Identification of Chemical Signals that Influence the Oviposition Behaviour of *Anopheles Gambiae* and Exploration of their Potential in Mosquito Control

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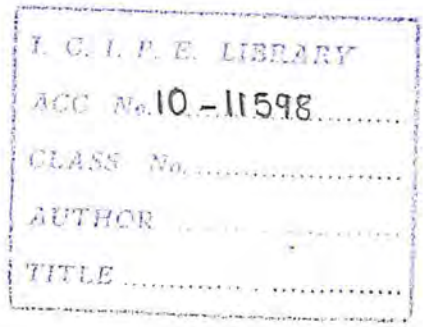

DECLARATION

This thesis is my original work and has not been presented before for a degree in any other University.

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To my dear husband Jesse Maina Kinyua and our wonderful son Morris Kinyua Maina.

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LIST OF ABBREVIATIONS

ARPPIS	African Regional Program in Insect Science
BCED	Behavioural and Chemical Ecology Department
DDT	Dichlorodiphenyltrichloroethane
GC	Gas Chromatograph
GC-EAD	Gas Chromatography-Electroantennographic Detection
GC-MS	Gas chromatography-mass spectrometry
ICIPE	International Centre of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KM	Kilometres
KU	Kenyatta University
MS	Mass spectroscopy
OAI	Oviposition Activity Index
RH	Relative Humidity
SAS	Statistical Analysis Systems
SII	The Netharlands International Institutes Programme
SNK	Student-Newmann-Keuls
WHO	World Health Organisation

ABSTRACT

The African mosquito species *Anopheles gambiae* and *An. funestus* are ranked high among the world's most efficient vectors of human malaria. Their juvenile stages develop in aquatic environments while adults are terrestrial. Chemical signals guide gravid females of these vectors to their egg-laying sites. Several attributes of a pond including presence of other organisms influence egg hatching success and larval survival. Gravid *An. gambiae* females strongly discriminate among potential egg-laying sites to ensure viability of their offsprings. This study is based on the hypothesis that gravid *An. gambiae* females use chemical cues from microbial activity and/or those associated with competitors as interspecific cues as well as intraspecific signals associated with their own eggs or larvae to select suitable habitats for oviposition.

The main aim of this study was to identify the chemicals that guide gravid *An. gambiae* to their oviposition site and to find out their effect on oviposition behaviour. To achieve this, behavioural responses of caged gravid *An. gambiae* on two choice assay of test water consisting of *Culex quinquifasciatus* egg rafts and/or larvae and test water as control were compared. We found out that *An. gambiae* is deterred or avoids laying eggs in the sites where there is *C. quinquifasciatus* egg rafts, larvae or both. *C. quinquifasciatus* larvae deterred the oviposition by gravid *An. gambiae* even at low density. Moreover, when both *C. quinquifasciatus* larvae and egg rafts were used with varying density of egg rafts and

constant number of larvae the deterrence was more than when the two were used separately.

Dynamic and static trapping systems were used to collect volatiles emanating from larvae, extract from test water with *C. quinquefasciatus* larvae, test water extract (supernatant of muddy soil mixed with double-distilled water and allowed to settle for 3-7days), *An. gambiae* egg extract, *C. quinquefasciatus* egg rafts extract, soil and cultured soil bacteria. Gas chromatograph-mass spectrometry (GC-MS) was used to characterize the chemical constituents of the volatiles.

Eleven compounds were identified from *C. quinquefasciatus* larval volatiles; dimethyl disulfide, dimethyl trisulfide, 3,5-dimethylbenzaldehyde, 2,4-bis(1,1-dimethylethyl)phenol, 1-chlorotetradecane, isopropyl myristate, isopropyl palmitate, 4-phenylmorpholine, 3-phenyl-1-azabicyclo[2.2.2]octane, eicosane and 2,4-bis(1-methyl-1-phenylethyl)phenol. Six compounds were identified from the extract of test water with *C. quinquefasciatus* larvae; 4-methylphenol, 4-(1,1,3,3-tetramethyl butyl)phenol, 4-(1,1-dimethylpropyl)phenol, 2[(4-hydroxyphenyl)methyl]phenol, N,N-dimethylthiocarbamoylphenyltrithiocarbonate, 2,4-bis(1-methyl-1-phenylethyl)phenol and (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane.

The test water was extracted with dichloromethane and nine compounds were identified; 2,4-bis(1,1-dimethylethyl)phenol, 4-(1,1-dimethylpropyl)phenol, 6,10,14-trimethyl-2-pentadecanone, 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol, 2,6-bis(1,1-

dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol, phytol, 2,4-bis(1-methyl-1-phenylethyl)phenol, (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene and 4-octyl-N-(4-octylphenyl)benzenamine.

Tetradecanoic acid, Z-11-hexadecenoic acid, n-hexadecanoic acid, (Z)-9-octadecenoic acid, octadecanoic acid, docosane, (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene were obtained from *An. gambiae* eggs extract. Z-11- hexadecenoic acid, n-hexadecanoic acid, (Z)-9-octadecenoic acid, octadecanoic acid, N-butyl-4,9-decadien-2-amine, arachidonic acid and 1,2,3-propanetriyl ester hexanoic acid were found in *C. quinquefasciatus* egg rafts extract.

Volatiles trapped from the muddy soil used for preparation of test water yielded eleven compounds; d-limonene, [3aR-(3a.alpha.,4.beta.,7.alpha.)]-2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-3H-3a,7-methanoazulene, 2,6-bis(1-methylethyl)benzeneamine, [1S-(1.alpha.,7.alpha.,8a.alpha.)]-1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)naphthalene, (1S-cis)-1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)naphthalene, [R-[R*,R*-(E)]]-3,7,11,15-tetramethyl-2-hexadecene, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, (1-methyldodecyl)benzene, 2[(4-hydroxy phenyl)methyl]phenol, 2-phenyl-2-(phenylmethyl)-1,3-dioxolane and 2,4-bis(1-methyl-1-phenylethyl)phenol. The same soil was cultured for bacteria and the trapped volatiles thereof yielded twelve compounds identified as: dimethyl disulfide, dimethyl trisulfide, 2-ethyl-1-hexanol, 2-phenoxyethanol, tetradecane, 2,6-bis(1,1- dimethylethyl)- 2,5-

cyclohexadiene-1,4-dione, hexadecane, octadecane, isopropyl myristate, 4-hydroxy-4-methyl-2-pentanone, 1-undecene and 4-phenylmorpholine.

Some of the compounds identified were evaluated for their effect on oviposition behaviour against gravid females of *An. gambiae* mosquitoes at different concentrations. Dimethyl dilsulfide and 1:1 mixture of N-hexadecanoic acid and octadecanoic acid had behavioural effect on gravid *An. gambiae*. At low concentrations the compounds showed positive oviposition response and as the concentration increased there was a negative oviposition effect. Erythro-6-acetoxy-5-hexadecanolide, previously isolated from *C. quinquifasciatus* egg rafts, showed a negative oviposition effect.

This study showed that interspecific chemical signals mediate the oviposition of gravid *An. gambiae* to a specific site. The presence of *C. quinqufasciatus* larvae and/or egg rafts in a pond deters gravid *An. gambiae* from ovipositing in that specific pond. The microorganisms in the soil influence to a great extent the decision of gravid *An. gambiae* to oviposit on a given site. The chemical cues believed to mediate oviposition behaviour by *An. gambiae* have been identified and characterized. This provides the basis of understanding the behavioural effect of individual and blended compounds and this may be used to develop alternative methods of controlling malaria vectors.

CHAPTER ONE

INTRODUCTION

1.0 General Introduction

Malaria causes high levels of human suffering and mortality. It is estimated that there are 247 million cases leading to nearly 881 000 deaths annually in sub-Saharan Africa alone (WHO, 2008). Malaria in Africa remains one of the most serious obstacles for development, with an estimated cost of $1.8 billion per annum. It represents 9% of the total disease burden (WHO, 2008). It is caused by four species of *Plasmodium*, protozoan parasites that are most common in the tropics, especially Africa, and are transmitted between humans by the bites of female *Anopheles* mosquitoes. Thus, the distribution of *Anopheles* mosquitoes is an important factor in determining the prevalence of *Plasmodium* infections in humans. At large spatial scales (100–1,000 Km), the distribution of malaria is best described by climate: warm, humid places with standing water support large mosquito populations and high malaria prevalence. At local scales (100 m to 1 Km), the risk of malaria is determined by mosquito behaviour and ecology, especially the distribution of blood-meal hosts and water for ovipositing. Female mosquitoes alternate between blood feeding and oviposition in water systems. However, suitable hosts and water systems are heterogeneously distributed (Giglioli, 1964). Thus, human biting reflects the mosquitoes' commute to complete its gonotrophic cycle, as well as inherent differences in the attractiveness, suitability and distribution of blood-meal hosts (Kelly, 2001).

Malaria is an important disease in sub-Saharan Africa and a serious public health problem in certain regions of South East Asia and South America (Mishra *et al.*, 1999). The problems of controlling malaria in these countries are aggravated by inadequate health care facilities, demographic and socio-economic factors. The situation has become even more complex over the last few years with the increase in resistance to drugs that are normally used to combat the malaria parasite. Widespread chloroquine resistance and general drug failure compelled African governments to adopt more expensive drugs as first-line treatments. Advances in molecular biology have led to the development of new vaccines and identification of genes that code for refractoriness of mosquitoes to infection with *Plasmodium* parasites. However, large-scale application of these techniques is not envisaged within the next two decades (Engers and Godal , 1998; Carlson *et al.*, 1995; Collins and Paskewitz, 1995).

Malaria transmission dynamics is highly variable throughout Africa. Depending on the area, as much as five different anopheline species can transmit parasites to human populations. Major vectors are *Anopheles gambiae*, *An. arabiensis*, *An. funestus*, *An. nili* and *An. mouchet*. They all belong to species complexes or groups of closely related species that are difficult to distinguish on morphological grounds. Malaria transmission in Africa is mainly due to *An. gambiae* and *An. funestus*.

An. gambiae is the principal vector of *Plasmodium falciparum*, the most dangerous of the malaria parasite in sub-Saharan Africa. For decades, the focus in malaria research was on *Plasmodium* and its relationship with vertebrate hosts. However in the past few years, the

mosquito vector has become the focus of many researchers which also involves the drive to understand the complex interactions between vector and parasite. Invaluable facts have been gathered about the insect's biology, behaviour, habitats, resting-places, breeding and feeding grounds. Tolerance and resistance of malaria vectors to a variety of insecticides has been documented (Curtis *et al.*, 1993; Georghiou, 1990). Large-scale trials with pyrethroid-impregnated bednets in Africa have demonstrated their impact on reduction of child morbidity and mortality (Lengeler *et al.*, 1996). It remains to be seen whether similar effects can be obtained and sustained in regions with intense perennial transmission. Moreover, these systems select for behavioural resistance in mosquitoes by inducing changes in their biting cycle and indoor/outdoor feeding behavior which may render bednets useless in the long run (Knols and Takken, 1998). Information on the dynamics of malaria vector populations in sub-Saharan Africa, their behaviour and chemical ecology, and how these affect transmission of disease is scant. The current malaria situation is critical since the development of alternative control strategies is slow, and existing methods are rapidly losing their efficacy. The situation calls for worldwide integrated efforts to prevent further deterioration of the malaria menace (Butler, 1997; Marsh and Snow, 1997). One such effort is the exploitation of what is known of the behaviour and general ecology of malaria mosquitoes to reduce contact with human hosts, similar to the development of control strategies for tsetse flies (*Glossina* spp.) based on simple odour-baited traps and targets (Willemse and Takken, 1994; Vale, 1993). Novel methods, based on the interruption of odour-mediated behaviours such as sugar feeding and oviposition are yet to

be developed since some of their basic principles are still unknown (Knols and Takken , 1997).

1.1 Malaria Vectors

The biology of the main African malaria vectors has been broadly known for more than 50 years (MacDonald, 1957). The description and identification of vector species was based on morphological characters and sub-divisions called sub-species, form, variety, race, and so on that have been described depending on distribution, biology, behavior and slight morphological differences. As early as the beginning of the 20th century, it became evident that in many cases, isolated genetic entities belonged to the same morphological species. The two most famous species complex are the *An. maculipennis* complex, with at least nine sub-species in Europe (Proft *et al.*, 1999), and the *An. gambiae* complex with seven sub-species in Africa. Very often, efficient malaria vectors and non-vector species are found within the same complex.

In Africa five different species are considered to be major vectors: *An. gambiae*, *An. arabiensis*, *An. funestus*, *An. nili* and *An. moucheti*. At least eight or nine other species are also secondary or locally important vectors. These include *An. paludis* in Democratic Republic of Congo (DRC) (Karch and Mouchet, 1992), *An. mascarensis* in some locations in South East Madagascar and *An. hancocki* in Cameroon (Wanji *et al.*, 2003; Fontenille *et al.*, 2000). Others are *An. pharoensis* in Egypt (Madwar, 1936); *An. melas* and *An. merus*, two halophilic species from the *An. gambiae* complex, in some coastal regions of West

Africa (Diop *et al.*, 2002; Akogbeto and Romano *et al.*, 1999), East Africa and Madagascar (Leong *et al.*, 2003), respectively.

