). Approximately 350 different chemical compounds are associated with human skin, of which L-lactic acid, ammonia, acetone, and carboxylic acids are integral kairomones during host seeking behaviour (Bernier *et al.* 2000; Steib *et al.* 2001; Bernier *et al.* 2003; Smallegange *et al.* 2005).

The saliva of mosquitoes contains various compounds that prevent haemostasis and the inflammatory response of the host (Rodriguez and Hernández-Hernández 2004). Antigenic compounds of the saliva cause an initial histamine-mediated immunological response resulting in the classic itchy bite, which disappears after a few hours, followed by a delayed cell-mediated response appearing 1-2 days later as the signature hive (Hill *et al.* 1996). Not only are the bites of mosquitoes a nuisance, the exchange of fluids between the mosquito and the host creates the opportunity for the transfer of pathogens that may cause disease. Mosquitoes are vectors of some of the most important disease-causing pathogens, including the protozoan parasite *Plasmodium* spp. (the causal agent of malaria), Western Equine Encephalitis virus, West Nile virus, Dengue Fever and Yellow Fever flaviviruses, and many more pathogens responsible for significant morbidity and mortality. As such, the mosquito has received the distinct notoriety as the most dangerous animal in the world.

1.3 Life History of *Aedes aegypti*

The cosmo-tropical anthropophilic yellow fever mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae), is a container or tree-hole breeding mosquito (Roberts and Janovy, 2000). As an exceptionally resilient species, *Ae. aegypti* breeds in virtually any small container of water, while their eggs can survive extensive periods of desiccation

(Malavige *et al*. 2004). Adults are diurnal and highly synanthropic, typically resting indoors in living rooms and bedrooms optimizing their vector capacity through increased biting opportunity (Mackenzie *et al.* 2004; Malavige *et al*. 2004). Consequently, *Ae. aegypti* is referred to as the most efficient of several *Aedine* species to vector disease-causing *flaviviruses*, including the Yellow Fever virus and Dengue Fever virus (Mackenzie *et al*. 2004; Malavige *et al.* 2004; Hill *et al.* 2005).

The detriment of yellow fever is immense. Even today, although an effective vaccine exists there are still an estimated 200,000 cases and 30,000 deaths reported annually, with nearly 90% occurring in Africa alone (Tomori, 2004). Dengue fever virus has also demonstrated its morbidity with an estimated 50-100 million cases worldwide, and a further 500,000 cases of Dengue Hemorrhagic Fever DHF, resulting in *circa* 24,000 deaths annually (Zanotto *et al*, 1996; Mairuhu *et al*, 2004). Although promising candidates are currently in research, there is no vaccine against DF viruses (Mackenzie *et al*. 2004; Malavige *et al*. 2004). For these reasons, dengue fever ranks as the most important mosquito-borne viral disease in the world.

Female *Ae. aegypti* deposit eggs singly on the inner walls of containers of standing water, and following a period of desiccation eggs hatch when submerged. Larvae are aquatic and proceed through four instars before metamorphosing into active non-feeding pupae. Pupation is relatively short with fully developed adults emerging through a split in the integument of the thorax after around 2-3 days. Under ideal conditions, males live up to one month while adult females live up to four or five months (Roberts and Janovy, 2000). Both males and females feed on nectaries and extra-floral nectaries of plants as a source of carbohydrates for energy, but only females feed on the

blood of humans to supplement their diet with protein for the developments of eggs. Several gonotropic cycles occur in a female's life and consequently blood feeding occurs several times on many different hosts, a behaviour responsible for their incredible vector capability.

1.4 History of Repellent Protection

The use of repellents for the protection from biting arthropods has a long history that predates humanity. Many animals utilize heterospecifics to procure substances for repelling predators and parasites. For example, capuchin monkeys anoint their pelage with odorous materials that repel ectoparasites. The black capped capuchin, *Cebus apella* (L.), exhibits "anting" behaviour. It rubs its fur with carpenter ants (*Camponotus* spp.) that then secrete the defensive semiochemical formic acid, which in turn repels the tick *Amblyomma cajennense* (F.) (Falotico *et al.* 2007). During peak mosquito activity, the wedge capped capuchins, *Cebus olivaceus,* rubs its fur with the millipede *Orthoporus dorsovittatus* (Order: Spirostreptida), anointing itself with the millipede's insect-repelling benzoquinone secretions (Weldon *et al.* 2003). The white faced capuchin, *Cebus capucinus,* is particularly fascinating in that fur rubbing can either be a solitary event, or a communal behaviour in which group members synchronously anoint their fur with plants that have repellent properties (Meunier *et al.* 2008). Other animals that anoint their pelage or plumage for protection from ectoparasites include the black-handed spider monkey, *Ateles geoffroyi*, and owl monkeys, *Aotus* spp. (Meunier *et al.* 2008), and various species of birds (Falotico *et al.* 2007). Defensive anointing is a behavioural adaptation believed to be an "extended phenotype" of these animals, as evidenced by the

anointing behaviour of captive-born capuchins in response to the presence of millipede benzoquinones (Weldon *et al.* 2003; Weldon 2004; Moore and Debboun 2006).

Haematophagous arthropods that annoy humans and other animals through their biting activity, and often vector disease-causing pathogens, comprise members of numerous insect taxa including, but not limited to, flies in the families Culicidae, Tabanidae, Psychodidae, Simuliidae, Muscidae, and Ceratopogonidae (all Diptera), bugs in the families Cimicidae and Reduviidae (all Hemiptera), lice in the order Anoplura, and fleas in the order Siphonaptera, as well as non-insectan arthropods, particularly ticks in the order Acarina.

The burning of various plants/plant oils and other materials to produce smoke as a spatial repellent is the oldest recorded method for repelling nuisance mosquitoes by humans. Records include the writings of Herodotus (484 BCE – ca. 425 BCE) reporting Egyptian fisherman burning oil from the castor-oil plant (Charlwood 2003), as well as the Geoponika (10th century) and the ancient Sanskrit *Yoga Ratnakara* (17th century) describing the burning of various plants, fish, bones, and dung (Moore and Debboun 2006). Smoke probably inhibits detection of semiochemicals by chemoreceptors and/or masks odors, thus preventing host location. It may also reduce gas exchange within the tracheal system, forcing movement away to a source of cleaner air. Furthermore, the fumigant toxicity of some insecticidal components in the smoke of burned plant materials results in an excitorepellent effect where low concentrations irritate the insects so that they escape before knockdown (White 2006).

Some of the earliest recorded topically applied repellent products included concoctions of vinegar, manna, and oil as described in the Geoponika, and infusions of

cow parsnip blossoms by the Southern Carrier Tribe of British Columbia, Canada (Moore and Debboun 2006). Prior to World War II the most effective topical repellent, and still one of the most popular, was the essential oil of citronella. From the grass family Poaceae various *Cymbopogon* species have been cultivated for the essential oil, which has been used in numerous forms for application. The Indian Army for instance used a formulation of citronella, camphor, and paraffin. It wasn't until after the US military had screened over 20,000 chemical compounds by 1956 that a suitable synthetic chemical became the most popular and most effective product still in use today. Several synthetic compounds were developed that provided reasonable efficacy, including dimethyl phthalate in 1929, indalone in 1937, and Rutgers 612 in 1939, which were subsequently combined to form the product 6-2-2. In 1991 it was removed from markets because of potential health hazards (Peterson and Coats 2001; Moore and Debboun 2006). In 1953 scientists with the US military discovered *N*,*N*-diethyl-3-methylbenzamide, DEET, the "gold standard" repellent of modern day. As a broad-spectrum repellent, DEET exerts its effects on many haematophagous arthropods including all genera of mosquitoes.

The mode of action of DEET has been a topic of controversy for some time. Dogan *et al.* (1999) hypothesized that the olfactory receptor neurons (ORN) for the attractant L-lactic acid, a major component of human sweat and skin emanations, is a direct target of DEET, interfering with the detection of L-lactic acid. They concluded that DEET is not a repellent but rather an L-lactic acid attraction inhibitor. Their work was supported by previous physiological studies showing that DEET inhibited detection of lactic acid by grooved peg sensilla (Bowen 1996; as cited in Dogan *et al.* 1999). However, a recent report has indicated that mosquitoes do in fact detect DEET, as

demonstrated by specific ORNs on the antennae of *Culex quinquefasciatus*, and avoid it even in the absence of L-lactic acid (Syed and Leal 2008).

DEET is highly effective but the Pest Management Regulatory Agency (PMRA) of Canada recommends that children generally not be protected with DEET products. Personal repellents with DEET concentrations exceeding 30% are not acceptable for registration due to the health risks associated (PMRA Re-evaluation Decision Document, 2002). Neurological effects such as seizures and encephalopathy in children have been reported in association with DEET, and the rapid skin penetration and bio-distribution have raised concerns on its potential toxicity (Omolo *et al.* 2004; Antwi *et al.* 2008). Furthermore, DEET is a solvent of some plastics, paints, varnishes, and synthetic fabrics (Trigg, 1996; Badolo *et al.*, 2004). Reasonably, people become more reluctant toward the use of DEET, and there is a strong desire for safer alternatives, typically with a preference for the more "natural" repellents.

In Canada, there are only four active ingredients registered for use as topical repellents. These include DEET, *p*-menthane-3,8-diol (PMD), soybean oil (though not widely marketed), and citronella. A 2004 re-evaluation of citronella indicated uncertainties in safety to human health, resulting in possible deregistration pending a final decision (PMRA 2004). With or without the phase-out of citronella, few active ingredients are available for use leaving very little choice for Canadians.

Globally there are four major DEET alternatives, only one of which, PMD, is registered in Canada. These alternatives include Picaridin (2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester; KBR 3023; Trade-name Bayrepel®), DEPA (*N*,*N*-diethyl phenyl acetamide), as well as the naturally occurring IR3535 (Ethyl

butyl acetyl aminopropionate) and PMD. IR3535 is derived from the amino acid β-alanine, whereas PMD, which became registered for use in 2002 (PMRA Regulatory Decision Document, RDD2002-04), is a monoterpene in the residue of steam distillates of lemon scented eucalyptus, *Corymbia citriodora* (Myrtaceae).

1.5 Present Investigation of Insect Repellents

Personal protection through topical application of repellent products provides obvious practical and economical benefits. Unfortunately, for those regions heavily burdened with debilitating vector-borne diseases, most already impoverished families are afflicted with the severe economic challenges of lost income and the cost of treatment, if accessible. The relatively high cost of synthetic repellents presents a further impediment to those most in need. Naturally derived plant-based insect repellents may offer accessible, affordable protection with reduced toxicity. In 1996, the U.S. Environmental Protection Agency (EPA) produced a list of substances, which included many essential oils that are exempt from registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Essential oils from such plants as andiroba, basil, catnip, cedar, citronella grass, clove, lemon scented eucalyptus, garlic, geranium, neem, rosemary, and thyme have all been identified as effective mosquito repellents. For most essential oils, it appears that repellency is most commonly due to the presence of terpenoids, which are often tested in repellency bioassays based on their status as the most abundant compounds within the oil (Barnard 1999; Traboulsi *et al.* 2005; Trongtokit *et al.* 2005; Moore *et al.* 2006; Zhu *et al.* 2006). Testing all compounds of essential oils for behavioural activity is time-consuming and laborious. Thus, electrophysiological recording may present important information useful in reducing the

number of compounds to be tested in behavioural experiments. Gas chromatographic-electroantennographic detection (GC-EAD) is a valuable tool to rapidly screen components detectable by insect antennae within complex volatile blends (Arn *et al.* 1975), thus allowing to select only antennal stimulatory constituents for behavioural study. Much of the electrophysiological research conducted with mosquitoes has focused on the action of DEET or the response to host volatiles and oviposition attractants. However, electrophysiology has hardly been applied to identify those chemicals in essential oils that may carry the repellent activity (Du and Millar 1999; Costantini *et al.* 2001; Qiu *et al.* 2004; Jhumur *et al.* 2007). It was my objective to apply GC-EAD to the screening of various essential oils for EAD-active components, which may cause repellency in behavioural experiments.

1.5.1 Soybean Oil

Of particular interest as a natural repellent is soybean oil, one of the main active ingredients of the commercially available BiteBlocker™. Several studies have documented the effectiveness of the BiteBlocker™ product, all reporting times comparable with mid-level (7-15%) concentrations of DEET (Fradin and Day 2002; Barnard and Xue 2004). The PMRA in 2000 granted full registration to Consep Soybean Oil Technical and the formulated line of Blocker™ products. Soybean oil contains common fatty acids such as palmitic, oleic, linoleic and stearic acids (PMRA 1999). However, the main active ingredients pertaining to repellency have yet to be reported.

1.5.2 Garlic

Historically, garlic has been consumed, hung, rubbed on fixtures, and topically applied for many therapeutic effects, principally according to folklore tales for protection from various evil sources including werewolves and vampires (McNally and Florescu 1994). It seems only natural that the perception of protection from evil bloodsuckers would transfer to mosquitoes and other biting arthropods. Systemic protection through garlic consumption has been shown false (Rajan *et al.* 2005), but there is evidence that topical application of garlic oil offers a certain level of protection (Bhuyan *et al.* 1974; Trongtokit *et al.* 2005). These reports, the anecdotal evidence and the many medicinal benefits (Block 1992) suggest the presence of ingredients that express repellence. To date however, no study has been conducted to identify semiochemicals responsible for repellency.

1.6 Research Objectives

My research objectives were:

1. To screen various plant essential oils by GC-EAD for antennal stimulatory components, using antennae of female *Ae. aegypti* as electroantennographic detector;
2. To determine the repellent efficacy and mode of action of soybean oil, and (if shown effective) to identify the repellent constituents therein; and
3. To determine the repellent efficacy of garlic oil, and (if shown effective) to identify the repellent constituents therein.

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2 FORTY THREE COMPOUNDS IN 11 BOTANICAL ESSENTIAL OILS ELICIT ANTENNAL RESPONSES FROM *AEDES AEGYPTI*[1]

2.1 Abstract

Essential oils of various plants are effective at repelling mosquitoes. The repellent constituents of these oils are often inferred just based on their dominant relative abundance. Our objective was to analyse plant essential oils by coupled gas chromatographic-electroantennographic detection (GC-EAD) on the premise that those compounds that can be perceived by antennae of the yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae), are candidate repellents. In the essential oils of catnip, cinnamon, citronella, cumin, eucalyptus, geranium, ginger, melissa, peppermint, rosemary, and thyme, 43 components induced antennal responses, the most ubiquitous of which were β-caryophyllene, linalool, 1,8-cineole, geraniol, and geranial. Only some of these compounds are known insect repellents, indicating that GC-EAD screening of plant essential oils and other sources is an appropriate viable technology to detect new quantitatively minor constituents, which could be potent repellents when tested at an appropriate dose.

[1] Mrs. Regine Gries provided significant assistance with the analytical chemistry of this chapter.

2.2 Introduction

Research on insect repellents has focused, in part, on the effect of botanical products, promoting the development of "natural" plant-based insect repellents. Essential oils from such plants as andiroba, basil, catnip, cedar, citronella grass, clove, lemon eucalyptus, geranium, neem, rosemary, and thyme have all been shown effective at repelling mosquitoes (Thorsell *et al*. 1998; Barnard 1999; Tawatsin *et al*. 2001). The chemical composition of essential oils is responsible for their biological activity (Rota *et al*. 2008). Most defensive chemicals in plants can be grouped into five categories: the nitrogenous compounds, terpenoids, phenols, proteinase inhibitors and growth regulators, and of these, terpenoids are most commonly responsible for the repellency of essential oils (Trongtokit *et al*. 2005; Moore *et al*. 2006). In the terpene group, monoterpenes are major constituents and thus the focus of many repellent studies (Moore *et al*. 2006). Many of these compounds have a pleasant fragrance, relatively low mammalian toxicity, and a vapour pressure almost ideal for action as a volatile spatial repellent.

Candidate semiochemicals in essential oils were typically identified and selected based on their relative abundance in gas chromatographic-mass spectrometric analyses. The concept that the most abundant compounds are responsible for an essential oil's repellent effect disregards minor constituents with potential significant repellency. Furthermore, not all major constituents of an essential oil elicit a behavioural response from insects. For example, major constituents of clove oil are eugenol, eugenol-acetate, and β-caryophyllene, but only eugenol is repellent to *Ae. aegypti* (Barnard 1999; USDA 1967, 1954). Thus, assessment of an oil's repellency should include the testing of

quantitatively minor constituents which at a higher dose may be very effective repellents (Barnard 1999).

Few publications describe the use of electrophysiology to identify host and oviposition site attractants for mosquitoes (Du and Millar 1999; Costantini *et al*. 2001), and no study has applied electrophysiology to determine (candidate) repellents in plant essential oils. Here we describe analyses of plant essential oils by gas chromatographic-electroantennographic detection (GC-EAD), using the antennae of the yellow fever mosquito, *Aedes aegypti* (L.), as the biological detector. This technique allows rapid screening of all the oils' volatile constituents. All antennal-stimulatory constituents can then be tested individually or in concert as candidate repellents.

2.3 Materials and Methods

2.3.1 Experimental Insects

The black-eyed Liverpool strain of *Ae. aegypti* (L.) was supplied by Dr. Carl Lowenberger (SFU). Adults were kept at 27^{o}C, a 65-70% relative humidity, and a 14:10 (light:dark) photoperiod. They were provisioned with a 10% sucrose solution (in water). Larvae were kept under the same conditions and fed a Nutrafin® Basix Staple Food fish diet. GC-EAD recordings were conducted between 0800-1600h, coinciding with the host-seeking period of *Ae. aegypti*.