1.1.1 *Anopheles gambiae* complex

The *An. gambiae* complex includes the most efficient malaria vectors on Earth. This is because they have adapted well to human habitation, and feed almost exclusively on human blood for egg production. In Sub-Saharan Africa, *Anopheles gambiae* Giles is the primary vector of human malaria. Immatures of this mosquito occur in small, temporary, sunlit pools such as barrow pits, hoof prints, tyre tracks, drainage ditches, and small puddles (Gimnig *et al.*, 2001; Minakawa *et al.*, 1999; Gillies and De Meillon, 1968). Wherever agriculture or gardening activities result in the collection of significant amounts of stagnant water, *An. gambiae* is there to take advantage. *An. gambiae* is a pioneer species and is able to colonize these habitats within a few days after the habitats are created (Minakawa *et al.*, 2005). The predation on *An. gambiae* larvae is less prevalent in temporary habitats than it is in large, permanent habitats, and competition is less common in newly created habitats (Sunahara *et al.*, 2002; Washburn, 1995; Service, 1977).

An. gambiae has a wide distribution, and usually occurs in large numbers wherever found. It is also highly susceptible to the parasite. The female bites mainly at night, but in several studies, 12% of bites occurred after sunrise. Because of its high degree of ecological adaptability, the vector species has become dominant in Africa. Investigations by the World Health Organization (WHO, 1996) revealed that under laboratory conditions, normal

development takes place when the pH varies from 4.0-7.8, as long as there is sufficient food (phytoplankton and zooplankton) while the maximum larval survival temperature is 41 °C. Rarely does this temperature occur in nature, even in the intense heat of equatorial Africa.

An. gambiae complex is split into seven distinct species, including two of the most efficient human malaria vectors worldwide: *An. gambiae sensu stricto* and *An. arabiensis*. Other recognized species of the complex are *An. melas*, *An. merus*, *An. bwambae*, *An. quadriannulatus* and *An. quadriannulatus B* (Hunt *et al.*, 1998). The last five species have limited or no role as malaria vectors, due to restricted geographical distribution and/or zoophily.

An. melas and *An. merus* are salt-water species that only develop in mangrove swamps along the west and east coast of Africa, respectively. *An. bwambae* is known from a single location in Uganda where its larvae develop in heavily mineralized water springs. Both species of *An. quadriannulatus* (still reffered to as *An. quadriannulatus* A in southern Africa and B in Ethiopia) are mostly zoophilic and therefore not involved in the transmission of human infecting parasites. On the other hand both *An. gambiae* and *An. arabiensis* have wide geographical distributions throughout sub-Saharan Africa and surrounding islands. They coexist widely over much of their range, although *An. gambiae* is usually predominant in humid environments while *An. arabiensis* is found in drier areas (Coetzee *et al.*, 2000). Both species appear highly dependent on humans for their feeding,

resting and to certain extent, breeding habits (Coluzzi, 2002; Gillies and Coetzee, 1987; Gillies, 1968).

1.2 Justification

Malaria is an important disease in sub-Saharan Africa and a serious public health problem in certain regions of South East Asia and South America (Mishra *et al.*, 1999). The problems of controlling malaria in these countries are aggravated by inadequate health care facilities, demographic and socio-economic factors. The situation has become even more complex over the last few years with the increase in resistance to drugs that are normally used to combat the malaria parasite.

An. gambiae the principal vector of *P. falciparum* is highly anthropophilic and the most dangerous of the malaria parasites in sub-Saharan Africa, where 90% of malaria cases occur. For decades, the focus of research has been on *Plasmodium* parasite and its relationship with vertebrate hosts. In the past few years, the mosquito vector has come again into the crosshairs of researchers – 40 years after the DDT campaigns. And with it came a desire to understand the complex interactions between vector and parasite.

Female mosquito demonstrates a series of characteristic behaviours for its survival and reproductive success, including mating, foraging and oviposition. These behaviours are regulated by internal and external factors. During these behaviour a wide range of semiochemicals plays a role for female mosquitoes to find their sugar sources, blood meal,

mating partners and oviposition sites. Invaluable facts have been gathered about the mosquito biology, behaviour and habitats (resting-places, breeding and feeding grounds). However one area of research that has been neglected is the oviposition behaviour of these mosquitoes. The cues used by *Anopheles* species to select suitable oviposition sites after blood meals remains poorly understood. The chemical ecology of these behaviours is still poorly understood. The knowledge and identification of the chemical signals that mediate oviposition could be exploited to develop new methods of monitoring and controlling mosquitoes.

1.3 Hypotheses

1. The characteristic tendency of *An. gambiae* to spread egg laying spatially may be regulated by intraspecific signals associated with eggs or larvae.
2. Gravid females *An. gambiae* use semiochemicals from microbial activity and/or those associated with competitors as interspecific cues to select suitable habitats for oviposition.

1.4 Objectives

1.4.1 General objective

The major objective of the project is to identify chemical signals that influence the oviposition behaviuor of *Anopheles gambiae* and exploration of their potential in mosquito control

1.4.2 Specific objective

1. To determine the oviposition responses by gravid *An. gambiae* to candidate oviposition pools in the presence/absence of *C. quinquefasciatus* larvae and egg rafts.
2. To determine the oviposition responses by gravid *C. quinquefasciatus* to candidate oviposition pools in the presence/absence of conspefic larvae and egg rafts.
3. To trap volatile compounds from oviposition pools and soils in objective 1 above.
4. To extract compounds from eggs and from water in oviposition pools in objective 1 above.
5. To identify the trapped volatiles and extracted compounds using GC-MS.
6. To investigate oviposition responses by gravid *An. gambiae* to the identified compounds.

CHAPTER TWO

LITERATURE REVIEW

2.0 The role of odours in insect behaviour

The atmosphere contains a complex mixture of millions of volatile compounds arising from different sources, and insects can detect odours specific to certain sources. The odour molecules are multi-dimensional in their nature, having different lengths, functional groups and chiralities. Insects have developed an extreme sensitivity to certain odours (Keller and Vosshall, 2003). Many insect species rely to a high degree on odour cues in their search for food, mating partners, hosts and suitable oviposition sites (Angioy *et al.*, 2003).

2.1 Semiochemicals

Chemical compounds that mediate interactions between organisms are called infochemicals or semiochemicals. The signals transmitted between individuals of different species are called allelochemicals, while those mediating between individuals of the same species are known as pheromones (Howse *et al.*, 1998; Gullan and Cranston, 1994). Pheromones are used for aggregation of organisms to favourable sites (for example food sources, shelter), as warning signals for danger (alarm pheromones), as indication of suitable oviposition sites and for mating. Allelochemicals are subdivided into three classes: allomones, kairomones, synomones and apneumones (Gullan and Cranston, 1994; Nordlund and Lewis, 1976). Allomones are beneficial to the producer but detrimental to the receiving organism. For instance, allomones include chemicals produced by prey animals to deter

predators. Kairomones form another group of semiochemicals in biotic interactions. They benefit the receiver, either evoking a behavioural or a physiological reaction, they affect foraging and oviposition behaviour (Dicke and Sabelis, 1988). Synomones benefit both the emitter and the receiver (Fig. 2.1).Apneumones are chemicals emitted by a non-living material on which one species is found to the detriment of the resident species and to the benefit of the receiver.

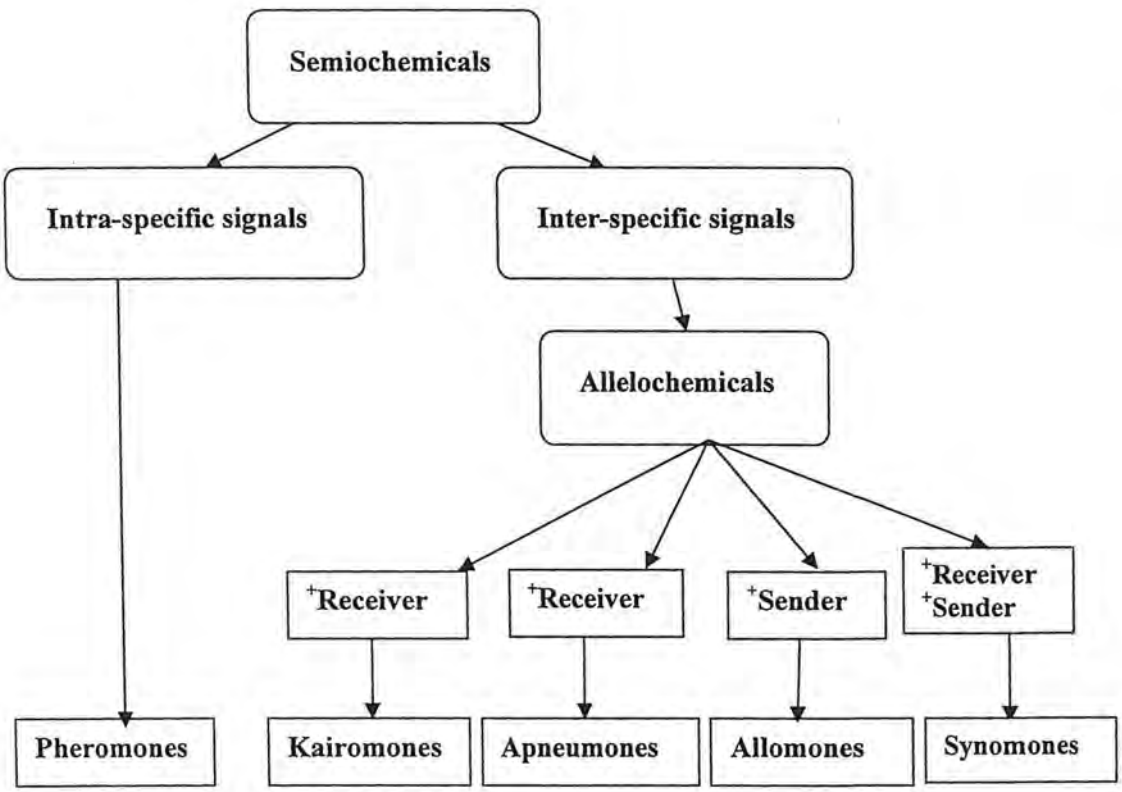


Figure 2.1. The classification of chemical compounds that mediate interaction between organisms, semiochemicals, according to the effect they have on the involved organisms. + = benefit

2.2 Mosquito behaviour

A female mosquito demonstrates a series of characteristic behaviours for its survival and reproductive success, including mating, foraging and oviposition. These characteristics are regulated by internal and external factors (Fig. 2.2). During these activities semiochemicals assist female mosquitoes to find their sugar sources, blood hosts, mating partners or oviposition sites (Takken and Knols, 1999).

2.2.1 Mating behavior

Mating involves a sequence of behaviour that brings males and virgin females into proximity, permit short distance location of the females by the male, and lead to engagement of the genitalia. Insects use a variety of stimuli in the coming together of males and females for mating. Of these stimuli, volatile sex pheromones and acoustic signals function over substantial distances, but many other stimuli are short range and are effective only after individuals of the two sexes have come into relatively close proximity by other means (Clements, 1999).

2.2.2 Host seeking behavior

Mosquito is guided to its human host predominantly by chemical cues discharged from the human body (Takken and Knols, 1999; Costantini *et al.*, 1996). Compounds emitted from hosts that include carboxylic fatty acids, lactic acid, ammonia, octenol and carbon dioxide are mosquito attractants (Dekker *et al.*, 2005; Dekker *et al.*, 2002; Braks and Takken, 1999; Kline *et al.*, 1990).

2.2.3 Sugar feeding

Female and male mosquitoes feed on nectar to increase their metabolic rate and to reserve more energy for taking flight prior to host seeking (Takken and Knols, 1999). In addition,

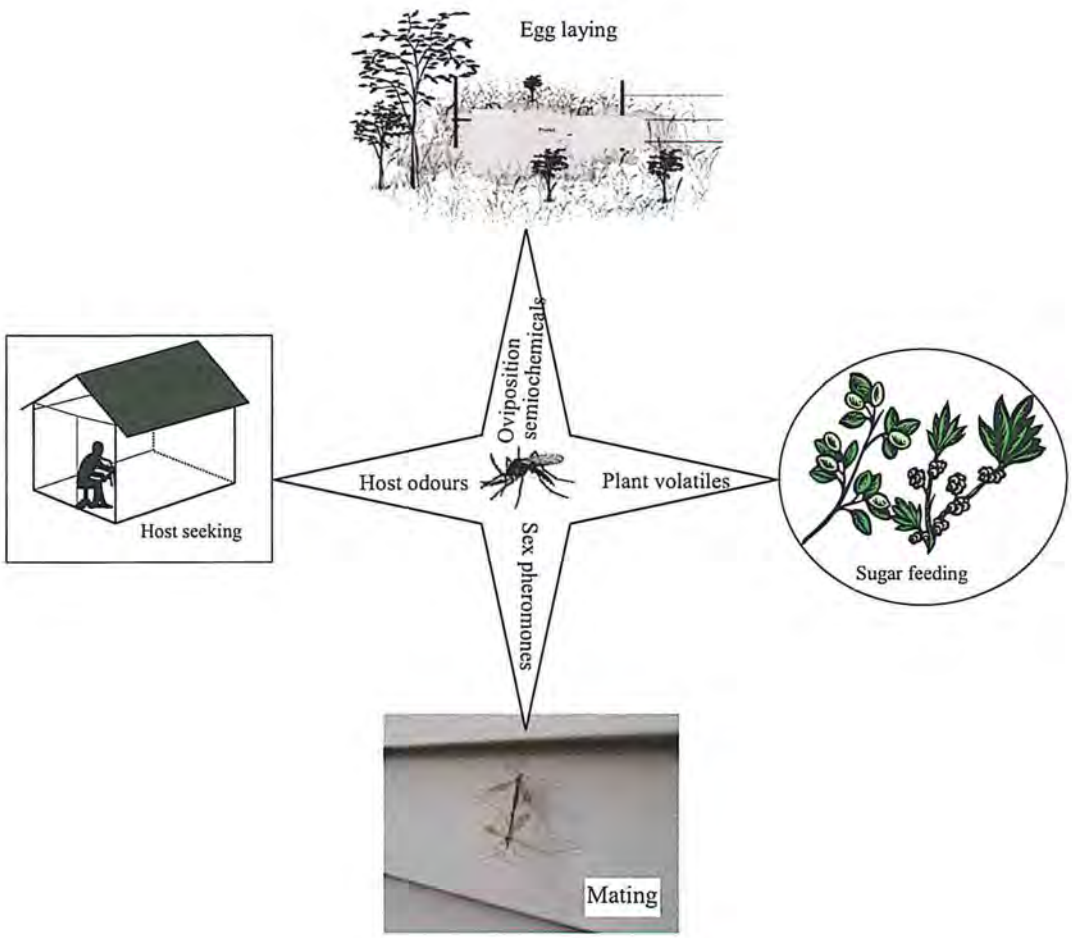


Figure 2.2. Mosquito behaviors are mediated by olfactory cues. Modified after Takken and Knols, 1999.

females need plant carbohydrates to develop eggs and to increase fecundity (Nayar and Sauerman, 1975). Orientation and attraction of mosquitoes to their host plant is mediated by volatiles given off from the plant (Figure 2.2). Mono- and bicyclic monoterpenes, such as thujone, are major components of the floral odors that attract mosquitoes. Also, certain green leaf volatiles, such as hexanal, 1-hexanol, and hexenol act as attractants for mosquitoes (Takken and Knols, 1999).