2.3.2 Analytical Methods

Essential oils and blends of authentic standards were analyzed by GC-EAD (Arn *et al*, 1975; Gries *et al*. 2002), using a Hewlett-Packard (HP) 5890 gas chromatograph equipped with a GC column (30 m × 0.32 mm ID) coated with DB-5 (J&W Scientific, Folsom, California, USA). Injector and detector temperatures were set to 250°C, and the temperature program was as follows: 50ºC held for 1 min, 20ºC per min to 300ºC. For GC-EAD recordings, the severed head of a female (with both antennae intact) was placed into the opening of a glass capillary electrode (1:0 × 0:58 × 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution (Staddon and Everton 1980). One antenna with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada) was placed into the recording capillary electrode. Aliquots of essential oils were tested with methyl salicylate and/or diallyl disulfide added as internal standards, because both elicit consistent antennal responses.

2.3.3 Essential oils

Eleven steam-distilled essential oils were obtained from Liberty Natural Products (20949 S. Harris Rd., Oregon City, Oregon, USA; (Table 2.1)). Aliquots of each essential oil were analyzed by GC-EAD, using at least three different mosquito antennae. Components of essential oils that stimulated the antennae were analyzed by coupled GC-mass spectrometry (MS), employing a Varian 2000 Ion Trap MS in full-scan electron impact mode fitted with the above-referenced DB-5 column or a DB-FFAP column (J&W Scientific, Folsom, California, USA). Molecular structures of antennal stimulatory constituents were assigned by comparing their mass spectra and retention indices (RI,

relative to *n*-alkane standards) with those reported in literature and/or those of authentic standards.

2.3.4 Authentic standards

Seven blends of authentic standards were prepared to confirm the identification of antennal stimulatory constituents, and (where applicable) to confirm the assignment of absolute configuration (Table 2.2). Each blend consisted of an assortment of compounds with distinctively different GC retention times. Benzaldehyde (>95% chemically pure) was purchased from Fisher Scientific (Ottawa, ON, Canada). (+/-)-Borneol and menthol (95%) were obtained from BDH Laboratory Supplies (Poole, Dorset, UK). (+)-Borneol (98%), (-)-borneol (98%), (+)-bornyl acetate, (-)-bornyl acetate (97%), (+)-camphene (80%), (-)-camphene (80%), (+)-camphor (95%), (-)-camphor (≥95%), (*R*)-(+)-limonene (97%), (*S*)-(-)-limonene (96%), (*R*)-(+)-β-pinene (98%), (*S*)-(-)-β-pinene (99%), citronellol (95%), (*R*)-(+)-β-citronellol (98%), (*S*)-(-)-β-citronellol (99%), citronellal (85-90%), menthyl acetate (≥97%), cumin aldehyde (98%), thymol (99.5%), citral (≥95%), β-caryophyllene (≥98.5%), 1,8-cineole (99%), (*R*)-(-)-α-phellandrene (~50%), (+/-)-linalool (97%), (-)-linalool (≥80%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). *cis*-Cinnamaldehyde (98%) and *trans*-cinnamaldehyde (98%) were obtained from MCB (Cincinnati, OH). DL-Menthone (97%) was purchased from ICN pharmaceuticals (Costa Mesa, CA). α-Terpineol (95%) was purchased from Alfa aesar (Ward Hill, MA). Isomenthone (98%) was synthesized from isomenthol (99%; Aldrich). Geranyl formate (95%) was synthesized from geraniol (98%; Aldrich). Nerol (≥90%; Fluka) and geranyl formate were tested individually because they had inadvertently been

omitted from blends. The internal standards methyl salicylate (99%) and diallyl disulfide (80%) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

β-Bisabolene as a constituent of ginger oil was obtained from Jocelyn Millar of the University of California Riverside, whereas authentic standards of α-Curcumene, Zingiberene, and β-Sesquiphellandrene were not obtainable. They were tentatively identified by comparing retention indices and mass spectra with components in the essential oil of turmeric, *Curcuma longa* (Zingiberaceae; Liberty Natural Products, Oregon City, OR), which is closely related to ginger (Kress *et al*. 2002), and known to contain several of these components (Martins *et al*. 2001; Babu *et al*. 2007).

Germacrene D was isolated from peppermint oil through high performance liquid chromatography (HPLC), employing a Waters LC 626 chromatograph equipped with a Waters 486 variable wavelength UV/visible detector set to 210 nm, HP Chemstation software (Rev.A.07.01), and a reverse phase Nova-Pak C18 column (60A°, 4μm; 3.9 × 300 mm) eluted with acetonitrile at 1 ml/min. Nuclear magnetic resonance (NMR) spectra of the isolated compound were taken with a Varian AS500 spectrometer at 499.77 MHz for ^{1}H, with chemical shifts reported in parts per million relative to tetramethylsilane (1H, δ 0.00).

2.4 Results and Discussion

Antennae of female *Ae. aegypti* responded to 43 components in eleven essential oils from seven different plant families (Tables 2.2 and 2.3), as follows.

2.4.1 Lamiaceae

Catnip (*Nepeta cataria*). Three major constituents of catnip oil elicited strong antennal responses (Table 2.3; Fig. 2.1a). Two components with identical mass spectra but distinctively different retention indices (DB-5, RI: 1380 and 1413, respectively, relative to *n*-alkane standards) (Regnier *et al*. 1967; Peterson *et al*. 2002; Schultz *et al*. 2004) were isomers of nepetalactone, and the other was β-caryophyllene (Adams 1989; Schultz *et al*. 2004; Eom *et al.* 2006). The identification of the nepetalactone geometrical isomers was based on the relative ratio (1:2) and elution order of the *Z,E*- and *E,Z*-isomers. That the ratio differed slightly from previous reports (Peterson *et al*. 2002; Schultz *et al*. 2004), is likely due to seasonal variation at the time of oil distillation (Schultz *et al*. 2004). The retention indices of the *Z,E* and *E,Z*-nepetalactone on a DB-WAX column (Liblikas *et al.* 2005) are very similar to those of our DB-FFAP column (which has very similar retention characteristics), further supporting the correct identification of the nepetalactone isomers.

Nepetalactone along with similar cyclopentanoid monoterpenes are thought to deter phytophagous insects, particularly since nepetalactone has been shown to be repellent to at least 13 insect families (Eisner 1964). German cockroaches, *Blatella germanica*, are repelled by catnip oil and by the two nepetalactone isomers, and most likely perceive them by chemoreceptors on the antennae (Peterson *et al*. 2002). Responses of *Culex pipiens pallens* to catnip oil in electroantennogram recordings (Zhu *et al*. 2006) support this observation. That a commercial catnip oil formulation was not repellent to *Ae. aegypti*, was likely due to an insufficiently low dosage (5%) of catnip oil, because in other studies with *Ae. aegypti* and *Ae. albopictus* catnip oil and the individual

nepetalactone isomers had a significant repellent effect (Zhu *et al*. 2006; Chauhan *et al*. 2005). β-Caryophyllene as another EAD-active constituent of catnip oil may or may not be repellent (USDA 1967; Jaenson *et al*. 2006; Gillij *et al*. 2008).

Thyme (*Thymus zygis*). Linalool, α-terpineol and thymol elicited responses from antennae (Table 2.3; Fig. 2.1a). Authentic (+/-) linalool (Blend 2, Fig. 2.2), (-)-linalool (Blend 7, Fig. 2.2), (+/-)-α-terpineol (Blend 1, Fig. 2.2), and thymol (Blend 5, Fig. 2.2) had identical mass spectral characteristics and elicited similarly strong responses from antennae, thus confirming the identification of these constituents. (*S*)-(+)-Linalool and the enantiomers of α-terpineol were not obtainable, thus disallowing us to assign the absolute configuration to these two constituents in thyme and other essential oils analyzed in this study.

The essential oil of the most common species of thyme, *Thymus vulgaris*, had a significant repellent effect on the northern house mosquito, *Culex pipiens* (Choi *et al*. 2002). It was further shown to provide protection for up to 135 min against *Ae. aegypti* at a 100% concentration (USDA 1954; Barnard 1999). Wild thyme, *Thymus serpyllum*, essential oil provided protection for up to 150 min in a complex formulation (Amer and Melhorn 2006). The repellent effect of *Thymus zygis* essential oil is expected to be similar to that of other thyme species, because the overall chemical compositions are markedly similar (Rota *et al*. 2008). Furthermore, thymol and linalool repel *Culex pipiens*, with thymol as efficacious as DEET (Park *et al*. 2005) and linalool being a spatial repellent for *Ae. aegypti* (Kline *et al*. 2003).

Rosemary (*Rosemarinus officinalis*). Ten compounds elicited responses from antennae, seven of which were identified as β-pinene, 1,8-cineole, camphor, borneol, α-terpineol, bornyl acetate, and β-caryophyllene (Table 2.3; Fig. 2.1a). The three remaining quantitatively minor compounds were tentatively identified as α-pinene, camphene, and limonene, but in blends of authentic standards neither (+)- nor (-)-α-pinene (Blend 4 & 5, Fig. 2.2), nor (+)- nor (-)-camphene (Blend 5 & 6, Fig 2.2), nor (+)-limonene (Blend 5, Fig, 2.2) were antennally active. The (+)- and (-)-enantiomers of borneol, camphor, and bornyl acetate each elicited responses from antennae, whereas only (-)-β-pinene and (-)-limonene were EAD active. The absolute configuration of α-terpineol eliciting a response remains unknown (see above).

With 87% and 68%, respectively, the relative abundance of (-)-borneol and (-)-β-pinene was higher than that of their antipodes, whereas camphor and α-terpineol occurred at or near racemic levels (Kreis *et al*. 1994; Mondello *et al*. 2006). The absolute configuration of bornyl acetate is not known. Linalool, thymol and geraniol occur in rosemary oil (Angioni *et al*. 2004) but were below detectable levels in this study.

Rosemary oil has insecticidal properties against larvae of two lepidopteran agricultural pests (Isman *et al*. 2008), and one species of thrips and its hymenopteran predator (Yi *et al*. 2006). Treatment with rosemary oil provided protection for hairless mice from *Culex pipiens* (Choi *et al*. 2002), and for humans for up to 60 min from *Ae. aegypti* at a 100% concentration (USDA 1954), or for 330 min at 20% in a complex formulation (Amer and Melhorn 2006). Except borneol and bornyl acetate, all antennally active constituents are reported in various mosquito repellent tests (USDA 1967; Park *et al*. 2005; Traboulsi *et al*. 2005; Jaenson *et al*. 2006). Interestingly, bornyl acetate has

been reported as repellent to the voracious highland biting midge *Culicoides impunctatus* (Stuart and Stuart 1998).

Peppermint (*Mentha piperita*). Eight components including β-pinene, 1,8-cineole, (-)-menthone, (-)-isomenthone, (-)-menthol, (-)-menthyl acetate, β-caryophyllene and Germacrene D elicited strong responses from female antennae (Table 2.3; Fig. 2.1a). Comparative GC and GC-MS analyses of the essential oil and authentic standards (Blends 1, 2, and 4) confirmed identification for all compounds except for Germacrene D, which instead was confirmed through ^{1}H-NMR analysis. Absolute configuration of the EAD-active compounds was inferred based on a previous report that the (+)-enantiomers are absent from peppermint essential oil (Askari *et al*. 1992; Ruiz del Castillo *et al*. 2004). Although isomenthol was not found in this study, it does occur in peppermint essential oil (Ruiz del Castillo *et al*. 2004), and thus was included as a standard (Blend 3), and proven to have antennal activity.

Peppermint essential oil provides protection for 1-2 h from *Ae. aegypti* and *Ae. albopictus*, depending on concentration and method of application (USDA 1954; Barnard 1999; Trongtokit *et al*. 2005; Yang and Ma 2005; Amer and Melhorn 2006). Menthone, β-pinene, and 1,8-cineole have previously been documented as repellents to mosquitoes (Traboulsi *et al*. 2005; USDA 1954). Germacrene D has strong fumigant toxicity to mosquitoes (Kiran and Devi 2007). Menthol is repellent to honey bees (Collins *et al*. 1996), but was not repellent to *Anopheles gambiae* (Barasa *et al*. 2002). There are no reports describing a behavioural effect of isomenthone and menthyl acetate, and the behavioural effect of β-caryophyllene remains undetermined.

Melissa (*Melissa officinalis*). Commonly referred to as lemon balm, leaves of this perennial herb are cultivated for their lemon flavour and for use in traditional folk medicine. Extracts of Melissa have been shown toxic to larvae of the African cotton leafworm *Spodoptera littoralis*, (Pavela 2005), and the essential oil is larvicidal to the house mosquito, *Culex pipiens* (Cetin 2006). Nerol, neral, geraniol, and geranial consistently elicited responses from *Ae. aegypti* antennae (Table 2.3; Fig 2.1a). Geraniol and citral (neral + geranial) are mosquito repellents with relatively high efficacy (USDA 1954; Omolo *et al*. 2004; Hao *et al*. 2008). Nerol, the *cis*-isomer of geraniol, has not been tested as a mosquito repellent but is repellent to other arthropods (Lwande *et al*. 1999; Wee *et al*. 2008). Acting as a juvenile hormone analogue (Singh and Upadhyay 1993), it is anticipated to be repellent to mosquitoes.

2.4.2 Lauraceae

Cinnamon (*Cinnamomum zeylanicum*). EAD-active components of cinnamon essential oil include benzaldehyde, α-phellandrene, linalool, *Z*-cinnamaldehyde, *E*-cinnamaldehyde, β-caryophyllene, *E*-cinnamyl acetate and *o*-methoxycinnamaldehyde. The first six components were identified based on comparative GC-MS analyses with authentic standards, whereas the last two components were identified based on information in the literature (Morozumi 1978; Zhu *et al*. 2006; Zhu *et al*. 2008).

Cinnamon oils and many of their chemical components including cinnamaldehyde, cinnamyl acetate, benzaldehyde, and β-caryophyllene are toxic to larvae of several mosquito species including *Ae. aegypti* (Cheng *et al*. 2004; Zhu *et al*. 2006).

Cinnamomum cassia essential oil was shown to elicit antennal responses from *Culex pipiens pallens* (Zhu *et al*. 2006), and to repel *Ae. aegypti*, as did *E*-cinnamaldehyde (Chang *et al*. 2006).

2.4.3 Poaceae

Citronella (*Cymbopogon nardus*). The eight antennal-stimulatory components of citronella essential oil were identified as limonene, linalool, citronellal, citronellol, neral, geraniol, geranial, and geranyl acetate (Table 2.3). Both synthetic (*R*)-(+)- and (*S*)-(-)-enantiomers of β-citronellol elicited a response. The (*R*)-(+)-enantiomer of citronellal prevails in the essential oil (~88%; Nhu-Trang *et al*. 2006) and likely induced the antennal response to it.

The *Cymbopogon* genus is responsible for some of the most widely used naturally-derived insect repellents worldwide. Discovered in 1901, citronella was the most widely used repellent prior to the 1940's, prior to the development of DEET (Katz *et al*. 2008). Citronella oil provides protection for 2 h against *Ae. aegypti* (USDA 1967; Trongtokit *et al*. 2005). Furthermore, except geranyl acetate, all antenna-stimulatory components are repellent to mosquitoes (USDA 1954, 1967; Kline *et al*. 2003; Omolo *et al*. 2004; Hao *et al*. 2008). Citronella candles and incense are marketed as spatial area repellents, but they are hardly effective in reducing mosquito bites. Citronella is registered in Canada as a topical insect repellent, but this registration is being phased-out due to concerns of potential risks to human health, because citronella contains the carcinogen methyleugenol (PMRA 2004).

2.4.4 Apiaceae

Cumin (*Cuminum cyminum*). Two components of cumin oil, cumin aldehyde and cumin alcohol, were EAD-active (Adams 1989; Li and Jiang 2004). Neither of them nor cumin oil have been tested as insect repellents. However, cumin has been implicated as a nonlethal repellent against the starling, *Sturnus vulgaris* (Clark 1997), and is a larvicide to *Culex pipiens pallens* and *Ae. aegypti* (Lee 2006).

2.4.5 Myrtaceae

Eucalyptus (*Eucalyptus polybractea*). 1,8-Cineole (eucalyptol) was the only component of eucalyptus oil that elicited antennal responses (Fig. 2.1b; Adams 1989). 1,8-cineole acts as an oviposition repellent to gravid female *Ae. aegypti* and is toxic to their larvae (Lucia *et al*. 2008; Waliwitiya *et al.* 2009). 1,8-cineole is also repellent to host-seeking *Culex pipiens molestus* (Traboulsi *et al*. 2005).

2.4.6 Geraniaceae

Bourbon Geranium (*Pelargonium x asperum*). Linalool, β-citronellol, geraniol, geranial, geranyl formate, β-bourbonene, β-caryophyllene, and germacrene D elicited strong and consistent antennal responses. The former five compounds were identified based on comparative GC-MS analyses with authentic standards (Table 2.3). β-Bourbonene, is the principal and characteristic sesquiterpene of several *Pelargonium spp.* (Babu and Kaul 2005; Juliani *et al*. 2006), and the molecular structure of germacrene D was confirmed by NMR analysis as described for peppermint oil. Both the (*R*)-(+)- and (*S*)-(-)- enantiomer of synthetic β-citronellol were EAD-active in blends of authentic

standards, even though bourbon geranium oil contains mainly the (*S*)-(-)-enantiomer (Ravid *et al*. 1992).

As a member of the *Pelargonium x asperum* complex, the citrosa plant *Pelargonium citrosum* is claimed to release fragrant citronella oil and is marketed as a spatial mosquito repellent although the composition and relative ratios of volatile constituents markedly differ from those of *Cymbopogon spp.* (Matsuda *et al*. 1996). *Pelargonium* plants failed to protect human volunteers seated next to them, though it was noted that the typical fragrance was detectable only when plants were disturbed, either by shaking or wind (Matsuda *et al*. 1996; Cilek and Schreiber 1994). Bourbon geranium oil provided protection for up to 2 h against *Ae. aegypti* (Barnard 1999; USDA 1954). The specific effects of β-bourbonene, β-caryophyllene, geranyl formate, and germacrene D remain unknown.