2.2.4 Oviposition Behaviour

Ovipositing insects need to select sites that improve the survival, growth, and reproductive potential of the offspring (Peckarsky *et al.*, 2000). The choice of an appropriate oviposition site has an important influence on maternal reproductive success in species with aquatic larvae (Millar *et al.*, 1994). Because several attributes of a pond influence hatching success and larval survival (Resetarits and Wilbur, 1989), there should be strong selection for females to discriminate among potential oviposition sites based on probable offspring viability (Petranka and Fakhourry, 1991). The 'decision' on where to oviposit is particularly important for maternal fitness in species such as mosquitoes, where juveniles are unable to move to a suitable habitat if conditions become unfavourable (Spencer *et al.*, 2002; Onyabe and Roitberg, 1997).

In keeping with these ideas, ovipositing female mosquitoes are known to choose among water bodies based on cues such as temperature, light, water depth, turbidity and the presence of competitors (Lee, 1991; Bentley and Day, 1989). Biotic factors can alter a mosquito's oviposition behaviour in several ways (Edgerly *et al.*, 1998). Mosquitoes may

avoid ovipositing where interspecific competitors are present (Blaustein and Kotler, 1993), but are attracted to sites where other conspecific larvae are present (Beehler and Mulla, 1995). Although there should be selective value in mosquitoes choosing sites where larval competition is low (Wilbur, 1997), the presence of conspecific larvae may provide a reliable cue that the pond offers conditions suitable for larval development (Stav *et al.*, 1999).

Many aspects of mosquito behaviour, including host location and oviposition, are mediated by detection of volatile semiochemicals (Gibson and Torr, 1999; Takken and Knols, 1999; Pickett and Woodcock, 1996). The selection of oviposition sites by many mosquitoes, is mediated by semiochemicals (Beehler *et al.*, 1992; Allan *et al.*, 1987). Chemical cues can originate from natural water bodies as breakdown products of bacterial origin or from the mosquito itself as oviposition pheromone (Bentley and Day, 1989). Both sources of stimuli result in the aggregation of eggs in sites suitable for larval development (McCall and Cameron, 1995).

Mosquitoes use chemical cues to detect ponds where conspecific larvae have previously been present (Takken, 1999; Millar *et al.*, 1994; Petranka and Fakhourry, 1991; Bentley and Day, 1989). They also use chemical and biological cues to detect the presence of larval predators and competitors in ponds (Spencer *et al.*, 2002; Beehler *et al.*, 1994b; Petranka and Fakhourry, 1991). For example mosquitoes may avoid ovipositing in water bodies where a fungus commonly associated with a competitor is present (Mokany and Shine,

2002). However, the oviposition response of mosquitoes to competitors varies among mosquito species. This variation may be related to different ecological requirements of larvae of different species (Mokany and Shine, 2002).

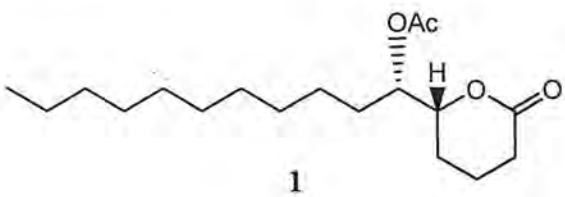
Studies show that *An. gambiae* actively selects habitats favorable for oviposition rather than randomly colonizing them (Minakawa *et al.*, 2004). Some mosquito species avoid ovipositing in habitats with predators and competitors (Blaustein et al., 2004; Kiflawi *et al.*, 2003; Mokany and Shine, 2003a). Munga *et al.* (2006) demonstrated that cues from backswimmers and tadpoles influence selection of oviposition site by gravid *An. gambiae* in cages and they suggested that gravid mosquitoes avoid habitats containing competitors and predators to reduce the risk of mortality of offspring. *An. gambiae* females are able to detect a chemical substance or blend released by the predator and competitors, or by microorganisms associated with them (Mokany and Shine, 2003b). However, gravid mosquitoes may be attracted to habitats with conspecific larvae, because presence of conspecific larvae may indicate suitable habitats for the species (Sumba *et al.*, 2008; Mokany and Shine, 2003b; Allan and Kline, 1998; Blaustein and Kotler, 1993). Studies done by Sumba *et al.* 2008 found out that presence of conspecific larvae influenced oviposition by gravid *An. gambiae* and preferences for ovipositing in water with larvae changed depending on the quality of water and density of larvae.

McCrae (1984) acknowledged the role of semiochemicals, in conjunction with visual stimuli, in the mediation of oviposition behaviour of *An. gambiae*. In his research he found

that *An. gambiae* preferred a dark over a light background as an oviposition substrate and that water from a natural breeding site attracted more ovipositing females than tap or distilled water. Studies at International Centre for Insect Physiology and Ecology (ICIPE) revealed that the peak oviposition time of *An. gambiae sensu lato* may be regulated by the light-dark cycle. However it was noted that the number of eggs laid during the peak oviposition time is affected by the suitability of the habitat (Sumba *et al.*, 2004b). Studies in western Kenya revealed that more eggs of *An. gambiae s.l.* were laid in bowls containing clean water and mud from known breeding sites than in clean water only (Minakawa *et al.*, 1999), thus corroborating McCrae's (1984) observations. The data suggests that soil factors that mediate olfactory oviposition behavior were present. Other studies at ICIPE revealed that the presence of live micro-organisms in the soil or water of a natural *An. gambiae* larval habitat affects choices of oviposition substrates by individual or groups of mosquitoes in the laboratory (Sumba *et al.*, 2004a). Volatile substances released from microorganisms may strongly affect oviposition site selection of anopheline mosquitoes (Rejmankova *et al.*, 2005; Knols *et al.*, 2004). It is difficult to imagine how nocturnal mosquitoes would locate ovipositing sites by abiotic and visual cues in the absence of chemical stimuli, particularly during the dry season when breeding sites become scarce. Studies by Sumba *et al.* (2008, 2004) have shown that *An. gambiae* use olfactory cues for selecting a better oviposition site, and it might be possible to isolate the substances that produce these olfactory cues, or to mimic the cues in some way to control mosquito behavior.

2.2.5 Oviposition Semiochemicals

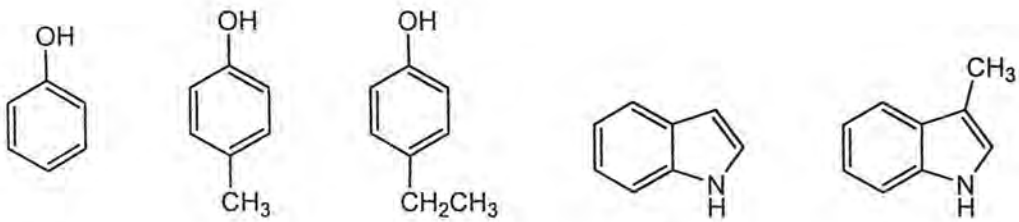
The culicine oviposition pheromone *erythro*-6-acetoxy-5-hexadecanolide (**1**) was extracted from the apical droplet left at the tip of the eggs by ovipositing *C. quinquefasciatus* (Laurence and Pickett, 1985; Laurence and Pickett, 1982). Gravid conspecifics as well as *Culex tarsalis* are highly attracted to the pheromone (Millar *et al.*, 1994; Pile *et al.*, 1993; Pile *et al.*, 1991; Otieno *et al.*, 1988). Mordue *et al.* (1992) demonstrated the presence of electrophysiological activity in *C. quinquefasciatus* in response to the pheromone. Oviposition pheromones from other mosquito species have not been identified, although Osgood (1971) reported a pheromone-like substance associated with the apical droplets of egg rafts of *C. tarsalis*. The chemical nature of this substance has not been elucidated, but available data suggest that it is related to *erythro*-6-acetoxy-5-hexadecanolide.



1

Culicines are attracted to chemical cues emitted by water with high organic content such as soakage pits and hay or grass infusions. Bacteria present in the organic-rich water were shown to produce chemicals that are highly attractive to gravid mosquitoes. Positive responses to hay infusions have been found in *Aedes aegypti, Ae. albopictus*, and *Ae. hendersoni* (Allan and Kline, 1995; Copeland and Craig, 1992; Reiter *et al.*, 1991) and *C. quinquefasciatus, C. tarsalis, C. stigmatosoma, C. pipiens*, and *C. restuans* (Lampman and

Novak, 1996; Isoe and Millar, 1995; Isoe *et al.*, 1995; Millar *et al.*, 1992). Gravid *C. molestus* is attracted to volatiles produced by the bacterium *Pseudomonas vesicularis*, which was isolated from water occupied by conspecific larvae (Dhileepan, 1997). Millar and co workers (1992) identified five chemical compounds, phenol (**2**), 4-methylphenol (**3**), 4-ethylphenol (**4**), indole (**5**), and 3-methylindole (skatole) (**6**), in the volatiles of hay infusions to which *C. quinquefasciatus* was attracted. These compounds are produced by bacteria present in the hay infusions (Beehler *et al.*, 1994b; Hasselschwert and Rockett, 1988; Hazard *et al.*, 1967).



2 **3** **4** **5** **6**

Significantly more egg rafts were deposited in water containing a synthetic blend of these compounds than in distilled water. 3-Methylindole (**6**) on its own elicited the strongest oviposition response. Attraction and oviposition occurred at concentrations from 0.01 – 1 $\mu g\ L^{-1}$ in water. At concentrations above $10\mu g\ L^{-1}$, 3-methylindole became repellent. Studies shows that 3-methylindole (**6**) and the egg raft pheromone had electrophysiological response (Blackwell *et al.*, 1993; Mordue *et al.*, 1992). In field experiments, significantly more egg rafts of *C. quinquefasciatus* were deposited in traps containing the mixture (compound **2**, **3**, **4**, **5** and **6**) than in untreated water (Beehler *et al.*, 1994a). In the same

study, *C. quinquefasciatus, C. tarsalis*, and *C. stigmatosoma* were all attracted to water containing 3-methylindole (**6**) only at 0.12 and 0.6 mg L^{-1}. Both *Ae. albopictus* and *Ae. aegypti* responded differently to these compounds. *Ae. albopictus* responded to only one concentration of 3-methylindole (**6**) while *A. aegypti* responded to phenol only (Allan and Kline, 1995).

Blends of the pheromone 6-acetoxy-5-hexadecanolide (**1**) and 3-methylindole (**6**) increased oviposition response, which was additive rather than synergistic (Millar *et al.*, 1994). The data suggest that the pheromone operates independently from the water-derived oviposition attractants. Similar results were obtained with a field study in Kenya (Otieno *et al.*, 1988). Field studies in Tanzania showed that there is a synergistic effect of the pheromone with volatiles from soakage pit water on oviposition response of *C. quinquefasciatus* (Mboera *et al.*, 2000). It is interesting that only one oviposition pheromone that is produced by one species only (*C. quinquefasciatus*) and acts cross-specifically in a number of congeneric *Culex* spp. has been identified. However, the water-derived attractants mediate oviposition behaviour in many culicine species.

CHAPTER THREE

EXPERIMENTAL

3.0 General Materials and Methods

3.1 Test soil

The muddy soil used to prepare test water for experiment was collected from oviposition ponds at Lwanda village in Mbita, Suba District, Western Kenya about 12 km from Mbita Point Field Station (MPFS) of the International Center of Insect Physiology and Ecology (ICIPE) and 500 km west of Nairobi, Kenya. Perforated 20 litres jericans were used to transport the soil from mbita to Nairobi and on arrival to the insectary in ICIPE the muddy soil was put in 100 litres bucket and stored in controlled condition of 26±2°C, 70-80% R.H.

3.2 Experimental water

To prepare the test water for the assay, 5 liters of muddy soil (collected from a pond previously colonized by eggs and larvae of *An. gambiae* at Mbita Point) and 20 litres of double-distilled water were mixed and allowed to settle for 3-7days. The supernatant (100 ml) from the mixture was used as the test water.

3.3 Experimental mosquitoes

Mosquitoes used for the experiments were obtained from established laboratory-reared colonies of *An. gambiae* s.s. (Mbita strain) from Mbita Point, Suba District, Western Kenya (November 2006). Larvae were reared in plastic trays (39 x 28 x 14 cm deep) in the

insectary at the ICIPE Duduville campus, Nairobi, Kenya at a density of about 500 larvae per 3000 ml of distilled water. Rearing room was maintained at 32±2°C, 52% R.H.. The larvae were fed daily on Tetramin® fish food. The adult mosquitoes were kept in cubic cages (30 x 30 x 30 cm) in a separate room maintained at 26±2°C, 70-80% R.H. and a normal photoperiod of 12:12 (L:D), the light being provided by a fluorescent lamp. Both male and female mosquitoes were kept in the same cage to allow for insemination. Mosquitoes were fed on 6% glucose solution and were starved for 12 hours prior to a blood meal. Three to five day-old female mosquitoes were fed on blood from a human volunteer's forearm for a 10-minute period on two or three consecutive days. Approval of use of human subjects was sought from Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1). Multiple blood meals have been shown to increase the chance of oviposition by females that are to lay their first batch of eggs (Briegel & Hörler, 1993). *C. quinquifasciatus* (Nairobi colony) was established in November 2006 and reared as outlined above for *An. gambiae*. Three-to-five-day-old female mosquitoes were fed on blood directly from an albino rat (*Rattus norvegigous*) for a 10-minute period on four to five consecutive days prior to setting up the oviposition assays.

3.4 Bioassay cages

The cage used for bioassay consisted of a metal wire frame (30 cm x 30 cm x 30 cm) with a solid metal base, covered with white mosquito netting. A sleeve opening was provided on one side of each cage to allow access into the cage. Oviposition site (Section 3.4) was set up in the cages. It was assumed that odour from the sites would reach gravid mosquitoes

through a concentration gradient. The cages were well ventilated to avoid mixing of odours from different oviposition sites.

3.5 The 'double cup' oviposition site set-up

In order to investigate the role of olfactory cues in the oviposition behaviour of *An. gambiae*, a 'double' cup oviposition set-up (Fig. 3.1), previously developed at ICIPE (Sumba, 2004) was used. This set-up consists of an outer black plastic cup (8 cm deep, 6 cm diameter) containing the test substrate and a smaller inner opaque plastic cup (2 cm deep, 4 cm diameter) containing the control substrate. The larger cup contained 100 ml of test water plus a known number of egg rafts or larvae or both larvae and egg rafts. The smaller cup contained 15 ml of control water lined with white filter paper (Whatman no.1) and placed floating in the larger cup.

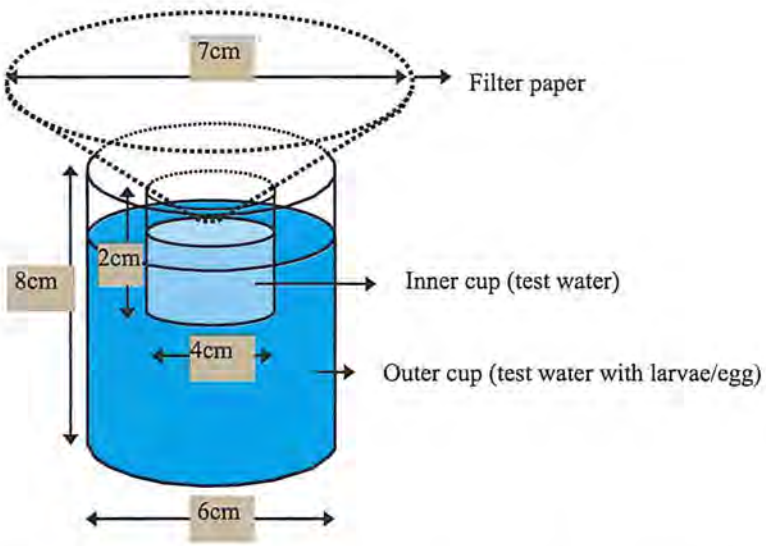


Figure 3.1. The 'double-cup' setup used to prevent mosquitoes touching test substrates prior to or during oviposition

A white cellulose filter paper placed in the inner cup prevented test mosquitoes from coming into direct tarsal contact with the source of the volatiles emanating from the substrate. In this assay, only odours from the test substrate permeated the filter paper and reached the ovipositing females. Thus, the females had no direct contact with eggs or larvae.

The same set up for double cup was used to test the effect of synthetic compounds but instead of the filter paper a glass microfibre filter (Whatman® 12.5 cm GF/A) cut in to a strip (1cm x 1 cm) was placed in an empty inner cup, and made to float on the surface of 100 ml test water in an outer cup (Fig. 3.2). The gravid *An. gambiae* laid eggs directly in the test water on the outer cup. A white polyester material was used to filter the eggs. It was assumed that glass microfibre filter would have a constant release rate of the compounds when all the other conditions are constant.

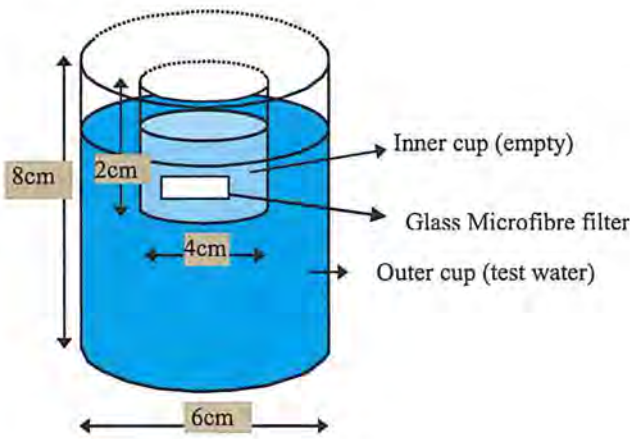


Figure 3.2. The 'double-cup' setup used to test the effect of identified synthetic compounds.

3.6 Bioassay set up

Five-day-old gravid females in groups of 20 were released into the experimental cages (30 × 30 × 30 cm) at 1530h to acclimatize them for one hour. Two artificial oviposition sites (cups) were then introduced into the cages. A cup with the test substrates was placed at one corner of the cage and another one with control water was positioned at diagonally opposite corners (Fig. 3.3). Egg rafts, larvae of *C. quinquifasciatus*, or both and synthetic compounds were used as the sources of volatiles. The cups were removed 14 hrs later and the number of eggs oviposited on the filter papers counted under a dissection microscope. Fresh gravid female mosquitoes and oviposition substrates were used for each replicate experiment.

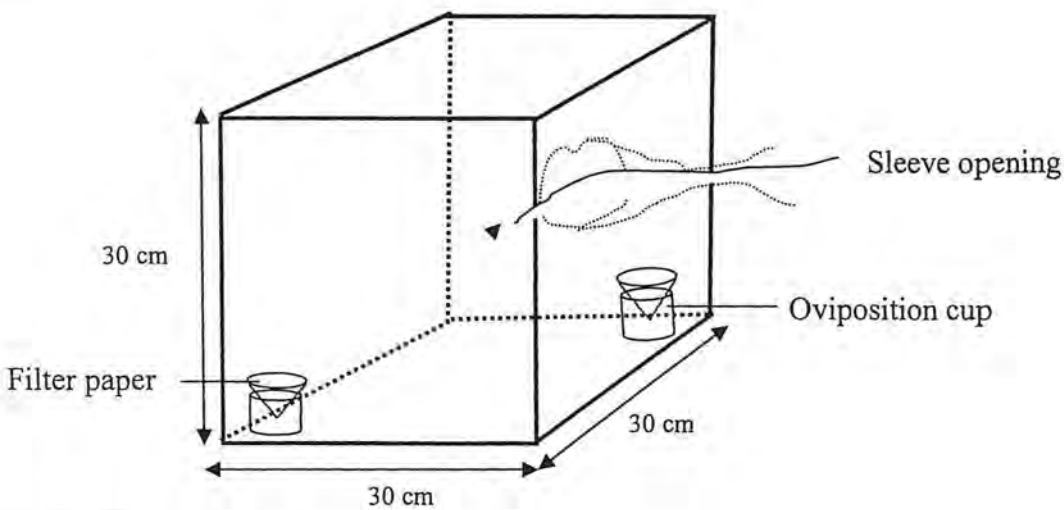


Figure 3.3. Bioassay setup.

3.7 Biological activity

3.7.1 Oviposition response of *An. gambiae* to hetero-specific egg rafts and larvae

The oviposition response of gravid *An. gambiae* female was investigated in response to:

1. Varying numbers (1, 5, 10, 15, 20, 25, 50 and100) of egg rafts of *C. quinquifasciatus* placed in the test water (in the outer cup);
2. Varying numbers (1, 5, 10, 15, 20, 25, 50 and100) of 2nd instar *C. quinquifasciatus* larvae in the test water; and
3. Varying numbers (1, 5, 10, 15, 20, 25, 50 and100) of egg rafts of *C. quinquifasciatus* each with 10 2nd instar larvae added into the test water.

The controls were test water without egg rafts and/or larvae in a similar arrangement of double cup. The eggs deposited were counted after 14hrs.

3.7.2 Oviposition response of *C. quinquifasciatus* to con-specific egg rafts and larvae

In this experiment gravid *C. quinquifasciatus* females were subjected to the treatments outlined in Section 3.7.1 above to evaluate their oviposition responses to volatiles from their own egg rafts and larvae, separately or together in the test water. Eggs laid were counted in the three treatments.

3.7.3 Oviposition response of gravid *An. gambiae* to synthetic compounds

One microlitre of dimethyl disulfide (7) (99% purity, Aldrich) was dissolved in 1 ml of hexane to make a solution of 1 ppm. Ten, 25, 50, 100, 200 and 250 µl corresponding to

10.6, 26.5, 53, 106, 212 and 265 µg respectively were pipetted on to 1 cm x 1 cm glass microfibre filter disc. The disc was allowed to dry by evaporation of the solvent and was floated in an inner cup on the surface of 100 ml test water contained in an oviposition cup of 6 cm diameter as shown in Fig. 3.2. The control cup was treated with hexane. The two set ups were placed diagonally in a 30 cm x 30 cm x 30 cm cage containing gravid *An. gambiae*. The number of eggs laid in both the test and control set ups were counted after 14 hours. The assay was carried out in a controlled temperature of 26±2 °C and relative humidity of 70-80%. The position of the test and control cups in the cage were alternated with each replicate.

3.8 Dynamic system of volatile collection

In dynamic trapping system (Fig. 3.4), air was pushed into the headspace volatile collection glass container at a regulated rate of 190 ml/min. Prior to entering the chamber, the air was cleaned by flowing through a purifying filter material (activated charcoal) that adsorbs impurities and then humidified by passing it through double distilled water. Inside the container, a uniform air flow over the sample was created. A portion of the air was pulled out of the chamber through an adsorbent trap connected to a vacuum pump. The flow rate of the outgoing air stream was regulated by a second flow meter (170 ml/min), allowing the collection of a defined percentage of the volatiles emitted by the sample. The adsorbent material (25 mg Super Q) was packed inside a narrow glass tubes in beds of approximately 2–50 mm between glass wool plugs or metal grids. Trapped volatiles were eluted from the adsorbing matrix under nitrogen into glass vials with 100 µl pure (HPLC grade)

dichloromethane. One µl of the sample was injected in GC-MS and in it was 1.08 nanogram of standard compound (ethyl nonanoate) for quantitative analysis.

Plate 3.1. Dynamic volatile collection system.

3.9 Static headspace volatile collection method

For static headspace analysis, the samples were enclosed in a container and the emitted volatiles were trapped onto an adsorbent (Fig.3.5). The air surrounding the sample remains 'static'. This method was used to trap the highly volatile organic compounds which due to their volatility would not be effectively trapped if the dynamic system was to be used.

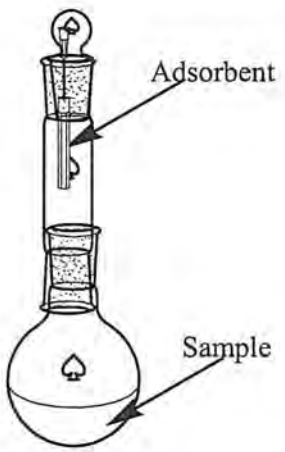


Figure 3.5. Static volatile collection system.

3.10 Volatile collection and extraction procedure

3.10.1 Volatile collection from *C. quinquifasciatus* larvae

Approximately 2000 *C. quinquefasciatus* larvae were placed in a glass container with 35 ml of test water (prepared as in section 3.5) and volatiles trapped using the dynamic system for 6 hours. The control consisted 35 ml of test water only. The adsorbent were eluted with dichloromethane and the respective samples analyzed by gas chromatograph-mass spectrometry (GC-MS). The gas chromatograms of the volatiles of *C. quinquefasciatus* larvae in water were compared with those of the control. Static trapping was run for 24 hours.

3.10.2 Extraction of compounds from test water containing *C. quinquifasciatus* larvae

Approximately 1000 larvae were placed in 25 ml of test water and left for 12 hours. The water was filtered to remove larvae using Whatman 12.5 cm filter paper. 10 ml of the water

was put in a vial, 1 ml of dichloromethane added, then thoroughly vortexed at 200/60 cycle for 5 minutes, then left to partition for ten minutes. 200 µl of organic phase was drawn with a syringe and put in a vial in ice and dried by gently blowing with nitrogen. The sample was then diluted for GC-MS analysis. The chromatogram obtained was compared with that from the test water extract (control), and the peaks due to test water extract were subtracted from the peaks obtained from test water with *C. quinquifasciatus* larvae extract.