2.4.7 Zingiberaceae

Ginger (*Zingiber officinale*). The four EAD-active components of ginger essential oil are α-curcumene, zingiberene, β-bisabolene, and β-sesquiphellandrene (Table 2.3; Fig. 2.1). Their retention indices were similar to those reported by Adams (1989), and Joulain and König (1998), and their mass spectra were entirely consistent with those reported in the literature. Moreover, retention time and mass spectrum of the β-bisabolene matched those of an authentic standard (Adams 1989; Joulain and König 1998; Qin *et al*. 2007). These four EAD-active components are also the predominant sesquiterpenes of ginger oil (Connell and Sutherland 1966; Akhila and Tewari 1984; Martins *et al*. 2001; Juliani *et al*. 2007). Many species within the Zingiberaceae family are repellent to mosquitoes and

other insects. Washings of macerated *Zingiber officinale* deter the Asian armyworm *Spodoptera litura* from feeding on shoots of groundnut, *Arachis hypogea* (Sahayaraj 1998). The aromatic turmeric *Curcuma aromatica* repels *Ae. togoi* up to 3 h (Pitasawat *et al*. 2003), and both *Curcuma longa* and *Z. officinale* repel *Ae. aegypti* for up to 1 h (Tawatsin *et al*. 2001; Trongtokit *et al*. 2005).

2.5 Concluding Remarks

The plant families Lamiaceae, Myrtaceae, and Poaceae represent some of the best-known species for plant-based insect repellents (Moore *et al*. 2006), likely due to some redundancy in their chemical composition. Of the oils tested here, only cumin and melissa were not previously reported to have mosquito repellent properties (Trongtokit *et al*, 2005; Amer and Mehlorn, 2006), and no previously determined behavioural effect could be found for 20 of the 43 antennal stimulatory constituents. Although the results of this study do not establish behavioural activity, they do provide the groundwork for future research that may. Many plant essential oils have relatively low mammalian toxicity, are classified as minimal risk pesticides, and are exempt from registration under FIFRA (FIFRA 40CFR Sec. 152.25 (g), EPA 1996). These include cinnamon, geranium, peppermint, rosemary, thyme, and citronella as well as some of their volatile constituents that were tested in this study.

Mosquitoes are haematophagous, but also obtain energy through sugar feeding on floral and extra-floral plant nectaries (Gary Jr. and Foster 2004; Moore *et al*. 2006). Plant chemicals are vital to the defense against insect herbivory, and terpenes are the largest and structurally most diverse group of plant chemicals that deter herbivores. All 43

EAD-active compounds identified in this study are terpenes, particularly monoterpene(oid)s and sesquiterpenes. There may be specific yet common moieties that convey the repellent effect. For example, Omolo *et al*. (2004) noted that effective repellency against *An. gambiae* was associated with compounds that had a 2,3-olefinic functionality and a hydroxyl group in a specific position. The sensitivity and selectivity of mosquitoes to olfactory cues emanating from feeding and oviposition sites can be attributed to many antennal olfactory sensilla (McIver 1982; Sutcliffe 1994). Studies of structure/activity relationships through single sensillum electrophysiological recordings may reveal specific functional moieties or entire molecules, and electronic and stereoelectronic requirements of potential olfactory receptors for optimal complementarity. Such studies may allow the development of synthetic repellent analogues that provide longer protection times whilst maintaining low toxicity and a pleasant fragrance.

2.6 Reference List

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Table 2.1 Eleven plant essential oils tested in coupled gas chromatographic – electroantennographic detection analyses, using an antenna of female *Aedes aegypti* as electroantennographic detector.

Common name	Scientific name	Family	Origin of plant
Catnip	*Nepeta cataria*	Lamiaceae	Canada
Cinnamon	*Cinnamomum zeylanicum*	Lauraceae	Sri Lanka
Citronella	*Cymbopogon nardus*	Poaceae	Indonesia
Cumin	*Cuminum cyminum*	Apiaceae	India
Eucalyptus	*Eucalyptus polybractea*	Myrtaceae	Australia
Geranium	*Pelargonium x asperum*	Geraniaceae	China
Ginger	*Zingiber officinale*	Zingiberaceae	Indonesia
Melissa	*Melissa officinalis*	Lamiaceae	France
Peppermint	*Mentha piperita*	Lamiaceae	USA
Rosemary	*Rosmarinus officinalis*	Lamiaceae	Tunisia
Thyme	*Thymus zygis*	Lamiaceae	USA

Table 2.2 Blend composition of authentic standards tested in coupled gas chromatographic-electroantennographic analyses using antennae of female *Aedes aegypti* as electroantennographic detectors.

Peak #	Blend composition[1]	Retention index[2]
	BLEND 1	
1	1,8-Cineole	1040
2	(+)-Camphor	1158
3	(+/-)-Borneol	1182
4	α-Terpineol	1201
5	Geraniol	1256
6	Menthyl Acetate	1296
7	Geranyl Acetate	1381
8	β-Caryophyllene (co-injected)	1439
	BLEND 2	
1	(+/-)-Linalool	1101
2	DL-Menthone	1168
3	Menthol	1185
4	Citronellol	1230
5	Neral (from citral)	1245
6	Geranial (from citral)	1274
7	(+/-)-Bornyl Acetate	1292
	BLEND 3	
1	Isomenthone	1173
2	Isomenthol	1185
3	*Z*-Cinnamaldehyde	1228
4	Cumin aldehyde	1253
5	*E*-Cinnamaldehyde	1267
6	Cumin alcohol	1298
	BLEND 4	
1	1(*R*)-(+)-α-Pinene	941
2	Benzaldehyde	960
3	1(*S*)-(-)-β-Pinene	980
4	(*R*)-(-)-α-Phellandrene	1006
5	(*S*)-(-)-Limonene	1035
	BLEND 5	
1	1(*S*)-(-)-α-Pinene	941
2	(-)-Camphene	959
3	1(*R*)-(+)-β-Pinene	980
4	(*R*)-(+)-Limonene	1035
5	Citronellal	1154
6	Thymol	1290

	BLEND 6	
1	(+)-Camphene	959
2	(+)-Borneol	1182
3	(*R*)-(+)-*β*-citronellol	1230
4	(+)-Bornyl acetate	1292
	BLEND 7	
1	(-)-Linalool	1101
2	(-)-Camphor	1158
3	(-)-Borneol	1182
4	(*S*)-(-)-*β*-Citronellol	1230
5	(-)-Bornyl acetate	1292
	INDIVIDUALLY	
	Geranyl formate	1300
	Nerol	1229

[1] Each chemical tested at 100 ng/μl with 2-μl aliquot injections
[2] DB-5 column, relative to *n*-alkane standards

Table 2.3 List of components that elicited antennal responses in gas chromatographic-electroantennographic detection analyses, using antennae of female *Aedes aegypti* as electroantennographic detector.

	Essential Oil	Retention index	Antennal stimulatory constituent	Area %	Method of identification (Ref.)
	LAMIACEAE				
A	Catnip	1380	*Z,E*-Nepetalactone	23.0	a (1, 2, 3) d
		1413	*E,Z*-Nepetalactone	49.9	a (1, 2, 3) d
		1439	β-Caryophyllene*	11.4	a (1), b
B	Thyme	1101	(+/-)-Linalool, (-)-Linalool	5.0	a (1, 4), b
		1201	α-Terpineol	1.1	a (1, 4), b
		1292	Thymol	49.1	a (1, 4), b
C	Peppermint	985	(-)-β-Pinene	1.4	a (1), b
		1040	1,8-Cineole	6.4	a (1), b
		1166	(-)-Menthone	21.4	a (1), b
		1173	(-)-Isomenthone*	6.0	a (1), b
		1180	(-)-Menthol	31.4	a (1), b
		1296	(-)-Menthyl acetate*	7.0	a (1), b
		1439	β-Caryophyllene*	3.0	a (1), b
		1500	Germacrene-D*	>1.0	a (1, 5), c, d
D	Rosemary	985	(-)-β-Pinene	9.2	a (1, 6), b
		1035	(*S*)-(-)-Limonene	3.4	a (1, 6), b
		1040	1,8-Cineole	35.1	a (1, 6), b
		1159	(+)-Camphor	12.4	a (1, 6), b
		1159	(-)-Camphor		
		1182	(+)-Borneol*	3.3	a (1, 6), b
		1182	(-)-Borneol*		
		1201	α-Terpineol	2.2	a (1, 6), b
		1291	(+)-Bornyl acetate*	1.0	a (1, 6), b
		1292	(-)-Bornyl acetate*		
		1439	β-Caryophyllene*	5.2	a (1, 6), b
E	Melissa	1229	Nerol*	2.2	a (1, 7), b
		1244	Neral	17.1	a (1, 7), b
		1256	Geraniol	2.7	a (1, 7), b
		1273	Geranial	22.1	a (1, 7), b
	LAURACEAE				
F	Cinnamon	968	Benzaldehyde*	0.5	a (1), b
		1010	(*R*)-(-)-α-Phellandrene	2.2	a (1), b
		1101	(+/-)-Linalool, (-)-Linalool	5.4	a (1), b
		1228	*Z*-Cinnamaldehyde*	11.8	a (1), b
		1267	*E*-Cinnamaldehyde	39.8	a (1), b
		1439	β-Caryophyllene*	7.0	a (1), b
		1451	*E*-Cinnamyl acetate*	3.5	a (1, 8), b
		1459	o-Methoxycinnamaldehyde*	>1.0	a (1, 8), b

	POACEAE				
G	Citronella	1035	(S)-(-)-Limonene	3.3	a (1), b
		1101	(+/-)-Linalool, (-)-Linalool	2.1	a (1), b
		1159	Citronellal	7.6	a (1), b
		1230	(+)-β-citronellol	6.3	a (1), b
		1230	(-)-β-citronellol		
		1245	Neral	5.4	a (1), b
		1256	Geraniol	41.9	a (1), b
		1274	Geranial	8.0	a (1), b
		1381	Geranyl Acetate*	9.5	a (1), b
	APIACEAE				
H	Cumin	1253	Cumin Aldehyde*	26.9	a (1, 9), b
		1298	Cumin Alcohol*	13.6	a (1, 9), b
	MYRTACEAE				
I	Eucalyptus	1040	1,8-Cineole	81.4	a (1), b
	GERANIACEAE				
J	Geranium	1101	(+/-)-Linalool, (-)-Linalool	5.1	a (1), b
		1230	(+)-β-citronellol	37.0	a (1), b
		1230	(-)-β-citronellol		
		1254	Geraniol	10.5	a (1), b
		1276	Geranial	15.3	a (1), b
		1300	Geranyl Formate*	2.9	a (1), b
		1401	β-Bourbonene*	1.0	a (1, 5), d
		1439	β-Caryophyllene*	1.5	a (1), b
		1500	Germacrene-D*	1.0	a (1, 5), c, d
	ZINGIBERACEAE				
K	Ginger	1492	α-Curcumene*	5.8	a (1, 2, 10, 11, 12), d
		1506	α-Zingiberene*	21.7	a (1, 2, 10, 11, 12), d
		1520	β-Bisabolene*	8.3	a (1, 2, 10, 11, 12), b (GC-MS only), d
		1538	β-Sesquiphellandrene*	10.9	a (1, 2, 10, 11, 12), d

*No previously reported behavioural effect found.

a Retention index and mass spectra, b GC-EAD & GC-MS with authentic standard, c NMR, d Literature

1 Adams 1989

2 Regnier *et al.* 1967

3 Peterson *et al.* 2002

4 Hudaib and Aburjai 2007

5 Joulain and König 1998

6 Angioni *et al*. 2004

7 Da Silva *et al.* 2005

8 Morozumi 1978

9 Li and Jiang 2004

10 Qin *et al*. 2007

11 Antonius and Kochlar 2003

12 Babu *et al*. 2007

2.7 Figure Captions

Figure 2.1 a) Representative example (n = 3) of gas chromatographic–electroantennographic detection (GC-EAD) analyses of various essential oils (A=catnip, B=thyme, C=peppermint, D=rosemary, E=melissa, F=cinnamon), using antennae of female *Aedes aegypti* as the electroantennographic detector. Compounds that elicited antennal responses are listed in Table 2.3. **IS**= internal standard.

Figure 2.1 b) Representative example (n = 3) of gas chromatographic–electroantennographic detection (GC-EAD) analyses of various essential oils (G=citronella, H=cumin, I=eucalyptus, J=geranium, K=ginger), using antennae of female *Aedes aegypti* as the electroantennographic detector. Compounds that elicited antennal responses are listed in Table 2.3. **IS**= internal standard.

Figure 2.2 Representative example of gas chromatographic–electroantennographic detection (GC-EAD) analyses of blends 1 to 7 (see Table 2.2) of authentic standards, using antennae of female yellow fever mosquitoes as the electroantennographic detector.

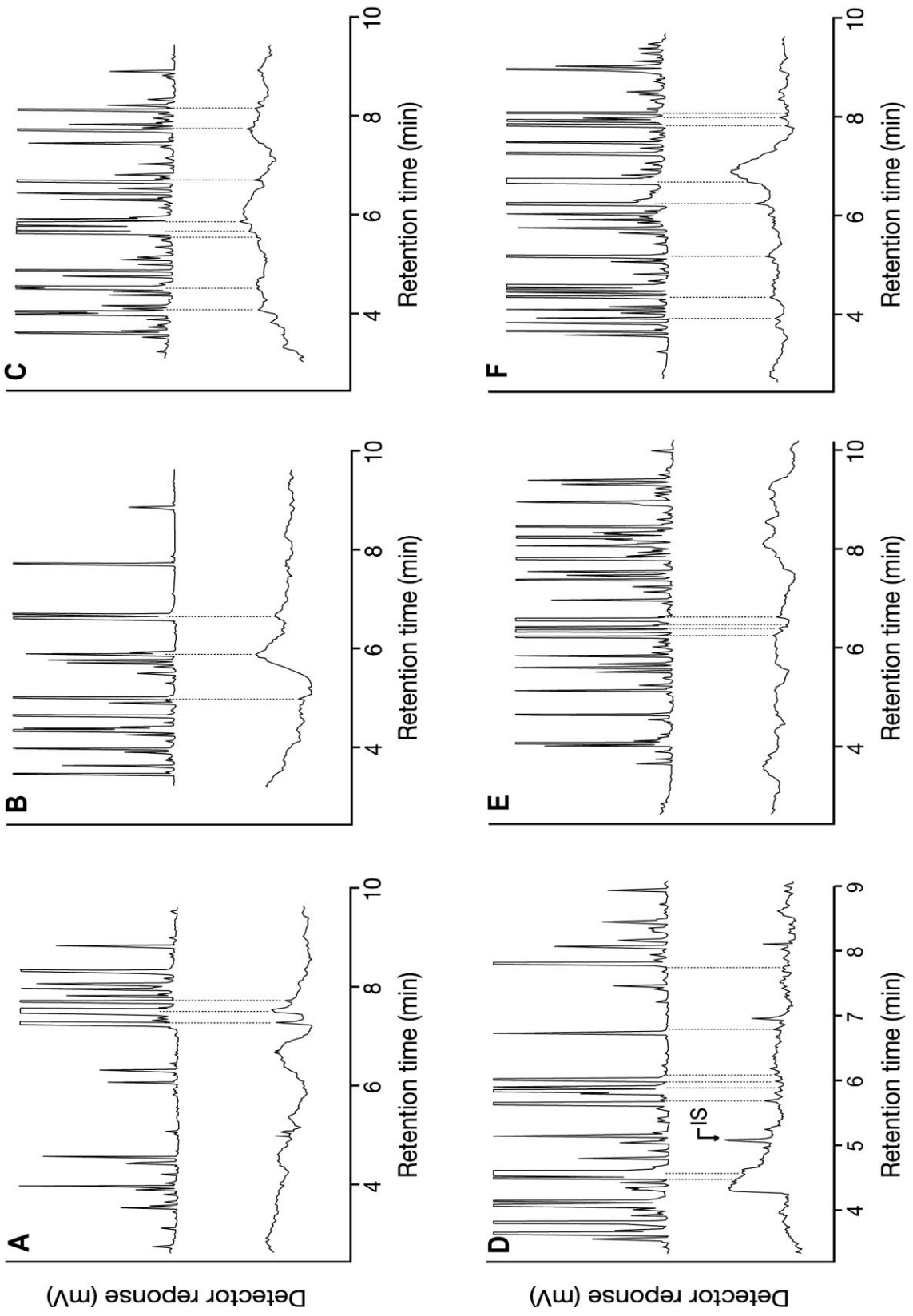

Figure 2.1 a)

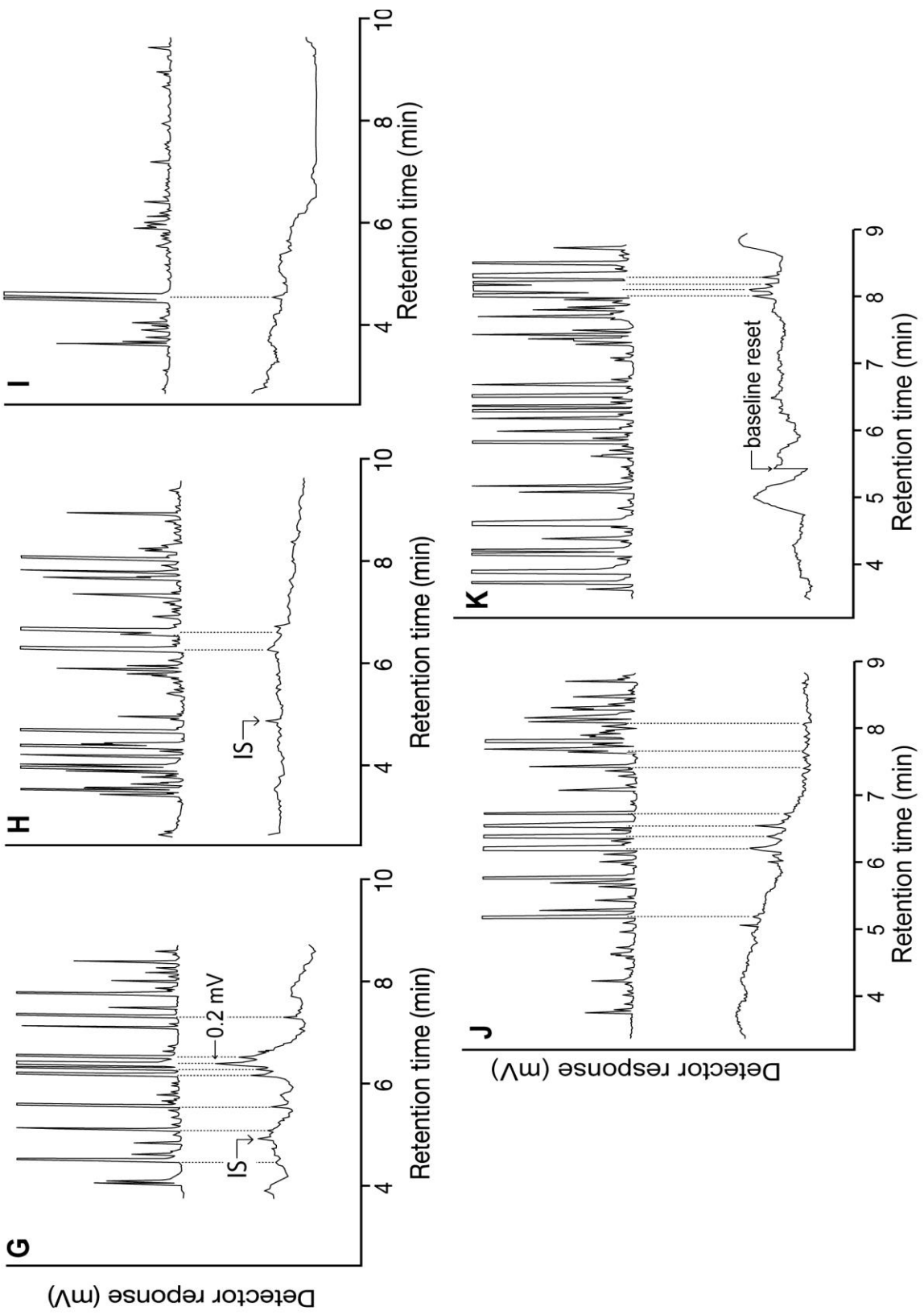
G
H
I
J
K
IS
0.2 mV
baseline reset
Detector response (mV)
Retention time (min)

Figure 2.1 b)

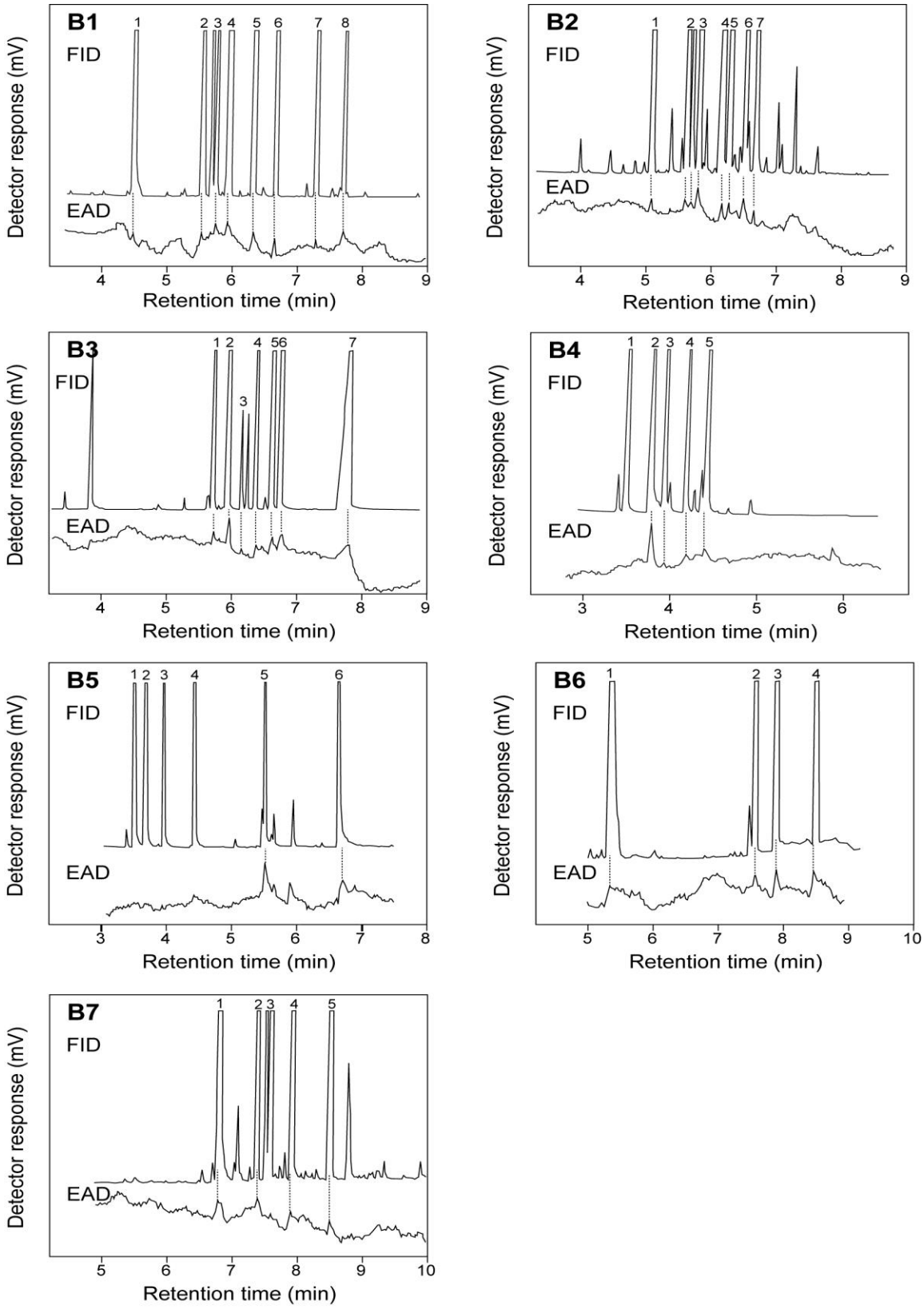
B1
FID
EAD
Detector response (mV)
Retention time (min)
B2
FID
EAD
Detector response (mV)
Retention time (min)
B3
FID
EAD
Detector response (mV)
Retention time (min)
B4
FID
EAD
Detector response (mV)
Retention time (min)
B5
FID
EAD
Detector response (mV)
Retention time (min)
B6
FID
EAD
Detector response (mV)
Retention time (min)
B7
FID
EAD
Detector response (mV)
Retention time (min)

Figure 2.2

3 IS SOYBEAN OIL AN EFFECTIVE REPELLENT AGAINST THE YELLOW FEVER MOSQUITO *AEDES AEGYPTI*?

3.1 Abstract

Soybean oil (SO) is considered an active ingredient in commercial BiteBlocker™ insect repellent products. Our objective was to test mechanisms by which SO exhibits repellency, using the yellow fever mosquito, *Aedes aegypti* (L.), as a representative of blood-feeding insects. Gas chromatographic – electroantennographic detection (GC-EAD) analyses of soybean oil were inconclusive. In dual-port glass-cage olfactometers, human hands treated with SO at various concentrations attracted as many mosquitoes as untreated hands, indicating that SO has no long-range repellent effect. In contrast, hands treated with *N,N*-diethyl-3-methylbenzamide (DEET) attracted significantly fewer mosquitoes than untreated control hands. In cage experiments with a section of a human forearm exposed to *Ae. aegypti*, sections treated with SO provided no protection against bites, whereas sections treated with DEET did. These results indicate that SO has no short-range or contact repellent properties. Both DEET and the BiteBlocker™ product yielded protection periods similar to those previously reported. Based on our data, the classification of SO as an active mosquito repellent should be re-considered.

3.2 Introduction

Insect repellents provide an important means of personal protection from nuisance and vector biting arthropods. Applied directly to the skin, commercial repellents

typically offer up to 8 h of protection, depending on active compound and formulation, arthropod species, application pattern, and environmental conditions. For over half a century, *N*,*N*-diethyl-3-methylbenzamide (DEET) has been the most recognized and most effective broad spectrum synthetic repellent. It is considered the "gold standard" against which all other repellents are measured (Costantini *et al*. 2004). It is generally deemed safe by regulatory agencies but adverse effects including neurological complications have been reported. Moreover, DEET is a solvent of some plastics, paints, varnishes, and synthetic fabrics (Trigg 1996; Qiu *et al*. 1998; Badolo *et al*. 2004). Many people are reluctant to use DEET for personal protection and instead seek naturally derived repellents such as plant essential oils. In Canada, the Pest Management Regulatory Agency (PMRA) in 2004 recommended the phase-out of products using citronella and related compounds, leaving Canadians with the limited selection of only three active ingredients repellent to insects. These consist of the synthetic repellent DEET (≤ 30%), the lemon eucalyptus derived *p*-menthane-3,8-diol (PMD), and the food product soybean oil (SO), *Glycine max*) (Fabaceae).

SO is claimed to be the main active ingredient of the commercially available repellent BiteBlocker™ (PMRA 1999; Fradin and Day 2002; Barnard and Xue 2004). Barnard and Xue (2004) report BiteBlocker™-mediated protection periods of 5.5 h against *Ae. albopictus*. Fradin and Day (2002) conclude that of seven possible natural repellents only BiteBlocker™ performed comparably to a 7% DEET formulation with an effective protection time of ca. 1.5 h against *Ae. aegypti*, and Amer and Melhorn (2006) report a protection period of 3 h with a 20% SO solution. SO is exempt from regulation in the U.S. (FIFRA 40CFR Sec. 152.25 (g), EPA 1996). In 2000, the PMRA granted full

registration to Consep SO technical 100%, and the formulated 2% SO BiteBlocker™ products (PMRA 2000). The proposed mode of action is that SO repellents disrupt the long- and short-range host-seeking behaviour of blood feeding insects by masking host odors and by cooling the temperature above the skin surface (PMRA 1999). SO was determined as the active ingredient by testing BiteBlocker™ products with and without SO in the end-use formulation. SO contains common fatty acids such as palmitic, oleic, linoleic and stearic acid. However, the main active ingredients and mode of action causing repellency have yet to be determined (PMRA 1999).

BiteBlocker™ is effective but GonE®, another SO-containing product, is not (Barnard and Xue 2004). Moreover, 100% SO had no repellent effect, raising doubt whether SO is an insect repellent (USDA 1954). Our objectives in this study were to investigate the mode(s) of action of SO. GC-EAD analyses of soybean oil were inconclusive. In dual-port olfactometers (Kline *et al.* 2003) we tested the potential of SO to mask long-range host odor. Furthermore, using the screened cage human bait method (Schreck and McGovern 1989; WHO 1996), we assessed short-range airborne and contact repellent properties of SO.

3.3 Materials and Methods

3.3.1 Experimental Insects

The black-eyed Liverpool strain of *Aedes aegypti* (L.) was supplied by Dr. Carl Lowenberger (SFU). Adults were kept at 27^{o}C, a 65-70% relative humidity, and a 14:10 (light:dark) photoperiod. They were provisioned with a 10% sucrose solution (in water). Larvae were kept under the same conditions and fed a Nutrafin® Basix Staple Food fish

diet. GC-EAD recordings and behavioural experiments were conducted between 0800-1600h, coinciding with the host-seeking period of *Ae. aegypti*.

3.3.2 Dual-Port Olfactometer Bioassay

SO as a volatile repellent against *Ae. aegypti* was tested according to methods developed by Kline *et al*. (2003). The bell-shaped glass olfactometer (Fig. 3.1) (25.0 × 14.5 cm i.d., ca. 5 L) had 2 opposite ports (each 10.0 × 4.0 cm i.d.), 8.0 cm above the base of the bell. A cone trap (15.0 × 4.0 cm o.d., 0.5 cm at narrowest part of cone) with a hand chamber (15.0 × 9.5 cm i.d.) attached distally was inserted into ports. A water aspirator was connected to the top of the bell, drawing air at 0.5 l/min through each of the hand chambers, the cone traps and finally through the bell. Hot plates approximately 10 cm below the joint of cone traps and hand chambers maintained the temperature of air entering the central chamber at 26^{o}C (±2). Screen mesh separated hand chambers from cone traps, thus preventing mosquitoes from contacting test stimuli. Ten nulliparous 5- to 8-d-old female *Ae. aegypti* were released into the olfactometer 30 min prior to each test. The density of mosquitoes in this cage represents a typical biting pressure experienced outdoors (Barnard *et al*, 1998; Fradin and Day, 2002). Each test was terminated when all mosquitoes were captured or 20 min had elapsed.

Both non-competitive and competitive bioassays were conducted. Non-competitive bioassays demonstrate the relative attractiveness of test stimuli by placing the stimuli in only one of two chambers and by assessing the mosquitoes' behavioural response to them. Test stimuli consisted of filter paper (Whatman No. 2, 1.27cm) which was saturated with 10 μl of a candidate repellent and inserted into the neck of a randomly assigned chamber. The opposite chamber remained empty. Experiments 1-6 (n = 6,

each) tested respectively 2, 5, 10, 25, 50, and 100% of research grade SO (Aldrich, Oakville, ON) in pentane. Experiments 7-10 (n = 6, each) tested respectively 5, 10, 25 and 50% of DEET (*N*,*N*-Diethyl-3-methylbenzamide; Aldrich, Oakville, ON) in ethanol. Experiment 11 tested a human subject's hand (n=23) for comparison of the stimuli's relative attractiveness. The numbers of mosquitoes captured in either cone trap or remaining in the central chamber were counted at the end of each replicate and were recorded as a percentage of total number of mosquitoes tested.

Competitive experiments tested the relative repellency of a candidate compound by combining it in one chamber with a test subject's hand (as the attractive source) while the other hand chamber contained the hand alone or in combination with an alternate repellent. The palm of a randomly assigned treatment hand received 10 μl of a repellent solution, whereas the control hand received the equivalent volume of solvent or alternate repellent compound. Experiments 12-17 (n = 6, each) tested hands treated with 2, 5, 10, 25, 50 and 100% of SO, respectively, *versus* control hands that were treated with pentane (except exp. 17). Experiments 18-21 (n = 6, each) tested hands treated with respectively 5, 10, 25 and 50% DEET in ethanol *versus* hands treated with ethanol. Experiments 22-27 (n = 6, each) were designed to compare the effect of two repellents. They tested hands treated with respectively 2, 5, 10, 25, 50 and 100% of SO in pentane *versus* hands treated with 10% DEET in ethanol. Experiment 28 (n = 24) tested untreated hands *versus* untreated hands to reveal any potential side bias. At the end of each replicate, numbers of mosquitoes captured in either chamber and in the central chamber were counted, and reported as a percentage of the total number of mosquitoes tested. Percentages of captured mosquitoes were grouped by repellent material and analyzed using the

Wilcoxon paired-sample test (Zar, 1999). The percentage of mosquitoes not captured in competitive bioassays (experiments 12-28) were pooled by treatment combination and subjected to analysis of variance (ANOVA) with means separation using Tukey's HSD test (SAS 9.1; SAS Institute 2008).

3.3.3 The Screened Cage Bioassay

Short-range and contact repellency of SO were tested in screened cage bioassays. These were designed to compare the protection time provided by SO with that of other repellents. (Schreck and McGovern, 1989; WHO, 1996). These bioassays used a wood-framed cage (27 × 27 × 42 cm; 30 L) with screened mesh top, back, and sides, a wood floor, and an acrylic front with a cotton stockinet sleeve for access. One hour prior to experimentation, 75 nulliparous, 5- to 8-d-old female *Ae. aegypti* were placed in a cage. The test subject's arm was covered with an elbow length polyethylene glove with a patch of 16.6 × 6 cm (100 cm^2) excised to expose the ventral forearm. Five min before the start of a test, the treatment stimulus was applied at a rate of 1.0 mg/cm^2. Experiment 29 (n = 6) tested neat (100%) SO *versus* light paraffin oil (PO) (EMD Chemicals, Gibbstown, NJ). Experiment 30 (n = 6) tested 2% SO in PO *versus* PO representing the concentration registered by the PMRA (PMRA, 1999). Experiment 31 (n = 6) tested BiteBlocker™ Herbal Insect Repellent Spray (containing 2% SO) (HOMS, LLC, Clayton, NC) *versus* PO. Experiment 32 tested 10% DEET in ethanol to gauge a possible repellent effect in preceding experiments. Biting pressure was assessed prior to each arm-insertion interval by inserting the hand of an untreated arm into the cage to receive at least 10 probings (without feeding) within 30 sec. For data acquisition, the treated arm was then inserted into the cage and the number of mosquitoes that landed and proceeded

to bite the skin in 3 min was recorded, repeating this procedure every 30 min. Two bites in one 3-min test, or one bite in one test followed by one or more confirmation bites in the subsequent test, constituted repellent failure. The complete protection time was recorded as the time from repellent application to repellent failure. On each day, only one candidate material was tested, ensuring that any residual material on the test subject and in the chamber had disappeared before the next bioassay.

Data were analyzed using SAS version 9.1 software (SAS Institute, Cary, NC). Differences in protection times (repellent efficacy) between treatment and control stimuli were assessed by classing protection times as intervals (e.g., 0, 33, 66 min) and by analyzing data with Fisher's exact test (α=0.05; PROC FREQ). To detect differences in protection effect between compounds, mean protection times were analyzed using the LIFEREG procedure for censored failure time data (α=0.05; PROC LIFEREG). This procedure is specifically designed to fit parametric models to censored failure time data (SAS 2008; Rutledge and Gupta 2006).