3.10.3 Extraction of compounds from *An. gambiae* eggs and *C. quinquifasciatus* egg rafts

Approximately 10000 eggs of *An. gambiae* and *C. quinquifasciatus* were placed in a vial and extracted with 5ml dichloromethane for 5 minutes. The extract was filtered with Whatman filter paper and concentrated under nitrogen. The extract was then diluted for GC-MS analyzes.

3.10.4 Volatile collection from soil

35 ml of muddy soil, sieved to remove debris was placed in a glass container and volatiles trapped using the dynamic system for 1 hour. The adsorbent was eluted with dichloromethane and the sample analyzed by GC-MS.

3.11 Bacteria culture

The soil collected from a pond colonized by *An. gambiae* in Mbita, which was used to prepare test water (section 3.5), was cultured for 24 and 48 hours using commercial nutrient

broth (Himedia Laboratories Pvt. Limited, Mumbai (Bombay)-400086, India). The nutrient broth was prepared by placing 8 grams in 1 litre of distilled water. 100 ml of liquid nutrient broth was put in eighteen 250 ml conical flasks, 0.1 g of the soil was added in six of the media containing flasks and all the flasks were sterilized for 20 minutes in an autoclave 225 EH (horizontal autoclave). The media was then removed from the autoclave and left to cool. 0.1 g of the soil was separately placed in 6 conical flasks containing the sterilized media to culture the bacteria. All the 18 conical flasks (6 conical flasks with sterilized nutrient broth only, 6 conical flasks containing sterilized soil plus sterilized nutrient broth, 6 conical flasks containing sterilized nutrient broth plus soil) were placed in a shaker-incubator (Incubator shaker series 25 from New Brunswick Scientific Co., Inc. Edision, New Jersey, U.S.A.) at 26°C and shaken at 200 rpm for 24 and 48 hours. Trapping of volatiles was carried out after 24 and 48 hours in 3 replicates for each sample. The volatiles were trapped using the dynamic trapping system for 1 hour and static trapping system for 36 hours. The samples were analyzed by GC-MS.

3.12 Identification of compounds

Volatile compounds were identified by comparison of gas chromatographic retention time (RT) and mass spectra (MS) with those of standards using an Agilent technology 7890A GC with 5975C MSD fitted with a 30 m HP-5 capillary column (0.25 mm i.d., 0.25 μm film thickness). Standards were obtained from Aldrich company. Routine analyses were carried out on a Hewlett Packard 5890 series II gas chromatograph fitted with a flame ionization detector (FID) and the same capillary column as above.

3.13 Gas chromatograph-mass spectrometry (GC-MS)

For gas chromatography-mass spectrometric identification of compounds, 1 µl of volatile samples were analyzed. The analysis was carried out on an Agilent technology 7890A GC with 5975C MSD. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV and an emission current of 34.6 µA. The temperature of the source was held at 230 °C (ion source) 150 °C (Quadruple) and the multiplier voltage was 1106 V. The pressure of the ion source was held at 7 X 10^{-6} mBar. The spectrometer had a scan cycle of 3 scans per 2 seconds. The mass range was set at m/z 1-1050 and the scan range for the samples from m/z 38-550. The instrument was calibrated using heptacosa (Perfluorotributylamine) $[CF_3(CF_2)_3]_3N$ (Apollo Scientific Ltd. UK). HP-5 GC capillary column, 30 m x 0.25 mm (i.d) x 0.25 µm (film thickness) supplied by J&W Scientific was used. The GC-MS was linked to a computer with MS data library (NIST & WILEY). The compounds were identified by comparing their MS with those of authentic samples or with library data and their fragmentation pattern.

3.14 Data analysis

An oviposition Activity Index (OAI) per replicate in the dual choice assays was calculated according to the formula by Kramer & Mulla (1979).

$$OAI = (N_t - N_s)/(N_t + N_s)$$

Where, N_t = the number of eggs on the test substrate (test water with larvae and/or eggs), N_s = the number of eggs on the control substrate (test water without larvae and/or eggs). The significance of each OAI was determined by one-sample Student's *t*-test and SNK Test (Student-Newman-Keuls).

The values are normally between +1 and –1. Positive numbers indicate that more eggs were laid on test substrate than control water while negative number indicates more eggs being laid on control water. According to Hwang (1980) and Poonam *et al.* (2002), a substance is considered active when OAI is +0.3 and above and repellent/deterrent when the value is –0.3 and below. Statistical analyses were carried out using SAS 9.1 and graphs drawn using Excel

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 General Chapter Overview

This chapter reports results of the oviposition assays on egg rafts and /or larvae of *C. quinquefasciatus* and synthetic compounds. It also gives gas chromatographic and profiles of detailed structure elucidation using mass spectrometry from seven samples; *Culex quinquefasciatus* larvae volatiles, extract from test water with *C. quinquefasciatus* larvae, extract of test water, *An. gambiae* egg extract, extract from *C. quinquefasciatus* egg rafts, soil and bacteria volatiles.

4.1 Oviposition bioassays

4.1.1 Oviposition response to hetero-specific egg rafts

In the cage bioassay, there was no positional bias (0AI=0.02) for oviposition by gravid *An. gambiae* when test water was presented silmutaneously in the two cups. However, the mosquitoes appeared to prefer ovipositing into the test water (OAI=0.17) over double distilled water when given a choice between the two. In the assays involving *C. quinquifasciatus* egg rafts in the test water, there was a small initial increase in OAI with number of egg rafts reaching a maximum at 5 egg rafts, which then dropped to negative values (Fig. 4.1). Further increase in *C. quinquifasciatus* egg raft numbers (20, 25 and 50) led to a dose dependent decrease in the number of eggs (OAI=-0.20, -0.34 and -0.49 respectively) deposited by *An. gambiae* females on test substrate and any subsequent increase did not cause any further change. The increase in OAI at 5 egg rafts/100 ml water,

the decrease in OAI at 25, 50 and 100 egg rafts/100 ml water were statistically significant ($p<0.05$, t-tests).

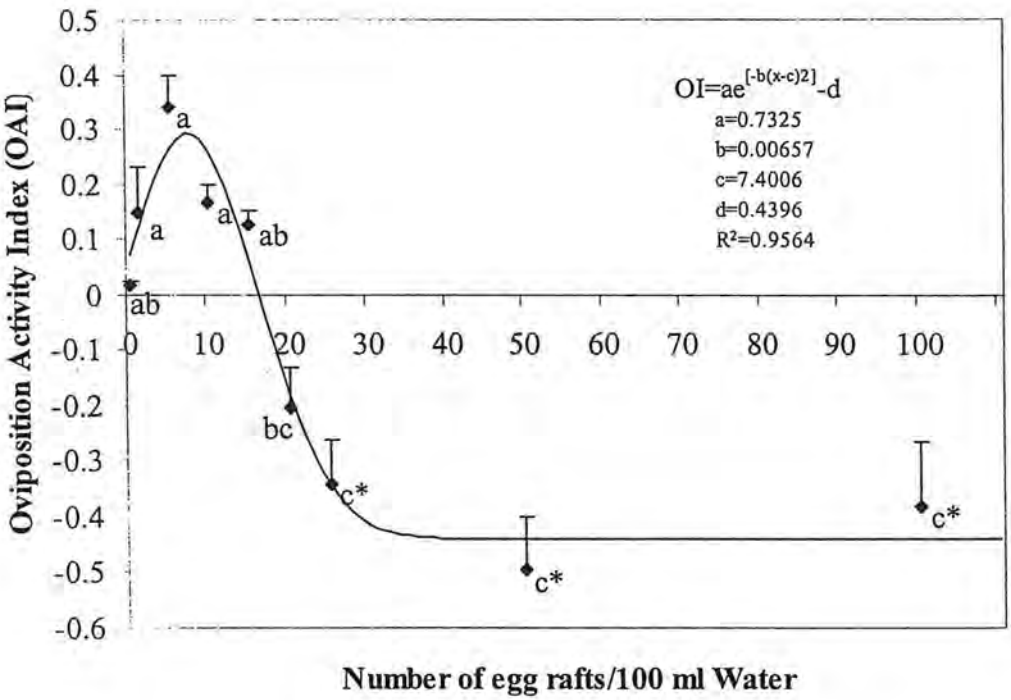


Figure 4.1. Oviposition indices (Mean±SE) for oviposition responses of gravid *An. gambiae* to *C. quinquifasciatus* egg rafts in test water.

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

4.1.2 Oviposition response to hetero-specific Larvae

Gravid females of *An. gambiae* laid increasingly fewer eggs in response to increasing numbers of larvae in test water and reached a minimum that levelled off at 50 larvae (OAI=

-0.46), statistically significant from zero (Fig 4.2). Any further increase in the number of larvae did not cause any change.

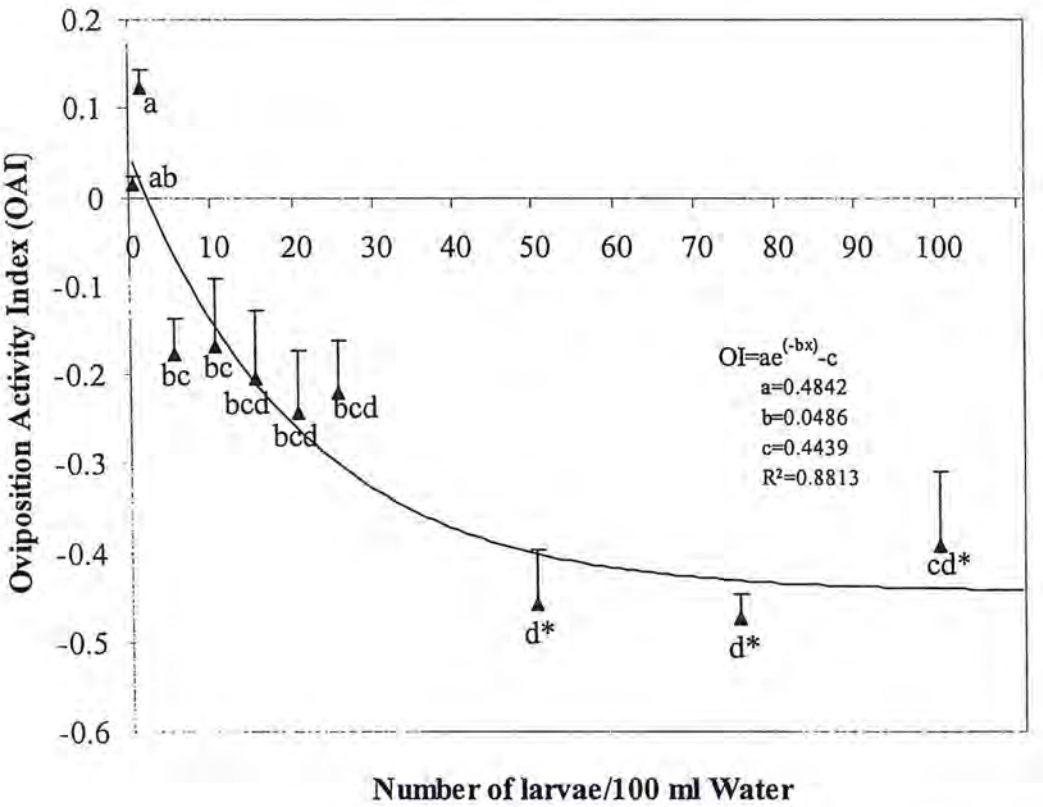


Figure 4.2. Oviposition indices (Mean±SE) for oviposition responses of gravid *An. gambiae* to presence of *C. quinquifasciatus* larvae in test water.

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

4.1.3 Oviposition response to hetero-specific egg rafts and larvae

The number of egg rafts deposited by *An. gambiae* females decreased with increasing number of *C. quinquefasciatus* egg rafts in test water while maintaining the larval number

at 10 (Fig. 4.3). A further increase in the number of egg rafts to 20 led to a sharp decline in the number of eggs laid (OAI= -0.42, which was statistically different from zero $p<0.05$) to a minimum that did not vary with further increase of egg rafts in the test water.

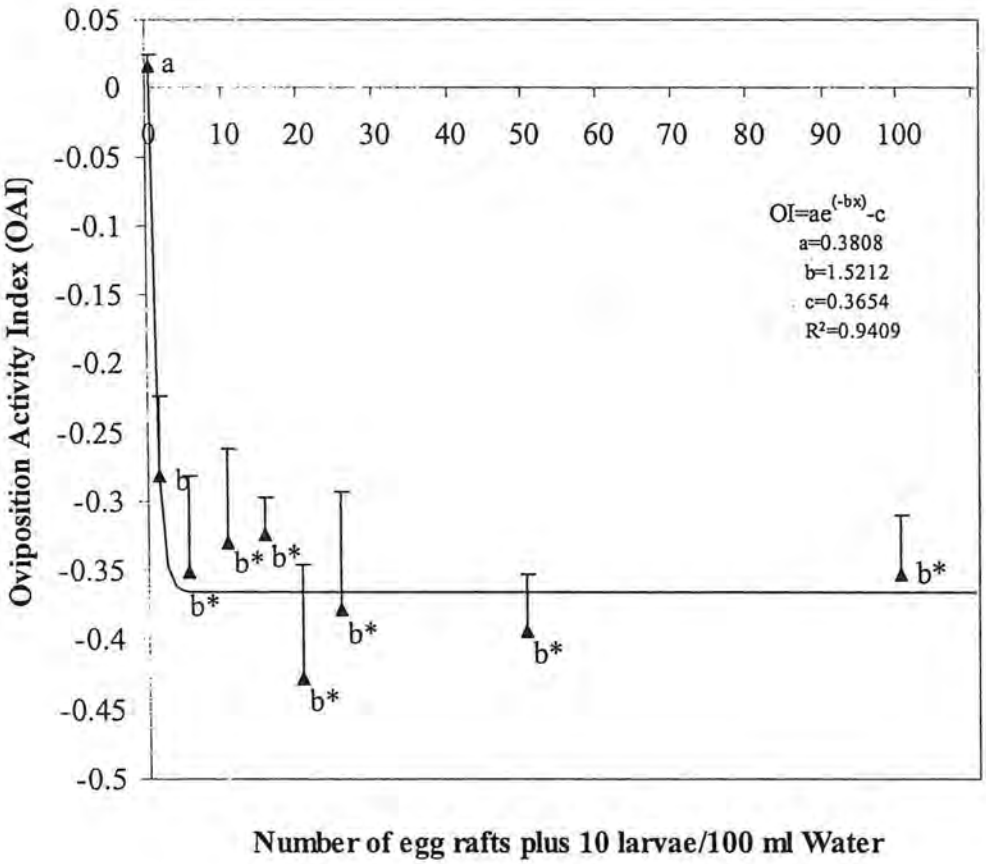


Figure 4.3. Oviposition indices (Mean±SE) for oviposition responses of gravid *An. gambiae* to egg rafts and a constant number (ten) of larvae of *C. quinquifasciatus* in test water.

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

4.1.4 Oviposition response by *C. quinquifasciatus* to volatiles from own egg rafts and larvae

Varying numbers of conspecific egg rafts and larvae were separately added into the test water. *C. quinquifasciatus* responded to volatiles emanating from these by laying more egg rafts (Figs. 4.4 and 4.5). The maximum number of egg rafts laid reached saturation at 25 egg rafts (OAI= +0.47) and 25 larvae (OAI= +0.4) in the 100 ml test water respectively. Further increase in either egg rafts or larvae in the test water did not significantly affect the oviposition response.

However, presence of both eggs and larvae in the test water altered the oviposition pattern (Fig. 4.6). With 10 larvae and increasing number of egg rafts, the oviposition response reached maximum at 25 egg raft/100 ml water. Higher numbers of egg rafts led to a decrease in the oviposition response that reached a minimum (OAI=0.12) at 50 egg rafts/100 ml test water with no further decline.

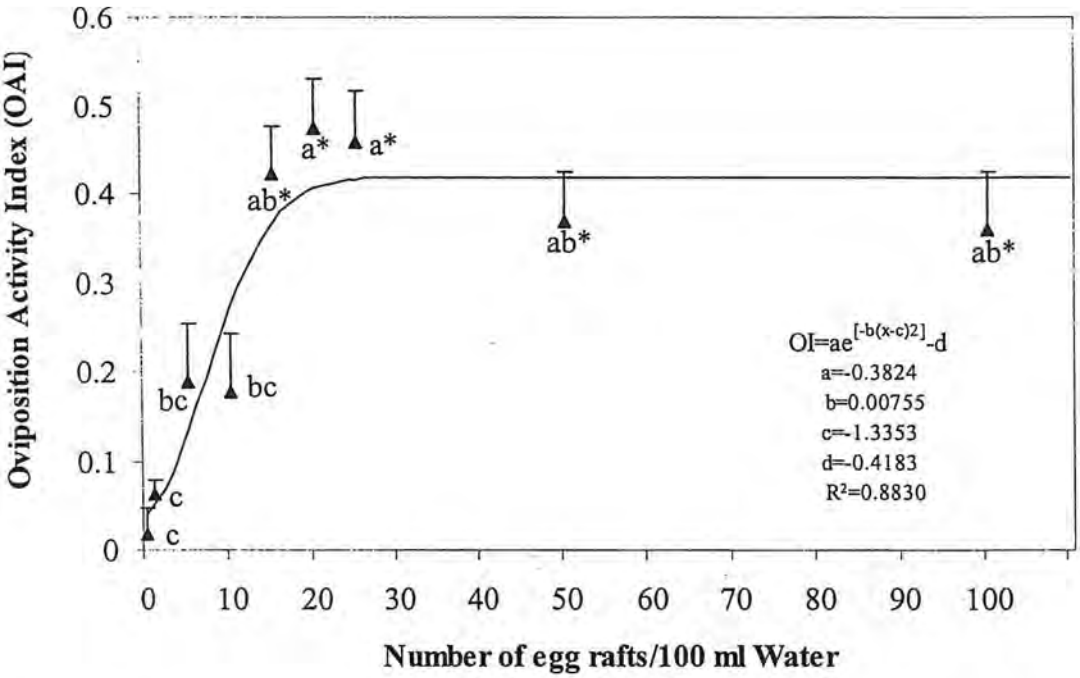


Figure 4.4. Oviposition indices (Mean±SE) for oviposition responses of gravid *C. quinquifasciatus* to conspecific egg rafts in test water.

*p<0.05 significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, p<0.05)

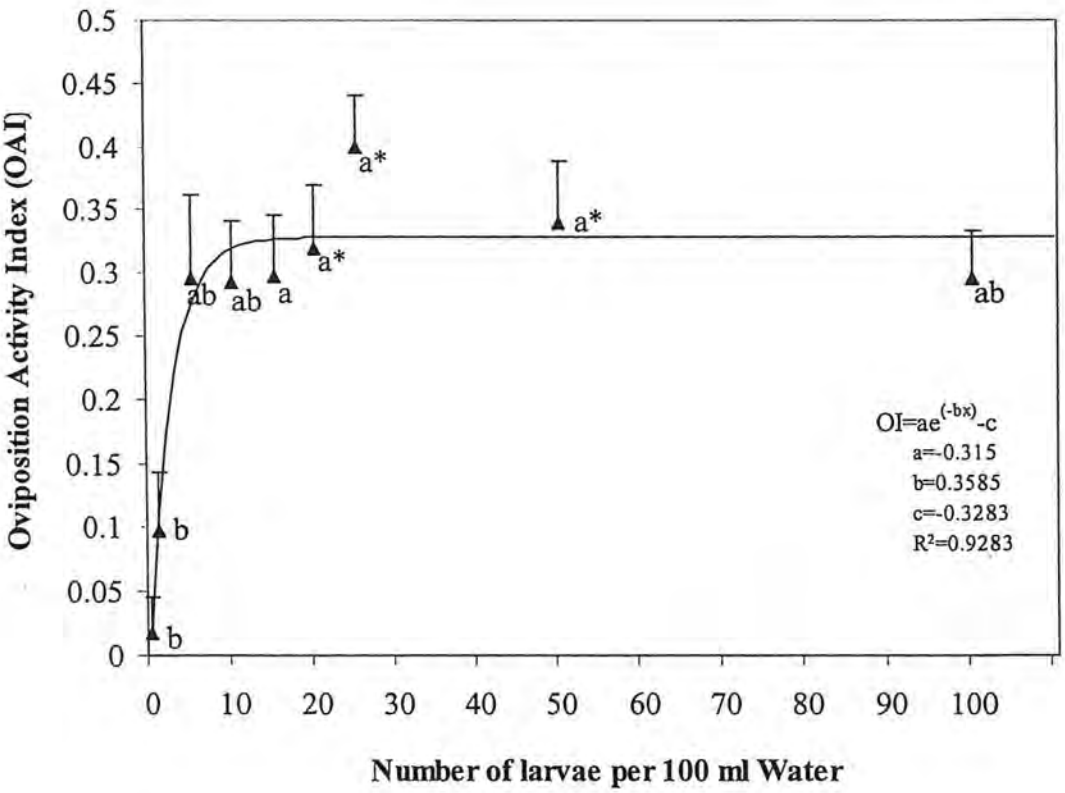


Figure 4.5. Oviposition indices (Mean±SE) for oviposition responses of gravid *C. quinquifasciatus* to conspecific larvae in test water.

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

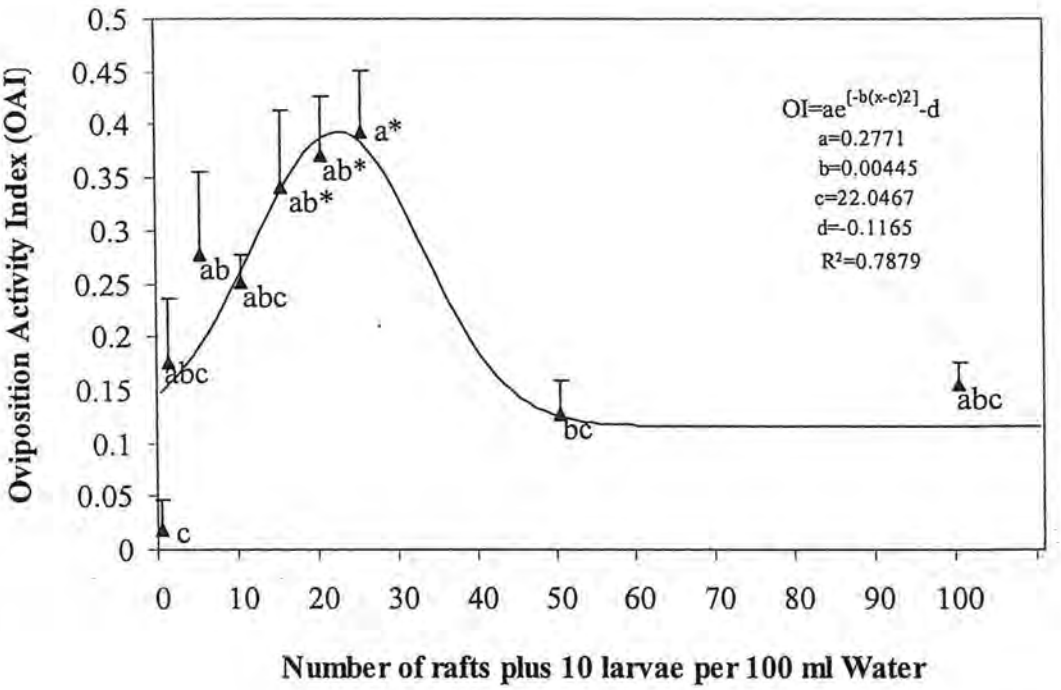


Figure 4.6. Oviposition indices (Mean±SE) for oviposition responses of gravid *C. quinquifasciatus* to conspecific egg rafts and a constant number (ten) of larvae in test water.

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

The results show that *An. gambiae* females exhibited varying oviposition response patterns depending on the numbers of larvae and/or eggs of *C. quinquifasciatus* present in the water. At low *C. quinquifasciatus* egg density, there was a small increase in oviposition, which was significant ($P<0.05$) at 5 egg rafts/100 ml. However, at higher egg densities, *An. gambiae* females were deterred in a dose dependent-manner. On the other hand, in the presence of larvae, gravid *An. gambiae* females were deterred from ovipositing at all

densities tested. Interestingly, in presence of a low density larvae (10/100 ml), *C. quinquifasciatus* egg rafts at all densities also deterred oviposition by *An. gambiae*. The incremental attraction of *An. gambiae* at low egg densities of *C. quinquifasciatus* may account for some co-presence of the two species in some situations in the field (Sumba, 2004), although the numbers of *An. gambiae* larvae are relatively small. However the results account for low avoidance of *C. quinquifasciatus* preferred pools by *An. gambiae* and suggest that two different but complementary volatile signals may be involved in deterring the females of this mosquito to lay in culicine pools. This accounts for the sharp avoidance by *An. gambiae* of the test substrate that contained even low densities of culex eggs and larvae.

As expected gravid *C. quinquifasciatus* laid more egg rafts on the treatment cup containing their own egg rafts than in control and at higher density of egg rafts there was significant difference ($p<0.05$) from zero at 20 egg rafts/100 ml. The same trend was observed when conspecific larvae was used against gravid *C. quinquifasciatus*. Gravid *C. quinquifasciatus* were attracted in dose dependent manner.

At low *C. quinquifasciatus* egg rafts and constant number of larvae there was increase in oviposition which was significant $p<0.05$ from zero at 25 egg rafts plus 10 larvae/100 ml water. Increase in egg rafts resulted in lesser attractiveness of the test substrate and there was no significant difference between zero and 100 egg rafts plus 10 larvae. This trend is puzzling and may suggest a complex pattern of oviposition choices made by *C.*

quinquifasciatus depending upon the relative proportion of conspecific eggs and larvae. Further studies are needed to elucidate the factors that underlie oviposition choices of this mosquito.