3.4 Results

Non-competitive experiments with hands (exp. 11) elicited both alightment and orientation behaviour towards the cone traps, resulting in a mean capture rate of 55.6% of mosquitoes (Table 3.1). This demonstrates that hands are effective in attracting mosquitoes in olfactometer experiments, and that they can serve as an attractive source for testing candidate repellent materials. All concentrations of SO and DEET failed to attract mosquitoes (Table 3.1). Competitive experiments demonstrated the relative long-range repellency of the material tested (Fig. 3.2). In experiments 13-17, hands treated

with SO or solvent attracted similar numbers of mosquitoes, indicating no long-range repellent effect by SO (Fig. 3.2). In experiments 18-21, hands treated with DEET in ethanol attracted significantly fewer mosquitoes than hands treated with just ethanol, indicating a long-range repellent effect by DEET (Fig. 3.2). Similarly, in experiments 22-27 hands treated with 10% DEET attracted significantly fewer mosquitoes than hands treated with SO, irrespective of the concentration tested (2, 5, 10, 25, 50, 100%) (Fig. 3.3). In experiment 28, either one of two untreated hands attracted similar proportions of mosquitoes (Fig. 3.3), indicating no side bias in the olfactometer. The presence of DEET in competitive experiments significantly reduced the overall number of mosquitoes responding to test stimuli, confirming its long-range repellent effect (Table 3.2).

In screen-cage experiments 29 and 30, 100% SO or 2% SO in paraffin oil failed to provide any protection against host-seeking *Ae. aegypti*, as did paraffin oil as control stimulus (Fig. 3.4). In screen-cage experiments 31 and 32, in contrast, Bite Blocker™ (containing 2% SO) or 10% DEET provided protection for 1.3 and 2.5 h, respectively, whereas their respective control stimuli failed to provide any protection (Fig. 3.4).

3.5 Discussion

Our data obtained in olfactometer and screened cage experiments provide evidence that SO acts neither as a long-range spatial repellent nor as a short-range or contact repellent. Unlike DEET, SO failed to prevent host-seeking mosquitoes from orienting toward and approaching attractive sources, confirming USDA (1954) findings. Because SO is typically obtained through solvent extractions, we speculated that the extraction processes may potentially remove repellent components from the oil. Therefore, we biossayed cold-pressed organic oil but the results (data not shown)

confirmed that SO alone is not effective in providing any level of protection against host-seeking mosquitoes.

The previously reported 3-h protection period provided by 20% SO (Amer and Melhorn 2006) may have been due to SO's preparation in a complex solvent formulation rather than due to SO itself. The use of excipients typically enhances the longevity of repellents by slowing down their dissipation (Bhuyan *et al*. 1974; Traboulsi *et al*. 2005; Amer and Melhorn 2006). Bioassaying repellents combined with excipients approximates the end-use formulation and allows us to determine the maximum protection time (Amer and Melhorn 2006).

Our tests of BiteBlocker™ yielded similar repellent effectiveness as previously reported (Fradin and Day 2002; Barnard and Xue 2004). As a constituent of BiteBlocker™, SO seems to contribute to the retention and delivery of other repellents rather than being a repellent itself. This conclusion is supported by results of a comparative study of insect repellents, revealing that the product GonE!® does not prevent biting from *Ae. aegypti* even though it contains SO as an ingredient (Barnard and Xue 2004). The authors speculate the repellent activity of BiteBlocker™ may be correlated with vanillin, which has a potentiating effect on other repellents (Barnard and Xue, 2004; Khan *et al*. 1975).

BiteBlocker™ is an emulsion consisting of glycerin and water, the emulsifier lecithin, soybean and coconut oil, citric acid, sodium bicarbonate, vanillin and geranium essential oil. The study submitted to the PMRA proclaims SO as an active ingredient based on experiments that removed it and other ingredients from formulations of the Blocker™ product (PMRA, 1999). Repellent properties of SO in Blocker^{TM} formulations

were inferred by findings that (*i*) a formulation lacking both SO and coconut oil was significantly less repellent than a formulation lacking SO, (*ii*) either of these two formulations was significantly less repellent than other formulations tested; and (*iii*) a formulation that contained SO but no coconut oil was as repellent as other formulations. We argue that SO may be necessary in the formulaic composition of the BiteBlocker™ products. SO consisting primarily of unsaturated fatty acids and coconut oil consisting primarily of saturated fatty acids may produce an effective dispersed phase of the oil-in-water emulsion matrix. This emulsion appears to yield an ideal viscosity and efficient method of delivery for insect repelling components. These may include the essential oil of geranium, which is repellent to mosquitoes (USDA 1954, 1967; Barnard, 1999; Trongtokit *et al*. 2005), and vanillin, which enhances the effect of other repellents (Barnard and Xue 2004; Khan *et al*. 1975) such as geranium oil in BiteBlocker™.

BiteBlocker™ provides a significant level of protection against *Ae. aegypti*. It is a useful addition to the limited arsenal of insect repellents available to Canadians. However, according to the definition of an active ingredient under the Pest Control Products Act (2002), our data suggest that SO does not qualify as such, because the observed repellency of BiteBlocker™ cannot be attributed to SO on its own. Instead, we suggest that the role of geranium oil in BiteBlocker™ products be examined more thoroughly, with the potential result of registering geranium oil as an active ingredient, and de-registering SO.

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Table 3.1 Mean percent of female *Aedes aegypti* captured in non-competitive experiments

			Mean (± SE) percent captured		
Exp. No.	Treatment	[ ]	Treatment trap %	Control trap %	% Non-responders
1	SO[a]	2%	0.0	0.0	100.0
2		5%	0.0	0.0	100.0
3		10%	0.0	0.0	100.0
4		25%	2.5(2.5)	0.0	97.5
5		50%	0.0	0.0	100.0
6		100%	0.0	4.3 (2.0)	95.7
7	DEET[b]	5%	0.0	0.0	100.0
8		10%	2.5 (2.5)	0.0	97.5
9		25%	5.0 (2.2)	0.0	95.0
10		50%	2.5 (2.5)	0.0	97.5
11	Hand		55.6 (4.0)	0.0	44.4

[a] Soybean oil

[b] *N*,*N*-diethyl-3-methylbenzamide

Table 3.2 Mean percent of mosquitoes not captured in competitive experiments 12-28 (n=17)

Treatment combination	Mean % (SE) not captured*
Hand + SO vs. Hand + DEET (exps. 22-27)	56.4 (1.7) a
Hand + DEET vs. Hand (exps. 18-21)	47.5 (5.4) a
Hand vs. Hand (exp. 28)	25.7 (2.7) b
Hand + SO vs. Hand (exps. 12-17)	24.7 (2.9) b

Data for treatment concentrations combined

*Means followed by the same letter are not significantly different (P>0.05) according to Tukey's HSD (SAS 2008)

3.7 Figure Captions

Figure 3.1 Top and lateral view of a dual-port Pyrex glass olfactometer for competitive and non-competitive experiments. Air was drawn at 0.5 L/min through the test stimulus chamber (10.0 × 4.0 cm i.d.), cone trap (15.0 × 4.0 cm o.d.) and central chamber (25.0 × 14.5 cm i.d., ca. 5L).

Figure 3.2 Mean (+ SE) percent of female yellow fever mosquitoes, *Aedes aegypti,* captured in experiments 12-21 (n = 6 each) in cone traps (see Figure 1) in response to a human hand to which was applied respectively soybean oil (SO) or *N,N*-diethyl-3-methylbenzamide (DEET) at various concentrations as the treatment stimulus or the equivalent volume of solvent as the control stimulus. In each of experiments 18-21, the asterisk indicates a significant preference for the control stimulus; Wilcoxon paired-sample test; $\alpha = 0.05$.

Figure 3.3 Mean (+ SE) percent of female yellow fever mosquitoes, *Aedes aegypti,* captured in experiments 22-27 (n = 6 each) in cone traps (see Figure 1) in response to a human hand to which was applied soybean oil (SO) at various concentrations as the treatment stimulus or 10% *N,N*-diethyl-3-methylbenzamide (DEET) as the control stimulus. Experiment 28 (n = 6) tested an untreated hand in each test stimulus chamber to reveal any potential side bias of the olfactometer. In each of experiments 22-27, the asterisk indicates a significant preference for the treatment stimulus; Wilcoxon paired-sample test; $\alpha = 0.05$.

Figure 3.4 Mean (+ SE) time elapsed in experiments 29-32 (n = 6 each) before soybean oil, BiteBlocker™ or *N,N*-diethyl-3-methylbenzamide (DEET) as the treatment stimulus, or paraffin oil or ethanol as the control stimulus, that were applied to a 100-cm^2 exposed area of a human forearm, failed to provide protection from bites by females of the yellow fever mosquito *Aedes aegypti*. In experiments 31 and 32, the asterisk indicates significantly longer protection due to the treatment stimulus than due to the control stimulus; Fisher's exact test; PROC FREQ, α=0.05. A different letter associated with the mean protection time of a treatment stimulus indicates a statistically significant difference; PROC LIFEREG; α =0.05.

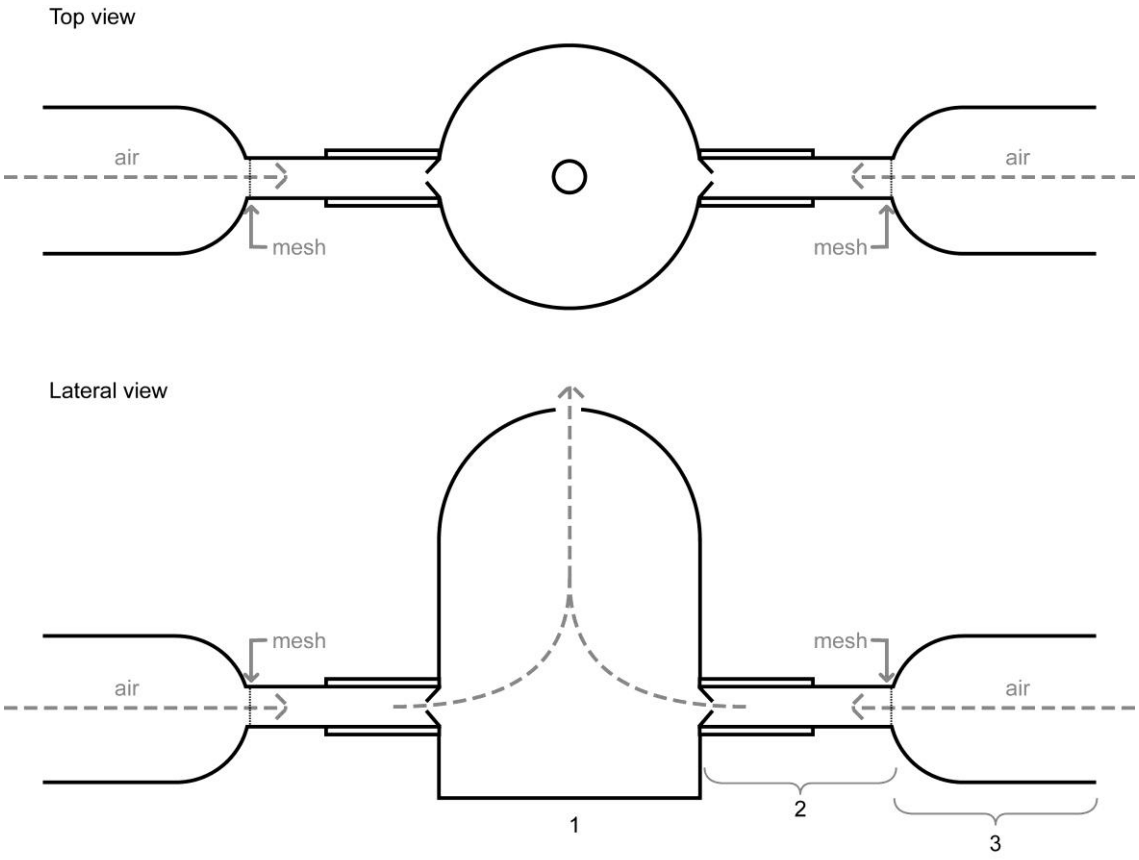

Figure 3.1

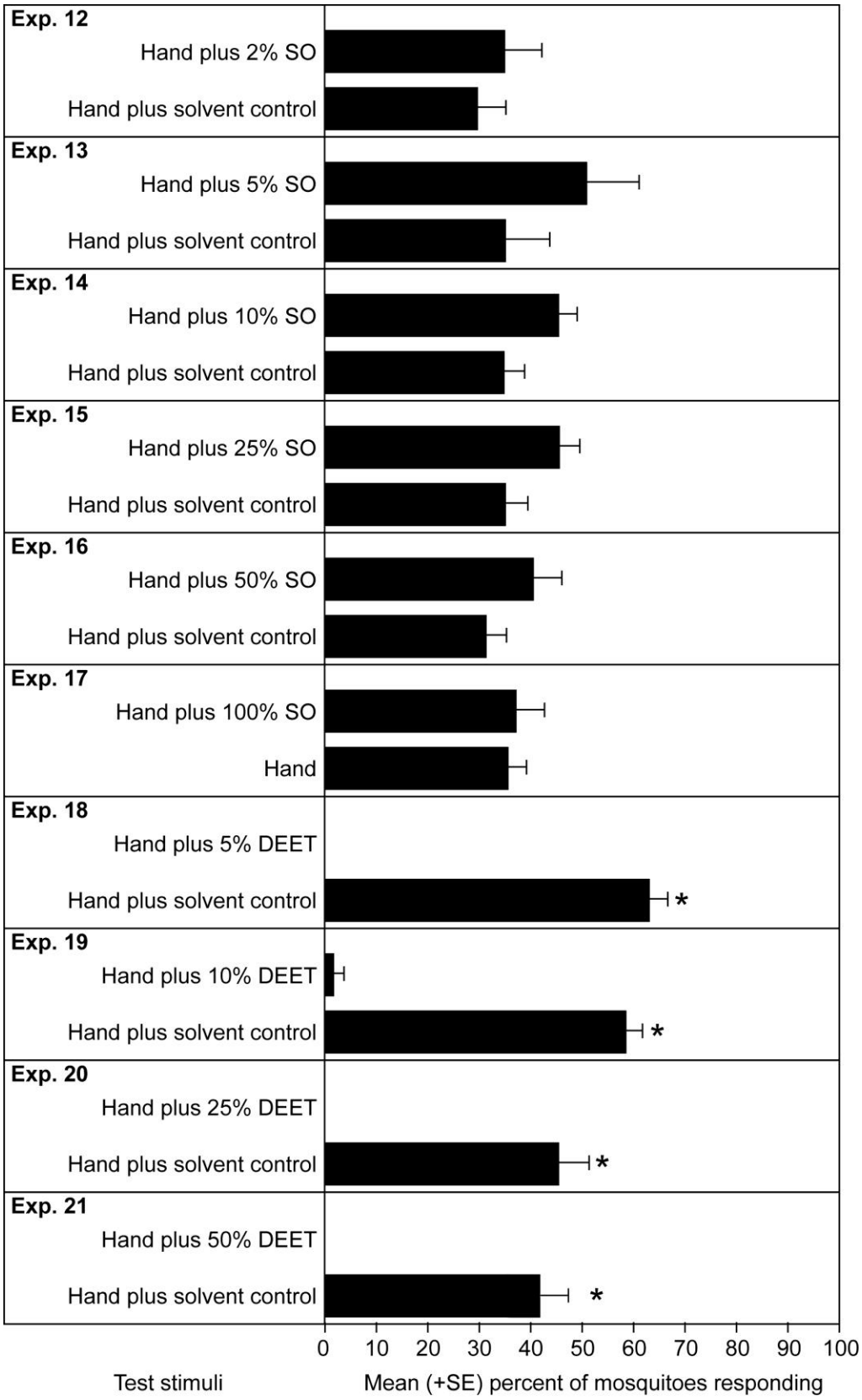
Exp. 12
Hand plus 2% SO
Hand plus solvent control
Exp. 13
Hand plus 5% SO
Hand plus solvent control
Exp. 14
Hand plus 10% SO
Hand plus solvent control
Exp. 15
Hand plus 25% SO
Hand plus solvent control
Exp. 16
Hand plus 50% SO
Hand plus solvent control
Exp. 17
Hand plus 100% SO
Hand
Exp. 18
Hand plus 5% DEET
Hand plus solvent control
*
Exp. 19
Hand plus 10% DEET
Hand plus solvent control
*
Exp. 20
Hand plus 25% DEET
Hand plus solvent control
*
Exp. 21
Hand plus 50% DEET
Hand plus solvent control
*
0 10 20 30 40 50 60 70 80 90 100
Test stimuli
Mean (+SE) percent of mosquitoes responding

Figure 3.2

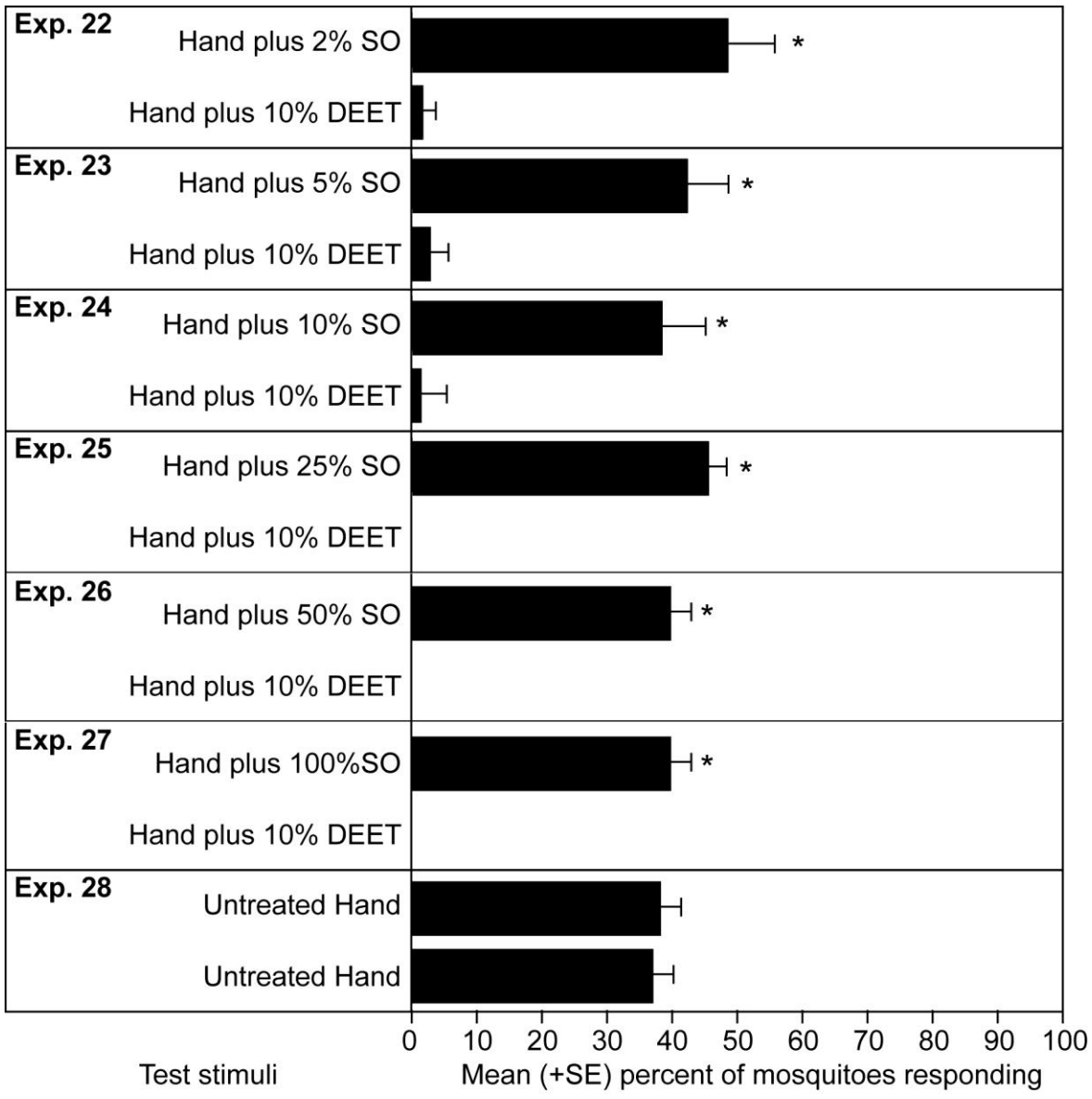
Exp. 22
Hand plus 2% SO
Hand plus 10% DEET
Exp. 23
Hand plus 5% SO
Hand plus 10% DEET
Exp. 24
Hand plus 10% SO
Hand plus 10% DEET
Exp. 25
Hand plus 25% SO
Hand plus 10% DEET
Exp. 26
Hand plus 50% SO
Hand plus 10% DEET
Exp. 27
Hand plus 100%SO
Hand plus 10% DEET
Exp. 28
Untreated Hand
Untreated Hand
*
0 10 20 30 40 50 60 70 80 90 100
Test stimuli
Mean (+SE) percent of mosquitoes responding

Figure 3.3

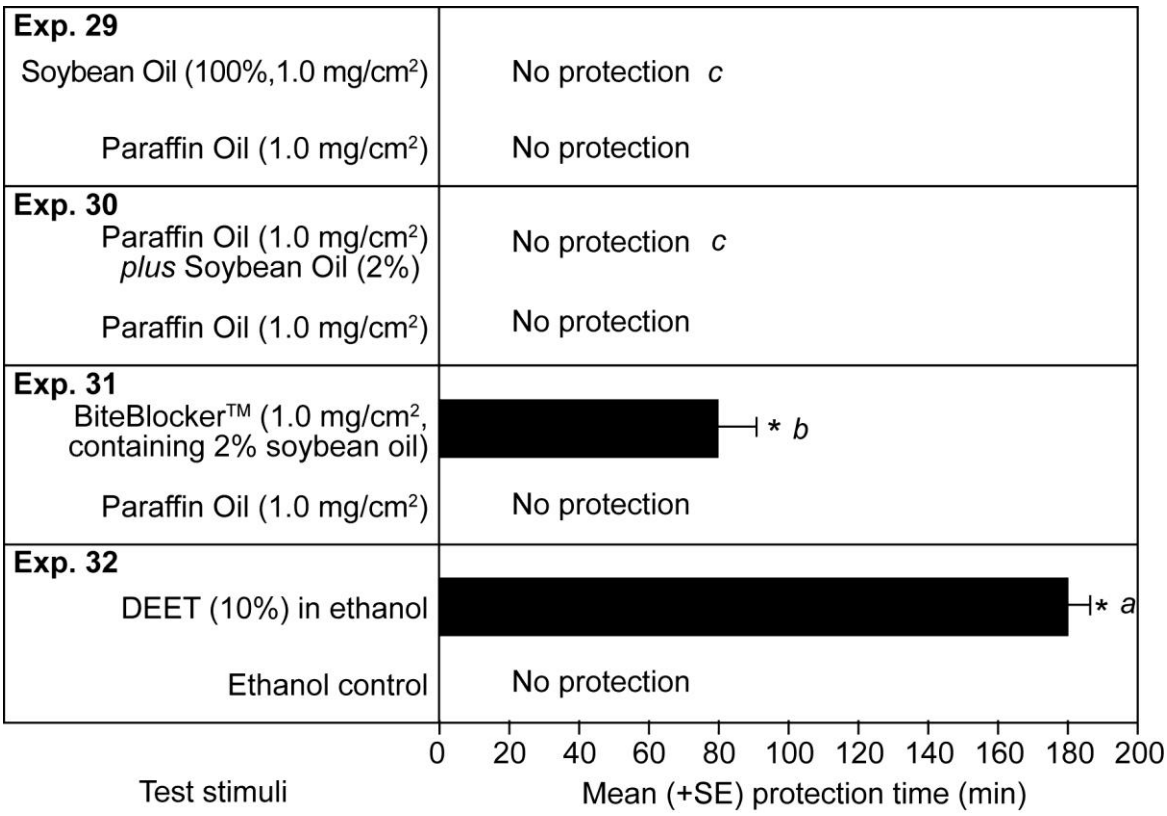
Exp. 29
Soybean Oil (100%,1.0 mg/cm²)
No protection c
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 30
Paraffin Oil (1.0 mg/cm²) plus Soybean Oil (2%)
No protection c
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 31
BiteBlocker™ (1.0 mg/cm², containing 2% soybean oil)
* b
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 32
DEET (10%) in ethanol
* a
Ethanol control
No protection
0 20 40 60 80 100 120 140 160 180 200
Test stimuli
Mean (+SE) protection time (min)

Figure 3.4

4 IDENTIFICATION OF ORGANOSULFUR CONSTITUENTS IN GARLIC ESSENTIAL OIL THAT ELICIT ANTENNAL AND BEHAVIOURAL RESPONSES FROM THE YELLOW FEVER MOSQUITO[2]

4.1 Abstract

Garlic (*Allium sativum*) and its essential oil have long been used for their distinct flavour, therapeutic effects, and as a topical and systemic insect repellent. We tested the hypothesis that the yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae), responds electrophysiologically and behaviourally to specific components of the steam distilled essential oil of garlic. In coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses of garlic oil, antennae of female *Ae. aegypti* responded strongly to 14 compounds. Seven of them [diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, 2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene, and 4,5,9,10-tetrathiatrideca-1,12-diene] were isolated or synthesized and tested for their ability to repel host-seeking female *Ae. aegypti*. A solution of diallyl trisulfide and diallyl tetrasulfide applied to a human forearm provided protection from female mosquitoes significantly longer than the paraffin oil control. All compounds had mean protection times significantly shorter than an equivalent dose of the "gold standard" DEET. Understanding the common moiety in organosulfur compounds that causes repellency

[2] Mrs. Regine Gries contributed significantly to the isolation and identification of compounds, and Dr. Grigori Khaskin synthesized compounds that could not be purchased.

could lead to the design of analogues that are more effective than their natural counterparts in repelling mosquitoes.

4.2 Introduction

Growing concern regarding the safety of synthetic insect repellents and the threat of various vector-borne diseases has driven the search for alternative botanically-derived insect repellents. Garlic, *Allium sativum* (L.), is well known for its antibacterial, antihelminthic, antitumor, and other medicinal properties (Block 1992). Garlic is also one of many botanical pesticides with deleterious effects on diverse insect pests. Garlic and its essential oil can be used for larvicidal control of *Anopheles* spp., *Aedes* spp., and *Culex* spp. mosquitoes (Amonkar and Reeves, 1970; Denloye *et al*. 2003). Garlic juice is insecticidal to cabbage root flies, *Delia radicum,* and houseflies, *Musca domestica* (Prowse *et al.* 2006). Intact, grated, and volatile extracts of garlic applied to brown rice repel the maize weevil, *Sitophilus zeamais*, and the red flour beetle, *Tribolium castaneum* (Rahman and Motoyama 2000).

Garlic is also used to repel mosquitoes. Although the consumption of garlic does not repel mosquitoes (Rajan *et al*. 2005), this method is still widely practiced in various parts of the world (Moore *et al*. 2006). Bassett (1998) claims that composition of garlic juice and hot pepper sauce repels mosquitoes, but the repellent contribution of each of these components remains unknown. Weisler (1989) reports that administration of a 1:20 composition of aneurine (Vitamin B_1) and garlic oil in the diet of domesticated animals can protect them from infestation by fleas and ticks. However, in a test with flea-infested dogs, neither component was effective alone, nor did Weisler (1989) disclose whether either aneurine or some component of garlic oil was actually present in the skin of the test

dogs. Therefore, the actual role of both components is uncertain. Moreover, Weisler (1989) erroneously teaches that allyl sulfide is the same as garlic oil, when in fact garlic oil is a complex mixture of many compounds.

Unlike systemic use, topical application of the essential oil of garlic has demonstrated relatively strong repellency. Trongtokit *et al*. (2005) found that 100% garlic oil offered 70 min of protection against *Aedes aegypti*, and Bhuyan *et al.* (1974) reported that a formulation of 1% garlic oil in petroleum jelly and beeswax provide protection for ~8 h against *Culex fatigans*. Nevertheless, no systematic experimental study has been conducted to determine the identity of compounds in garlic oil that express repellence to blood-feeding insects or arthropods. Our objective was to analyze garlic oil electrophysiologically by coupled gas chromatographic-electroantennographic detection (GC-EAD) using the yellow fever mosquito, *Aedes aegypti* (L.) as the electroantennographic detector, and to bioassay antennal-stimulatory component(s) for their ability to repel *Ae. aegypti*.

4.3 Materials and Methods

4.3.1 Experimental Insects

The black-eyed Liverpool strain of *Ae. aegypti* (L.) was supplied by Dr. Carl Lowenberger (SFU). Adults were kept at 27^{o}C, a 65-70% relative humidity, and a 14:10 (light:dark) photoperiod. They were provisioned with a 10% sucrose solution (in water). Larvae were kept under the same conditions and fed a Nutrafin® Basix Staple Food fish diet. GC-EAD recordings and behavioural experiments were conducted between 0800-1600h, coinciding with the host-seeking period of *Ae. aegypti*.

4.3.2 Analytical Methods

Aliquots of garlic oil (*Allium sativum* – Mexico; Clearwater Soap Works, Box 1775 RR1, Clearwater, BC V0E 1N0, Canada) were analyzed by gas chromatography and by GC-EAD (Arn *et al*. 1975; Gries *et al*. 2002) using a Hewlett-Packard (HP) 5890 gas chromatograph equipped with a GC column (30 m × 0.32 mm ID) coated with DB-5 (J&W Scientific, Folsom, California, USA). Injector and detector temperatures were 275°C, and the temperature program was as follows: 50ºC held for 1 min, 10ºC per min to 160ºC, then 20ºC per min to 300ºC. For GC-EAD recordings, a severed female insect head (with both antennae intact) was placed into the opening of a glass capillary electrode (1:0 × 0:58 × 100 mm) (A-M Systems, Inc., Carlsborg, Washington, USA) filled with saline solution. One antenna with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia, Canada) was placed into the recording capillary electrode. Antennally active compounds of garlic oil were identified by coupled GC-mass spectrometric (MS) analyses employing a Varian 2000 Ion Trap MS fitted with the above-referenced DB-5 column. Assignments of molecular structure for antennal stimulatory constituents were confirmed by comparing their retention time and mass spectra with those of authentic standards or with those reported in literature [Methyl allyl disulfide, methyl allyl trisulfide: Yu *et al*., 1989; 3*H*-1,2-dithiolene, 4*H*-1,2,3-trithiin: Chen and Ho, 1998; 5-methyl-[1,2,3,4]-tetrathiane, methyl allyl tetrasulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, 2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene, 4,5,9,10-tetrathiatrideca-1,12-diene: Block *et al*., 1988; Block, 1992].

High performance liquid chromatography (HPLC) employed a Waters Delta 600 HPLC fitted with a reverse phase Prep Nova-Pak HR C18 column (19mm × 300mm), using a Waters 2487 Dual λ Absorbance detector set to 210 nm and a Waters 746 Data Module integrator. For experiments 2-8, individual EAD-active constituents of garlic oil were isolated with a 20 ml/min flow of acetonitrile/water (70:30). Constituents with longer GC retention times were isolated by first heating aliquots of garlic oil in Petri dishes at 40^{o}C under vacuum for 12 h to enrich their absolute and relative abundance prior to HPLC separation.

4.3.3 Analyses of Heat-Labile Constituents

Garlic oil is known to contain some thermally labile organosulfur constituents (Block *et al*. 1988; Yu *et al*. 1994; Chen and Ho 1998). Suspecting that some of them rearrange in the 275^{o}C hot GC injection port and then elicit antennal responses, garlic oil was also analyzed by cold (175^{o}C) or on-column injection. Temperature programming was 50^{o}C for 1 min, followed by an increase of 10^{o}C per min to 280^{o}C.

4.3.4 General Bioassay Procedures

Short-range and contact repellency of garlic oil and antennal stimulatory constituents were tested in screened cage bioassays, which were designed to compare mean protection times (Schreck and McGovern, 1989; WHO, 1996). These bioassays used a wood framed cage (27 × 27 × 42 cm; 30 L) with screened mesh top, back, and sides, a wood floor, and an acrylic front with a cotton stockinet sleeve for access. One hour prior to each test, 75 nulliparous, 5- to 8-d-old female *Ae. aegypti* were placed in a cage. The test subject's arm was covered with an elbow length polyethylene sleeve with

a patch of 16.6cm × 6cm (100 cm^2) excised to expose the ventral forearm. Five min before the start of a test, the treatment stimulus was applied at a rate of 1.0 mg/cm^2. Biting pressure was assessed prior to each arm-insertion interval by inserting a hand into the cage to receive at least 10 probings (without feeding) within 30 sec. For data acquisition, the treated arm was then inserted into the cage and the number of mosquitoes that landed and proceeded to bite the skin in 3 min was recorded, repeating this procedure every 30 min. Two bites in one 3-min test, or one bite in one test followed by one or more confirmation bites in the subsequent test, constituted repellent failure. The complete protection time was recorded as the time from repellent application to repellent failure. On each day, only one candidate material was tested, ensuring that any residual material on the test subject and in the chamber had disappeared before the next bioassay.

4.3.5 Specific Experiments

Experiment 1 re-investigated the effect of steam distilled garlic oil by testing the behavioural response of mosquitoes presented with an arm treated with 0.1 mg/cm^2 of garlic oil in paraffin oil and an arm treated with paraffin oil alone.

Experiments 2-7 tested the hypothesis that one or more antennal stimulatory components of garlic oil (Figure 4.2) repelled mosquitoes. Test compounds were isolated from garlic oil through preparative HPLC, synthesized or purchased (Table 4.1). They included diallyl disulfide (99% chemically pure by GC; exp. 2), diallyl trisulfide (99%; exp. 3), diallyl tetrasulfide (70%; exp. 4), 2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran (51%) and 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran (25%) in combination (exp. 5), 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene (75-81%; exp. 6), and 4,5,9,10-tetrathiatrideca-1,12-diene (≥92%; exp. 7). The cyclic compounds

2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran and 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran) eluted in the same HPLC fractions and were thus tested together. All compounds were prepared as a 10% (w/v) solution in paraffin oil for a dosage of 0.1 mg/cm^2. For comparison, experiment 8 tested a 10% ethanolic solution of DEET (*N*,*N*-diethyl-3-methylbenzamide, 97%, Aldrich, Oakville, ON, Canada).

4.3.6 Syntheses of 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene (75-81%) (13) and 4,5,9,10-tetrathiatrideca-1,12-diene (≥92%) (14).

See Figure 4.1. Thiourea (2.90 g) was added to a solution of 3.00 ml of allyl bromide (35 mmol) in 100 ml of ethanol. The mixture was refluxed for 4 h, cooled and 80 mmol of KOH pellets were added with 0.2 ml of water. This mixture was then heated for 1.5 h. After cooling to 0^{o}C, 10 mmol (1 ml) of 1,3-propanedithiol was added, followed by the addition of 15 g NaOH in 150 ml. The mixture was stirred 10 min, after which 55 mmol of $K_3Fe(CN)_6$ in 60 ml of H_2O were added during 1h. The mixture was kept at 0^{o}C for another 2 h. An ether/hexane (1:1) mixture (100 ml) was added and the organic ingredients were extracted. The extract was washed with water, brine, and then dried ($MgSO_4$) with solvents evaporated *in vacuo*, yielding a 3.6 g mixture of products. This mixture contained 10% 4,5,9,10-tetrathiatrideca-1,12-diene (**14**), (GC, cold injection, yield 14%). Compound **13** was produced analogously utilizing 1,2-propanedithiol as a synthetic intermediate.

An alternative synthesis eliminated the use of the highly offensive 1,3,-propanedithiol (or 1,2-propanedithiol) and the separate formation of allylisothiuronium salt from allylbromide and thiourea. Instead, we formed in the same flask two di-isothiuronium salts, one from allylbromide and the other from double excesses of

thiourea and 1,3-dibromopropane. Hydrolyses with KOH yielded a mixture of allylthio- and 1,3-dithio propane thiolates. Oxidation of this mixture with $K_3Fe(CN)_6$ afforded a blend with 8% of 4,5,9,10-tetrathiatrideca-1,12-diene (**14**). 6-Methyl-4,5,8,9-tetrathiadodeca-1,11-diene (**13**; 8% yield) was produced similarly using 1,2-dibromopropane. Compounds were purified by preparative HPLC.