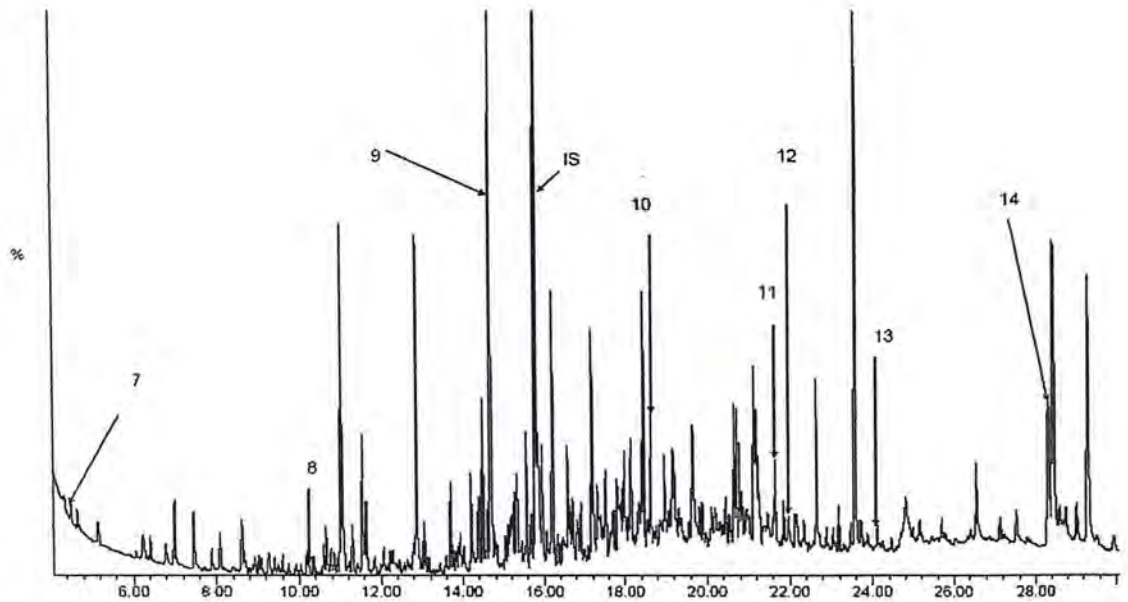
4.2 Compounds identified from the volatiles of water containing *C. quinquefasciatus* larvae

In the experiments carried out with *C. quinquifasciatus* larvae it was found out that *An. gambiae* laid more eggs in control cups than in treatment cups consisting of *C. quinquifasciatus* larvae. Volatiles emanating from *C. quinquifasciatus* larvae mediate the oviposition behaviour of gravid *An. gambiae*. In search for these compounds that mediate oviposition behaviour volatiles were trapped from *C. quinquifasciatus* larvae in test water. One µl of the sample was injected in to GC-MS and in it was 1.08 nanogram of internal standard [**(IS)** ethyl nonanoate] for quantitative analysis. A total of eleven compounds were detected and characterized from these volatiles, obtained by both dynamic and static trapping systems (Fig. 4.7, 4.8, 4.9, 4.10, Table 4.1). The compounds detected were identified as dimethyl disulfide **(7)**, dimethyl trisulfide **(8)**, 3,5-dimethylbenzaldehyde **(9)**, internal standard (ethyl nonanoate) **(IS)**, 2,4-bis(1,1-dimethylethyl)phenol **(10)**, 1-chlorotetradecane **(11)**, isopropyl myristate **(12)**, isopropyl palmitate **(13)**, 4-phenylmorpholine **(14)**, 3-phenyl-1-azabicyclo[2.2.2]octane **(15)**, eicosane **(16)** and 2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**. Five compounds (**7**, **8**, **12**, **14** and **15**) were obtained in the two trapping systems and four of these compounds were found in more concentration in static trapping system than in dynamic system.

Table 4.1. Compounds identified from the volatiles of *C. quinquefasciatus* larvae

Compound	Retention time (Min)	Dynamic system concentration (ng)	Static system concentration (ng)
Dimethyl disulfide, **(7)**$^{+}$	4.498	0.0048	0.9134
Dimethyl trisulfide **(8)**$^{+}$	10.366	0.0248	0.0458
3,5-dimethyl Benzaldehyde **(9)***	14.666	0.5836	-
2,4-bis(1,1-dimethylethyl) Phenol**(10)***	18.631	0.7662	-
1-chloro Tetradecane **(11)***	21.657	0.5329	-
Isopropyl Myristate **(12)**$^{+}$	22.124	0.4167	trace
Isopropyl Palmitate **(13)***	24.118	0.8906	-
4-phenyl Morpholine **(14)**$^{+}$	28.306	0.7915	1.0637
3-phenyl-1-Azabicyclo[2.2.2]octane **(15)**$^{+}$	24.185	trace	1.0086
Eicosane **(16)****	26.559	-	1.7961
2,4-bis(1-methyl-1-phenylethyl) Phenol **(17)****	28.888	-	1.5500

$^{+}$Appears in sample obtained by both dynamic and static trapping system
*Appears only in sample obtained by dynamic trapping system
** Appears only in sample obtained by static trapping system



IS= Internal standard

Figure 4.7. Gas chromatogram of volatiles obtained from *C. quinquefasciatus* larvae in test water by dynamic trapping system

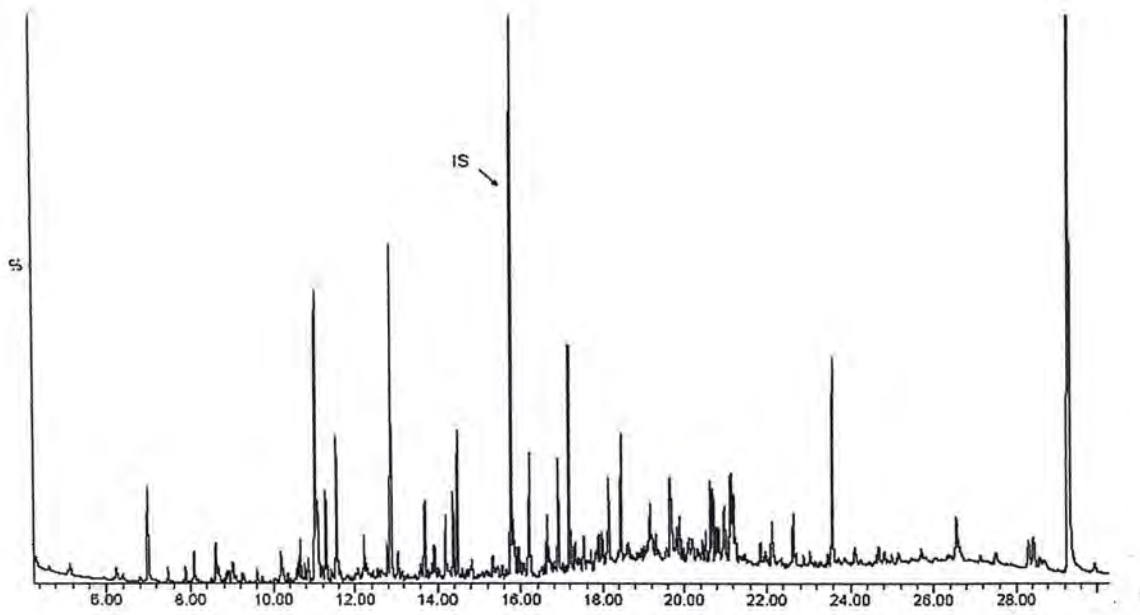


Figure 4.8. Gas chromatogram of volatiles of test water (control) obtained by dynamic trapping system

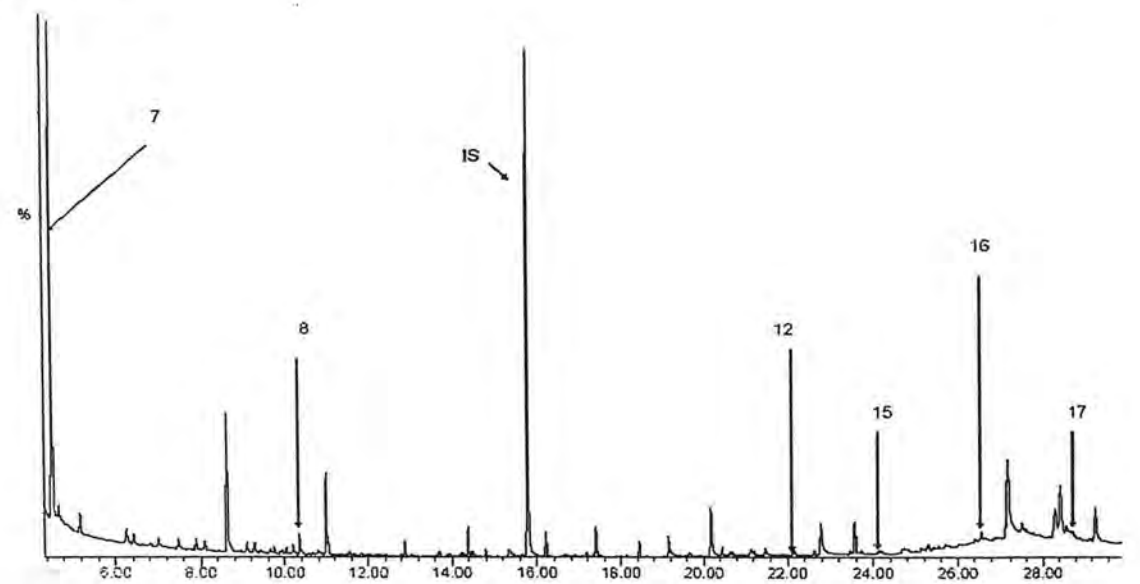


Figure 4.9. Gas chromatogram of volatiles obtained from *C. quinquefasciatus* larvae in test water by static trapping system

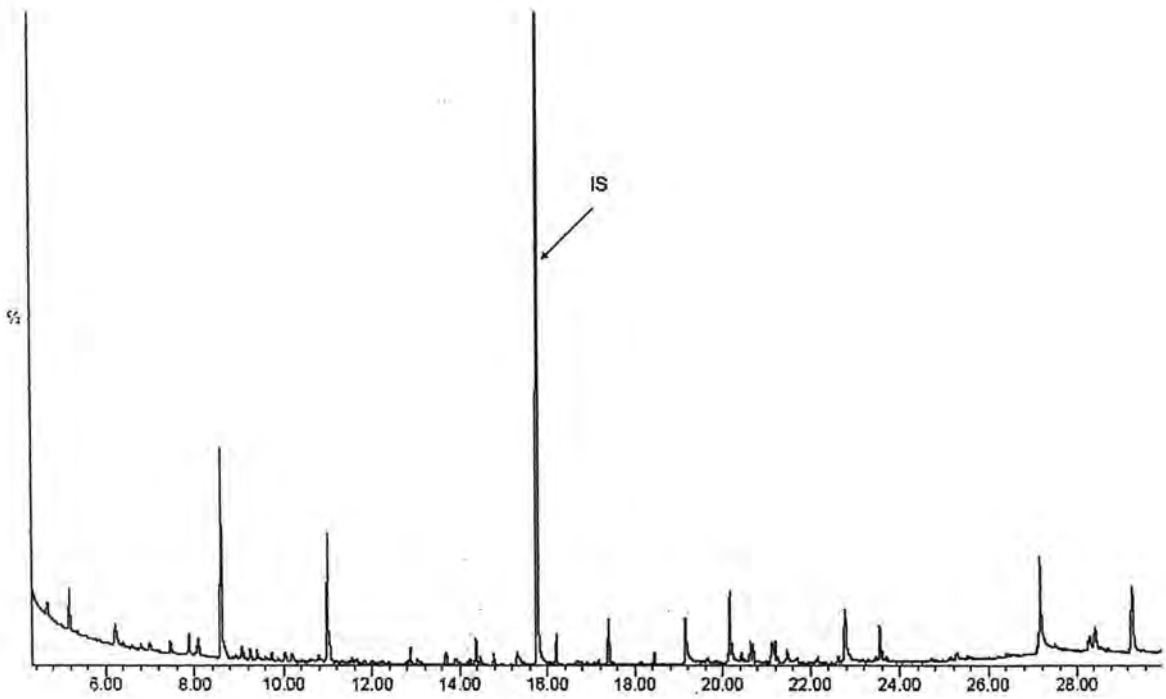
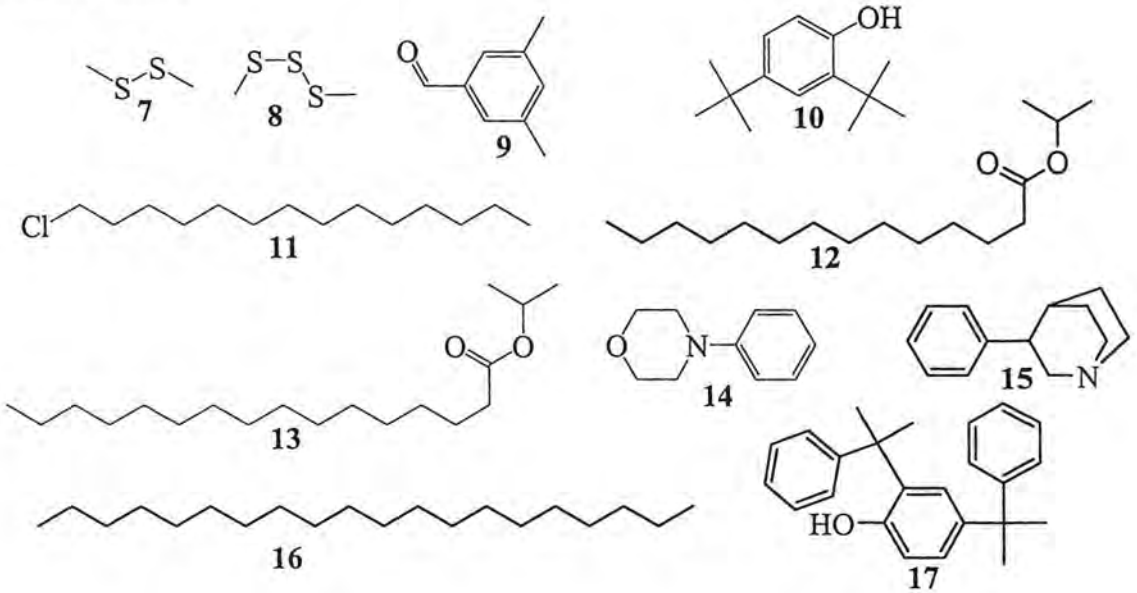


Figure 4.10. Gas chromatogram of volatiles of test water (control) obtained by static trapping system



4.2.1 Dimethyl disulfide (7) and Dimethyl trisulfide (8)

Compound **7** was found in trace amounts when the dynamic trapping method was applied. This could be attributed to the volatility of the compound. Compounds **7** and **8** were characterized on the basis of GC-MS spectra. The molecular ion peak (M^+) appeared at m/z 94 which was also the base peak. Loss of a methyl radical gave a peak at 79 further loss of methyl radical gave a peak at m/z 64 ($S{=}S^+$), while loss of one $CH_3S^{\cdot}$ yielded m/z 47 (Fig.4.11). The MS of compound **8** revealed M^+ peak at m/z 126. Loss of a methyl radical gave a peak at m/z 111 while loss of one $CH_3S^{\cdot}$ gave a peak at m/z 79 and loss of $CH_3SS^{\cdot}$ gave m/z 47 (Fig 4.12).