4.3.7 Data Analyses

Experiments 1-8 (n = 6, each) were conducted with a single human test subject, randomly selecting the compound to be tested each day. Data were analyzed using SAS version 9.1 software (SAS Institute, Cary, NC; SAS 2008). Differences in protection times (repellent efficacy) between treatment and control stimuli were assessed by classing protection times as intervals (e.g., 0, 33, 66 min) and by analyzing data with Fisher's exact test (α=0.05; PROC FREQ). To compare the repellent effect of compounds, their mean protection times were analyzed using the LIFEREG procedure for censored failure time data (α=0.05; PROC LIFEREG). Because failure times obtained are censored interval values, the Weibull distribution is more appropriate than the normal distribution, and the PROC LIFEREG procedure is designed to fit parametric models to such censored failure time data (SAS Institute 2008; Rutledge and Gupta 2006).

4.4 Results

In experiment 1, aliquots of 0.1 mg/cm^2 garlic oil in paraffin oil applied to a human arm provided an estimated mean protection time (eMPT) of 44 (±7) min. Paraffin oil alone afforded no protection (Fig. 4.2).

GC-EAD analyses of garlic oil revealed 14 components (Fig. 4.3) that consistently elicited responses from female antennae. These were methyl allyl disulfide (**1**), 3*H*-1,2-dithiole (**2**), diallyl disulfide (**3**), methyl allyl trisulfide (**4**), 4*H*-1,2,3-trithiin (**5**), diallyl trisulfide (**6**), 5-methyl-1,2,3,4-tetrathiane (**7**), methyl allyl tetrasulfide (**8**), 5*H*-1,2,3,4-tetrathiepine (**9**), diallyl tetrasulfide (**10**), 2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran (**11**), 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran (**12**), 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene (**13**), and 4,5,9,10-tetrathiatrideca-1,12-diene (**14**).

The temperature of the GC injection port affected the presence and relative abundance of antennal stimulatory constituents in garlic oil (Fig. 4.4). Heating of the injection port reduced the relative abundance of straight chain diallyl and methyl allyl tri- and tetrasulfides **4**, **6**, **8** and **10** by 42, 62, 75, and 94% respectively. Conversely, this increased the relative abundance of methyl allyl disulfide (**1**) and diallyl disulfide (**3**) by 115% and 102%, respectively, while concurrently forming the four cyclic polysulfides **2**, **5**, **7**, and **9**. The relative abundance of the higher molecular weight compounds **11**-**14** remained unaffected.

Of the 14 antennal stimulatory components (Fig. 4.4), seven (**3**, **6**, **10, 11** *plus* **12**, **13**, and **14)** were isolated from garlic oil by HPLC or synthesized and tested for their ability to repel host-seeking *Ae. aegypti* from a human host. Of these seven compounds, diallyl trisulfide (**6**) diallyl tetrasulfide (**10**) provided protection times significantly different from that of a paraffin oil control (Fig. 4.2). All compounds had protection times significantly shorter than those of DEET tested at the same concentration.

4.5 Discussion

Our data provide evidence that garlic oil, and specific constituents isolated therefrom, repel host-seeking *Ae. aegypti*. However, at a dose of 10% active ingredient (0.1 mg/cm^2), none of the antennal stimulatory constituents was as effective as the "gold standard" DEET in repelling *Ae. aegypti*. Nonetheless, these compounds and their repellency provide impetus for designing molecules that will be more repellent, but less "smelly" than their natural counterparts in garlic.

GC-EAD analyses of garlic oil revealed 14 organosulfur compounds that elicited strong responses from female *Ae. aegypti* antennae. Both garlic and garlic oil contain thermally labile organosulfur compounds (Block 1992). Thus, we expected one or more antennal stimulatory compounds in GC-EAD analyses to have formed by thermal rearrangement in the 275^{o}C hot GC injection port. Similarly, allyl isothiocyanate in mustard isomerizes due to high injection port temperatures (Chen and Ho 1998). Gas chromatography of solvent extracts of garlic results in the dehydration of allicin (allyl 2-propenethiosulfinate) to form two disulfides (Yu *et al*. 1989). At hot injection port temperatures, we observed the rearrangement of aliphatic tri- and tetrasulfides to mono- and disulfides as well as cyclic polysulfides.

Between 80^{o}C and 150^{o}C the pyrolysis of **3** generates mainly **6** and smaller quantities of **10**-**14** (Block *et al*. 1988). Room temperature extracts of garlic contain primarily the thermally labile allicin (2-propene-1-sulfinothioic acid S-2-propenyl ester), whereas its thermal rearrangement products diallyl, dimethyl, and methyl allyl polysulfides are present in the steam-distilled essential oils of garlic (Block *et al*. 1988). Steam distillation of garlic is likely to convert not only allicin to **1, 3**, **4**, **6**, and **8**, but also

to convert **3** to **4**, **6**, **8** and **10**-**14**. This is evidenced by the presence of compounds **1**, **3**, **4**, **6**, **8**, **10**, **11**, **12**, **13**, and **14** in analyses of garlic oil by on-column injection, which minimizes thermal degradation processes (Fig. 4.4).

Chromatographic analyses of garlic oil with cold and hot injection demonstrated that temperatures >175^{o}C induced the formation of the cyclic polysulfides **2**, **5**, **7**, and **9**, indicating that these antennal stimulatory constituents were not present in garlic oil. Some of these and similar compounds also form following heating (180^{o}C) of alliin (*S*-allylcysteine *S*-oxide) and deoxyalliin (*S*-allylcysteine) (Yu *et al*. 1994). Compound **2** is the oxidation product of 1,2-dithiacyclopentane, which is produced when allyl mercaptan (2-propene-1-thiol), a volatile component of garlic oil, and hydrogen sulfide interact (Yu *et al*. 1989; Yu *et al*. 1994). Compound **7** reportedly forms when one molecule of allyl mercaptan interacts with three molecules of hydrogen sulfide (Yu *et al*. 1994). Similar to the formation of **2**, we contend that **5** and **9** are oxidation products of 1,2,3-trithiacyclohexane and 1,2,3,4-tetrathiepane, respectively. According to Yu *et al*. (1994), 1,2,3-trithiacyclohexane is formed when one molecule of allyl mercaptan interacts with two molecules of hydrogen sulfide, and 1,2,3,4-tetrathiepane is formed when one molecule of allyl mercaptan interacts with three molecules of hydrogen sulfide.

Seven of the 14 antennal stimulatory constituents were selected for behavioural studies. Compounds **1**, **4**, and **8** were excluded from testing because they were too difficult to isolate or purify after synthesis. In contrast, the diallyl polysulfide homologues **3**, **6**, and **10** represent predominant compounds in garlic oil, and could be isolated by preparative HPLC in relatively high purity. Test compound **10** was more likely ca. 92% rather than 70% because the impurities **3** and **6** would have formed during

the GC analysis. The structural isomers **11** and **12** were tested in combination because they eluted in the same HPLC fraction, and could not be synthesized in quantities sufficient for behavioural studies. Compounds **13** and **14** were more readily synthesized than isolated.

The terminal allyl sulfide group of garlic oil constituents is likely the functional moiety that elicits responses from mosquito antennae (Figure 4.4). This group is common to 10 of the 14 antennal stimulatory compounds. Unlike their diallyl (**3**, **6**, **10**) and methyl allyl (**1**, **4**, **8**) counterparts, the dimethyl polysulfide analogues are not electrophysiologically active, indicating that the allyl sulfide group is essential for binding with olfactory receptors. It follows that compounds **2**, **5**, **7**, and **9** interact with other receptor proteins, or their molecular conformation somehow allows them to fit the same receptor responsible for binding the other ten.

Both steric and electronic properties of semiochemicals interact with olfactory receptors (Gupta and Bhattacharjee 2006). Structure-activity investigations revealed the specificity of olfactory receptors in recognizing semiochemicals or their analogues. For example, single-sensillum recordings with the turnip moth, *Agrotis segetum,* have shown reduced receptor binding responses to various structural analogues of the sex pheromone molecule (Bengtsson *et al*. 1987; Bengtsson *et al*. 1990; Jonsson *et al*. 1991; Jonsson *et al*. 1992; Gustavsson *et al*. 1997). Apparently, steric and electronic properties of analogues preclude their optimal complementary binding with the receptor protein cavity, but do not necessarily prohibit some degree of receptor stimulation (Wu *et al*., 1993; Gustavsson *et al*. 1997; Campanacci *et al*. 2001). Instead, functional moieties such as the acetate group and double bond position of the turnip moth sex pheromone bind and

stimulate the pheromone receptor with varying degrees of complementarity and receptor affinity (Jonsson *et al*. 1992; Gustavsson *et al*. 1997). Moreover, analogues binding a pheromone receptor are capable of eliciting behavioural responses sometimes similar to that of the pheromone, depending on the receptor binding affinity (Wu *et al*. 1993).

The protection time of an insect repellent is proposed to be a function of its (*i*) molecular length (with respect to steric and electronic effects in receptor binding affinity), (*ii*) lipophilicity, and (*iii*) vapor pressure (Suryanarayana *et al*. 1991). These factors would account for variability in protection time observed for individual constituents of garlic oil. Compounds with great receptor affinity require fewer molecules to activate an olfactory receptor (Jonsson *et al*. 1992), and when tested at a high dose would theoretically result in long protection times. However, this concept is applicable only to those compounds that are sufficiently volatile to evaporate and enter antennae.

GC-EAD analyses of garlic oil revealed compounds that bind olfactory receptors in mosquito antennae, but do not reveal the optimal molecular conformation for binding. Antennal responses to individual compounds vary according to compound abundance and deterioration of the antenna through the course of an antennogram. Single-sensillum recordings might be more appropriate to reveal the complementarity of an olfactory receptor and organosulfur constituents of garlic oil (Jonsson *et al*. 1991; Jonsson *et al*. 1992).

Intact garlic cloves contain alliin and the enzyme alliinase, which converts alliin to allicin when the clove is injured (Stoll and Seebeck 1948). Allicin causes the pungent "burning" sensation of cut raw garlic. Together with homologues and other sulfur

compounds, allicin has evolved as the plant's defense mechanism for protection from pathogens and predators (Block 1992; Macpherson *et al*. 2005). Most extant species of mosquitoes blood-feed to obtain protein for egg development, and sugar-feed to obtain energy, especially within the first days of emergence (Foster and Takken 2004; Moore *et al*. 2006). Being highly synanthropic and anthropophilic, *Ae. aegypti* has a significant fitness advantage in exclusively blood-feeding (Scott *et al*. 1997; Scott *et al*. 1998). However, that sugar-feeding persists must have some competitive advantage, particularly in rural areas where humans are less accessible (Martinez-Ibarra *et al*. 1997; Foster and Takken 2004). Persistent sugar-feeding implies an ability to detect semiochemicals from plants, including those with insecticidal and deterrent properties such as garlic (Amonkar and Reeves 1970; Rahmann and Motoyama 2000; Denloye *et al*. 2003). The presence of olfactory receptors on mosquito antennae for organosulfur compounds might be adaptive in detecting and avoiding potentially harmful feeding sites.

This pilot study has screened constituents of garlic oil for potential insect repellency. The experiments conducted with a single human subject do not account for potential interpersonal variability and limit inferences to the test subject. Nonetheless, the experimental protocol used a standardized attractive source, which permitted a comparative evaluation of compounds and their repellent activity. The results obtained warrant further testing of compounds at various concentrations on multiple test subjects.

In light of desired properties of ideal insect repellents, such as long-lasting and odorless, the antennally active constituents in garlic oil are of little value as commercial repellents (see Garson and Winnike 1968). They possess a strong offensive odor, defy facile isolation or synthesis, and compare unfavourably with DEET's efficacy.

Nonetheless, all compounds except **1** have a molecular weight within the range (146-257 g/mol) of the most effective repellents evaluated by the USDA (USDA 1954; Rayner and Wright 1966; Garson and Winnike 1968). It seems feasible to develop organosulfur analogues with stronger efficacy and less offensive smell. Such designer molecules may become the next generation of potent insect repellents.

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Table 4.1 Source of antennal stimulatory constituents of garlic oil tested in experiments.

Antennal stimulatory constituent	**Source**
Diallyl disulfide	80% Sigma Aldrich (Oakville ON, Canada); isolated from garlic oil by prep-HPLC
Diallyl trisulfide	99% LKT Laboratories Inc. (St. Paul MN, US); isolated from garlic oil by prep-HPLC
Diallyl tetrasulfide	Isolated from garlic oil by prep-HPLC
2-(2,3-Dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran & 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran	Isolated from garlic oil by prep-HPLC
6-Methyl-4,5,8,9-tetrathiadodeca-1,11-diene	Synthesized (Figure 4.1)
4,5,9,10-Tetrathiatrideca-1,12-diene	Synthesized (Figure 4.1)

4.7 Figure Captions

Figure 4.1 Scheme for the synthesis of 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene (**13**) and 4,5,9,10-tetrathiatrideca-1,12-diene (**14**).

Figure 4.2 Mean (+ SE) protection times against bites from the yellow fever mosquito provided by (a) garlic oil (10%) in paraffin oil in experiment 1 (n = 6), (b) each of seven garlic oil constituents (10% each) in paraffin oil in experiments 2-7 (n = 6, each), and (c) DEET (10%) in ethanol applied at 1 mg/cm^2 to a 100-cm^2 exposed area of a human forearm. The paraffin oil or ethanol control stimulus provided no protection. In each of experiments 3 and 4, an asterisk indicates a significant difference between test stimuli (Fisher's exact test; PROC FREQ; α=0.05). In experiments 2-8, means followed by the same letter are not significantly different (PROC LIFEREG; α=0.05).

Figure 4.3 Flame ionization detector (FID) and electroantennographic detector (EAD: female *Aedes aegypti* antennae) responses to aliquots of garlic oil. Chromatography: Hewlett Packard (HP) gas chromatograph equipped with a GC column (30 m × 0.32 mm ID) coated with DB-5; injector and detector temperature: 250°C; temperature program: 50ºC (held for 1 min), 10ºC per min to 160ºC, then 20ºC per min to 300ºC. The molecular structure and name of EAD-active compounds 1-10 that elicited responses from antennae are depicted above the GC-EAD trace. EAD-active components 11, 12, 13 and 14 elute later than 13.5 min in the gas chromatogram. Trace components 11-14 are detectable by FID and EAD particularly after heating garlic oil in open Petri dishes at 40°C for 12 h, thus enhancing their absolute and relative abundance.

Figure 4.4 Effect of mode of injection and resulting temperature on the percent abundance of antennal stimulatory compounds in garlic oil. Chromatography: Hewlett Packard (HP) gas chromatograph equipped with a GC column (30 m × 0.32 mm ID) coated with DB-5; temperature program: 50^{o}C for 1 min, 10^{o}C per min to 280^{o}C. [a]Retention indices (RI) relative to *n*-alkane standards. **tr** indicates trace presence, below integrable levels.

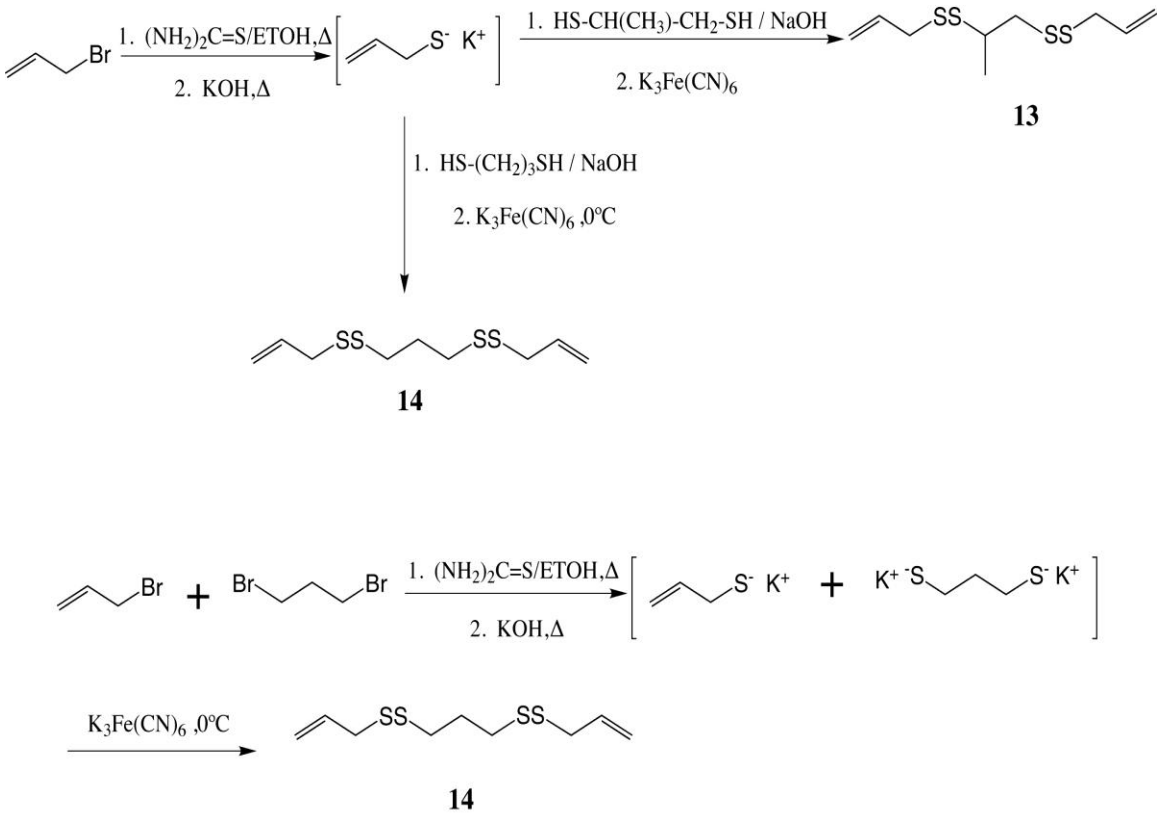
1. (NH2)2C=S/ETOH,Δ
2. KOH,Δ
1. HS-CH(CH3)-CH2-SH / NaOH
2. K3Fe(CN)6
13
1. HS-(CH2)3SH / NaOH
2. K3Fe(CN)6 ,0ºC
14
1. (NH2)2C=S/ETOH,Δ
2. KOH,Δ
K3Fe(CN)6 ,0ºC
14

Figure 4.1

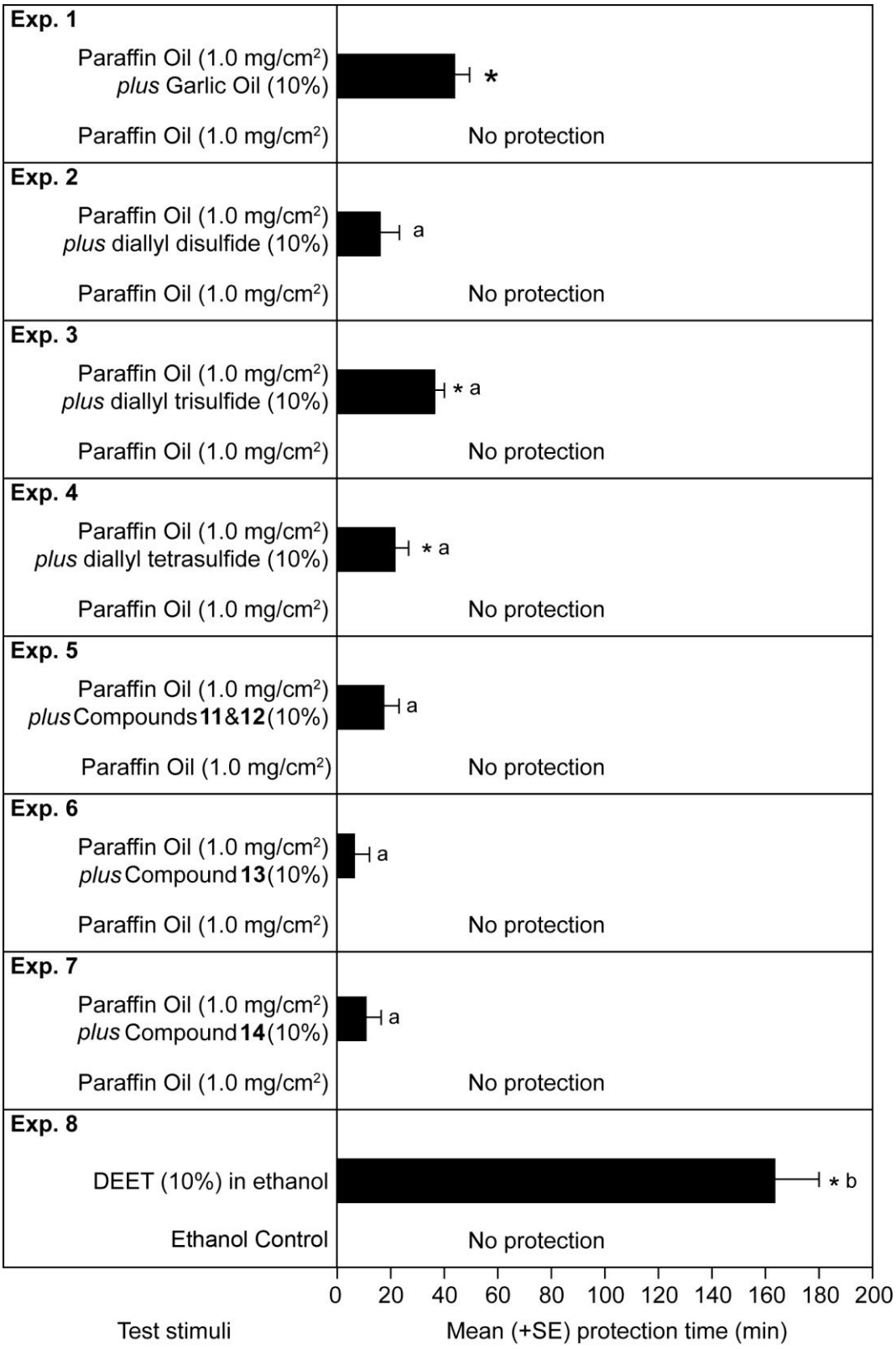
Exp. 1
Paraffin Oil (1.0 mg/cm²) plus Garlic Oil (10%)
*
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 2
Paraffin Oil (1.0 mg/cm²) plus diallyl disulfide (10%)
a
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 3
Paraffin Oil (1.0 mg/cm²) plus diallyl trisulfide (10%)
* a
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 4
Paraffin Oil (1.0 mg/cm²) plus diallyl tetrasulfide (10%)
* a
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 5
Paraffin Oil (1.0 mg/cm²) plus Compounds 11&12 (10%)
a
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 6
Paraffin Oil (1.0 mg/cm²) plus Compound 13 (10%)
a
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 7
Paraffin Oil (1.0 mg/cm²) plus Compound 14 (10%)
a
Paraffin Oil (1.0 mg/cm²)
No protection
Exp. 8
DEET (10%) in ethanol
* b
Ethanol Control
No protection
0 20 40 60 80 100 120 140 160 180 200
Test stimuli
Mean (+SE) protection time (min)

Figure 4.2

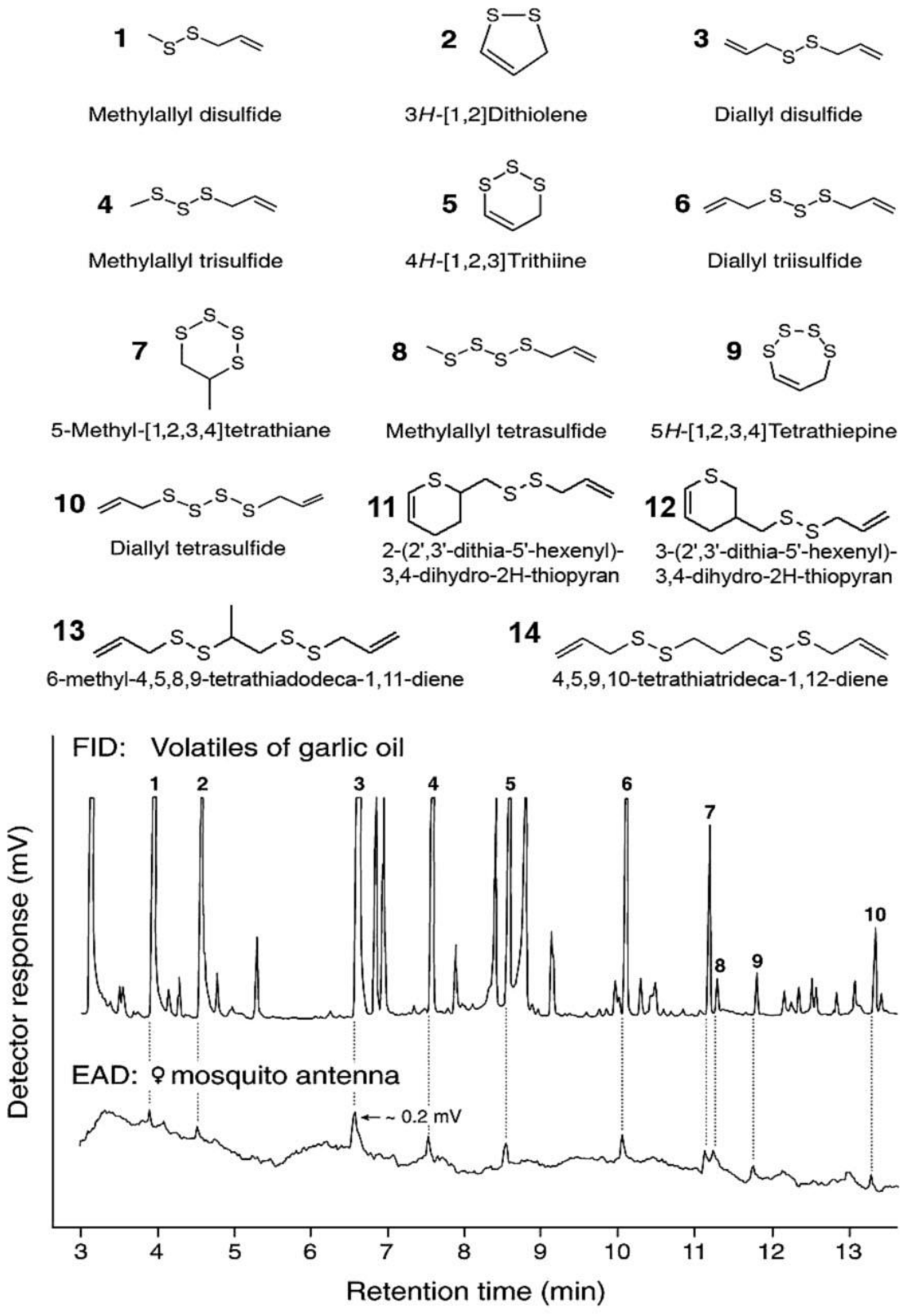
1
Methylallyl disulfide
2
3H-[1,2]Dithiolene
3
Diallyl disulfide
4
Methylallyl trisulfide
5
4H-[1,2,3]Trithiine
6
Diallyl triisulfide
7
5-Methyl-[1,2,3,4]tetrathiane
8
Methylallyl tetrasulfide
9
5H-[1,2,3,4]Tetrathiepine
10
Diallyl tetrasulfide
11
2-(2',3'-dithia-5'-hexenyl)-
3,4-dihydro-2H-thiopyran
12
3-(2',3'-dithia-5'-hexenyl)-
3,4-dihydro-2H-thiopyran
13
6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene
14
4,5,9,10-tetrathiatrideca-1,12-diene
FID: Volatiles of garlic oil
EAD: ♀ mosquito antenna
~ 0.2 mV
Detector response (mV)
Retention time (min)

Figure 4.3

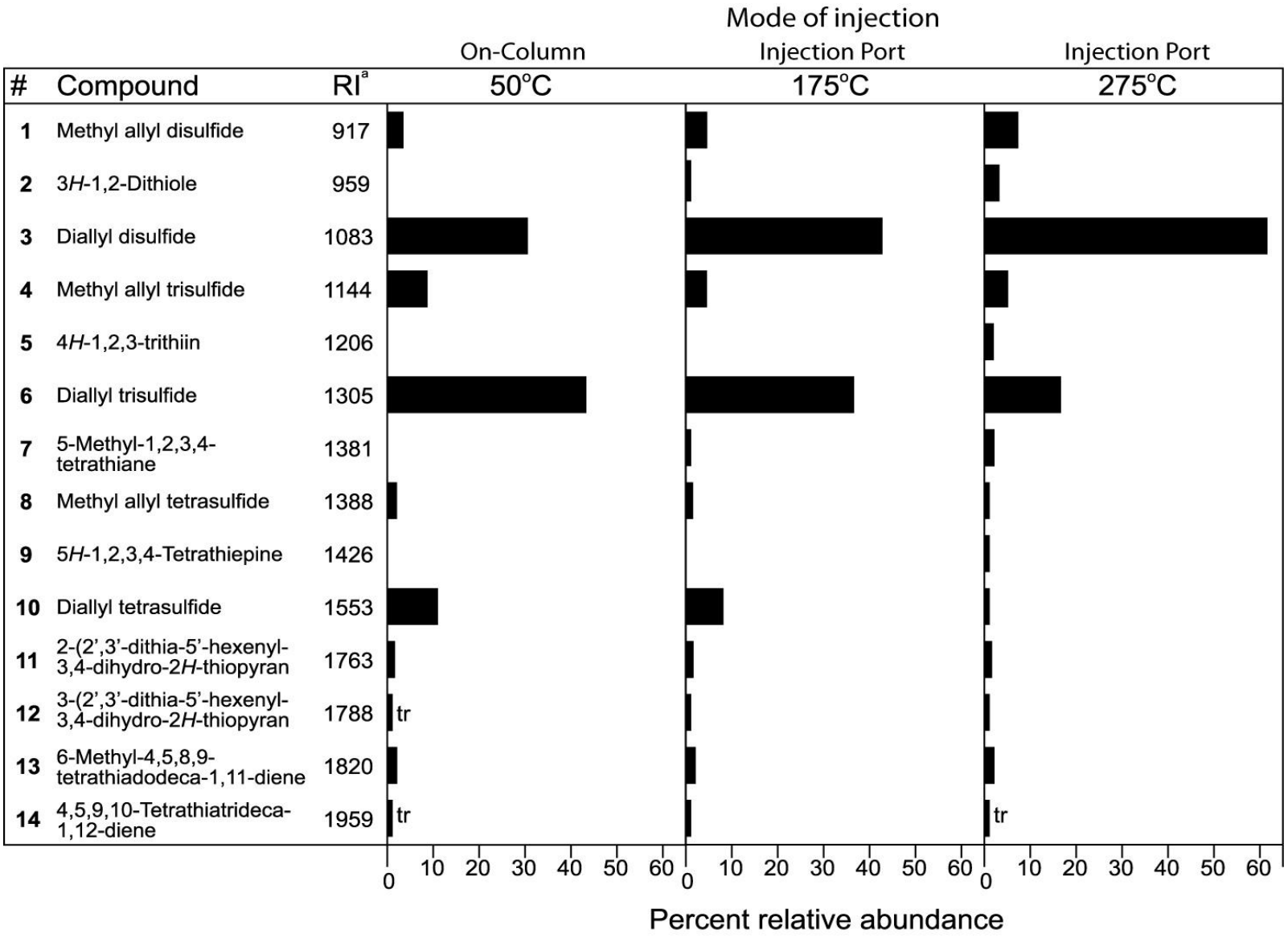
Mode of injection
On-Column
Injection Port
Injection Port
Compound RI[a] 50°C 175°C 275°C
1 Methyl allyl disulfide 917
2 3H-1,2-Dithiole 959
3 Diallyl disulfide 1083
4 Methyl allyl trisulfide 1144
5 4H-1,2,3-trithiin 1206
6 Diallyl trisulfide 1305
7 5-Methyl-1,2,3,4-tetrathiane 1381
8 Methyl allyl tetrasulfide 1388
9 5H-1,2,3,4-Tetrathiepine 1426
10 Diallyl tetrasulfide 1553
11 2-(2',3'-dithia-5'-hexenyl-3,4-dihydro-2H-thiopyran 1763
12 3-(2',3'-dithia-5'-hexenyl-3,4-dihydro-2H-thiopyran 1788 tr
13 6-Methyl-4,5,8,9-tetrathiadodeca-1,11-diene 1820
14 4,5,9,10-Tetrathiatrideca-1,12-diene 1959 tr
tr
tr
0 10 20 30 40 50 60
0 10 20 30 40 50 60
0 10 20 30 40 50 60
Percent relative abundance

Figure 4.4

5 CONCLUDING SUMMARY

5.1 Conclusions

In my thesis, I have investigated plant oils and their constituents as candidate repellents for the yellow fever mosquito. Gas chromatographic-electroantennograpic detection (GC-EAD) is a valuable tool to rapidly screen components detectable by insect antennae within complex volatile blends, thus allowing the selection of only antennal stimulatory constituents for behavioural study. According to my data, the following conclusions can be drawn:

1. Antennae of adult female *Ae. aegypti* are stimulated by 43 terpene(oid) compounds from 11 essential oils. Many of these compounds have been demonstrated previously as having repellent activity, while no previously reported behavioural correlation for 20 constituents could be found.
2. Soybean oil, the claimed active ingredient of the commercial repellent product BiteBlocker™ does not repel adult female *Ae. aegypti* significantly from a human host.
3. Soybean oil probably acts as a formulation ingredient of the Blocker™ products.
4. In screened cage behavioural experiments, a 0.1-mg/cm^2 dosage (10%) of garlic oil in paraffin oil has a protection period of circa 44 min which is significantly greater than that of a paraffin oil control.
5. Antennae of adult female *Ae. aegypti* are stimulated by 10 constituents of garlic oil. These 10 constituents along with 4 thermal degradation products are methyl

allyl disulfide, 3*H*-1,2-dithiole, diallyl disulfide, methyl allyl trisulfide, 4*H*-1,2,3-trithiin, diallyl trisulfide, 5-methyl-1,2,3,4-tetrathiane, methyl allyl tetrasulfide, 5*H*-1,2,3,4-tetrathiepine, diallyl tetrasulfide, 2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, 6-methyl-4,5,8,9-tetrathiadodeca-1,11-diene, and 4,5,9,10-tetrathiatrideca-1,12-diene.

6. Seven of the 14 antennal stimulatory constituents of garlic oil could be isolated for behavioural tests of their repellent activity. At a dosage of 0.1 mg/cm^2 (10%), diallyl trisulfide and diallyl tetrasulfide provided an estimated mean protection time significantly greater than that of a paraffin oil control. Adult females appeared to be repelled by diallyl disulfide and the combination of 2-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran *plus* 3-(2,3-dithia-5-hexenyl)-3,4-dihydro-2*H*-thiopyran, but the results were not statistically significant. 6-Methyl-4,5,8,9-tetrathiadodeca-1,11-diene and 4,5,9,10-tetrathiatrideca-1,12-diene showed activity hardly different from that of control stimuli.

5.2 The Future

A significant portion of research not included in this thesis has provided the basis for a new mosquito repellent that has recently been patented: Gries, R.M., Campbell, C., Khaskin, G., Avelino, N., and Gries, G.G. Compounds, compositions and methods for repelling blood feeding arthropods and deterring their landing and feeding. US Patent 12/057960. This repellent product is presently being considered for registration.