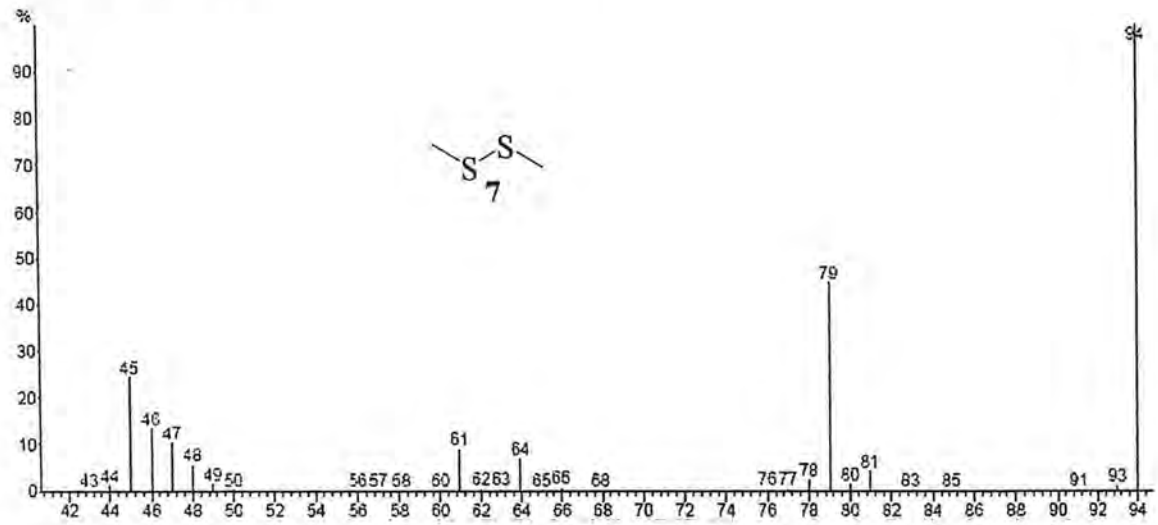


Figure 4.11. Mass Spectrum of dimethyl disulfide (**7**)

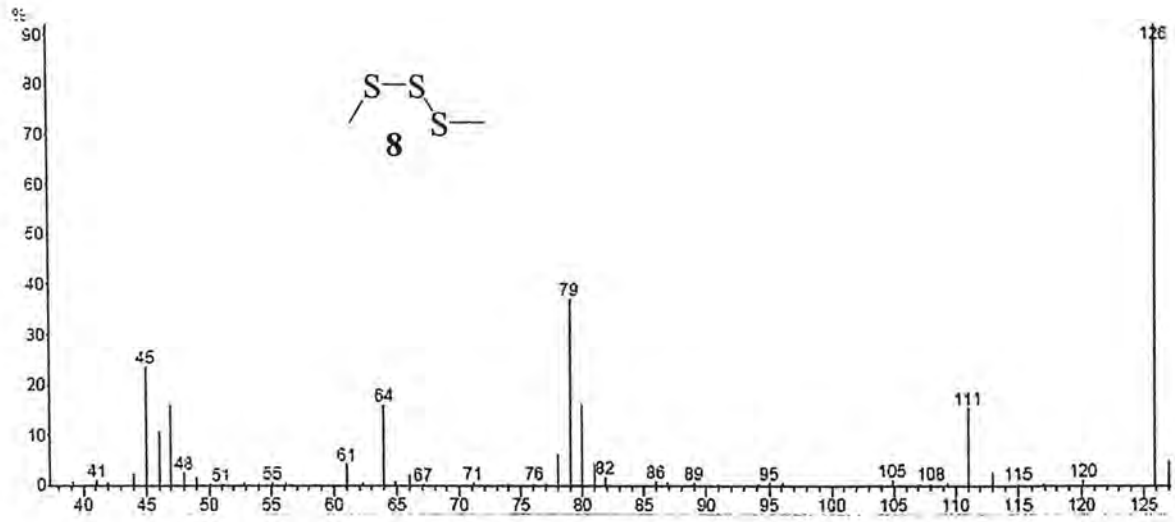


Figure 4.12. Mass Spectrum of dimethyl trisulfide **(8)**

4.2.2 3,5-dimethylbenzaldehyde (9)

The mass spectrum of compound **9** (Fig. 4.13) exhibited molecular ion peak at m/z 134, corresponding to the formula $C_9H_{10}O$. The base peak at m/z 133 was due to the loss of a hydrogen atom (M^+-1). The loss of a methyl radical gave a peak at m/z 119. The loss of two methyl substituents led to the peak at m/z 105. Loss of –CO and -2CH_3 gave the peak at m/z 77.

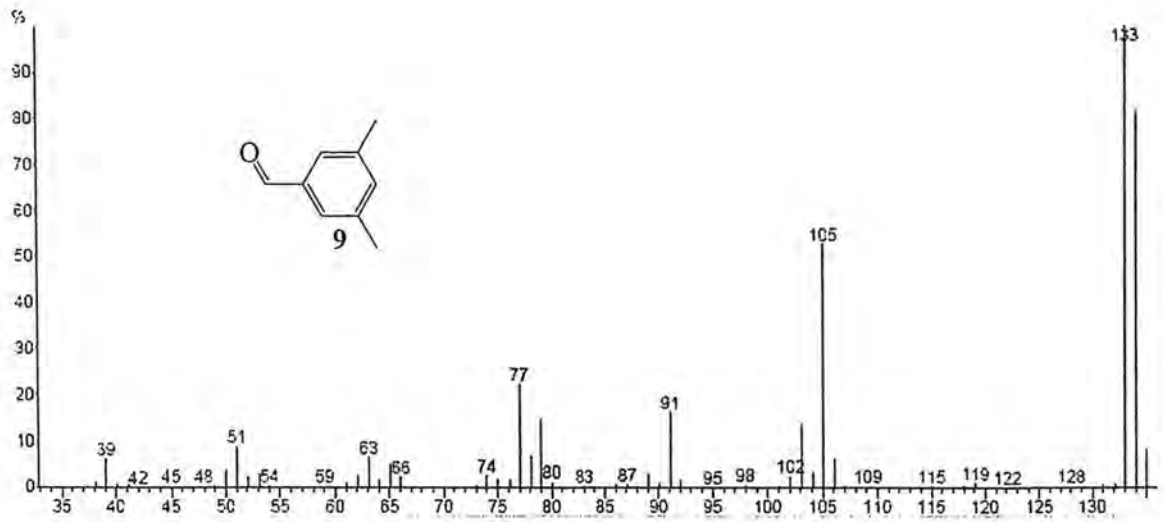


Figure 4.13. Mass Spectrum of 3,5-dimethylbenzaldehyde **(9)**

4.2.3 2,4-bis(1,1-dimethylethyl)phenol (10)

The mass spectrum of compound **10** (Fig. 4.14) revealed M^+ peak at m/z 206 corresponding to molecular formula $C_{14}H_{22}O$. The base peak at m/z 191 ($C_{13}H_{19}O^+$) was due to loss of methyl group M-CH_3. Subsequent loss of water gave a peak at m/z 175. The loss of three methyl groups from one of the substituents (1,1-dimethylethyl) gave a peak at m/z 163 ($C_{11}H_{15}O^+$).

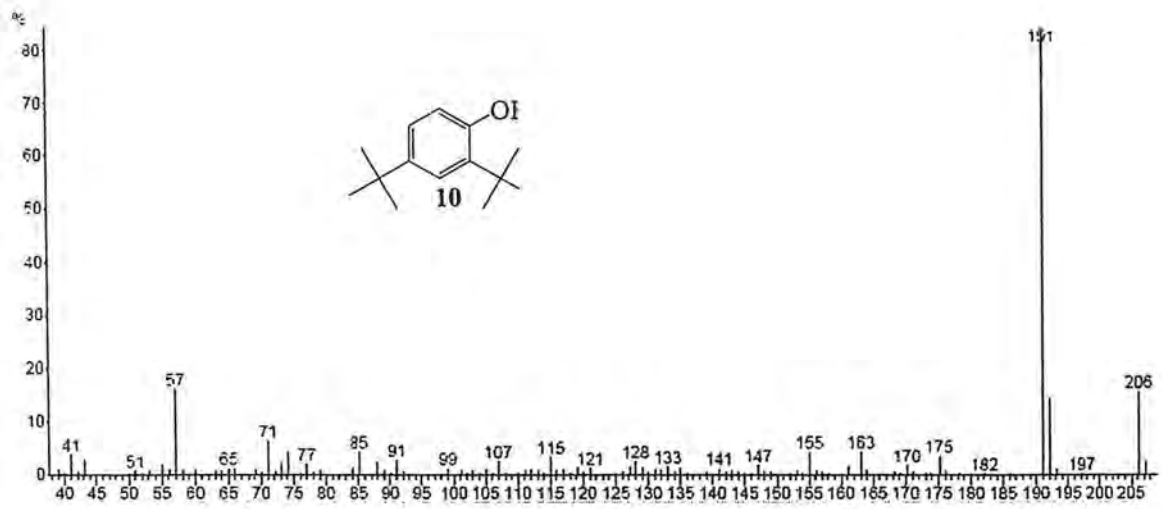
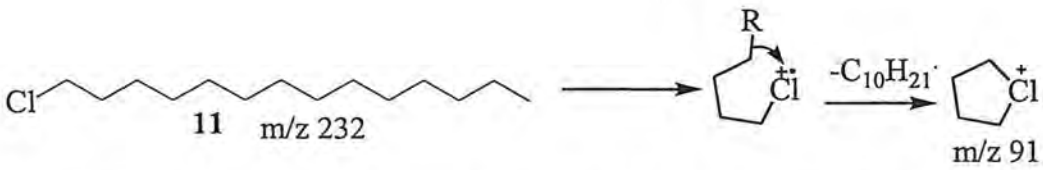


Figure 4.14. Mass Spectrum of 2,4-bis(1,1-dimethylethyl)phenol **(10)**

4.2.4 1-chlorotetradecane (11)

Molecular ion peak of compound **11** at m/z 232 ($C_{14}H_{29}Cl$) was weak (Fig. 4.15). Straight chain chlorides longer than C6 give $C_4H_3Cl^+$ m/z 91 and $C_5H_{10}Cl^+$ m/z 105 ions. The $C_4H_8Cl^+$ ion forms the most intense (sometimes the base) peak (Silverstein *et al.*, 2005). The formation of five member cyclic structure $C_4H_8Cl^+$ ion at m/z 91 (Scheme 4.1) explains the peak stability. MS profile of compound **11** gave hydrocarbon fragmentation peaks at m/z 85, 71, 57 and 43 corresponding to the molecular formulae $C_6H_{13}^+$, $C_5H_{11}^+$, $C_4H_9^+$ and $C_3H_7^+$, respectively. The peak at m/z 57 forms the base peak. Fragmentation of compound **11** at chloride side gave peaks at m/z 147, 133, 119, 105 and 91 with the first three peaks corresponding to molecular formulae $C_8H_{16}Cl^+$, $C_7H_{14}Cl^+$ and $C_5H_{10}Cl^+$, respectively.



Scheme 4.1. Formation of five member cyclic of 1-chlorotetradecane **(11)**

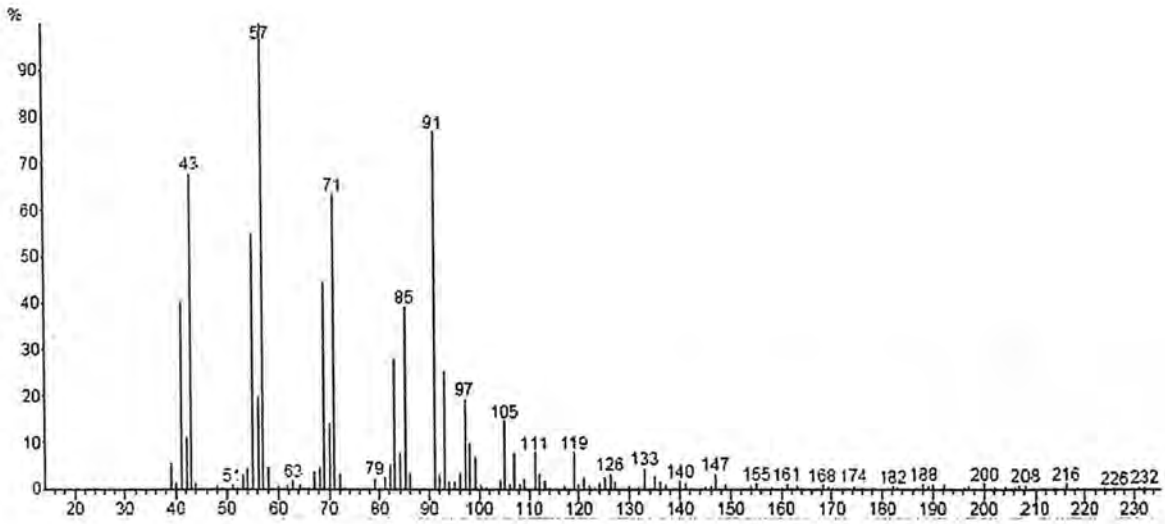
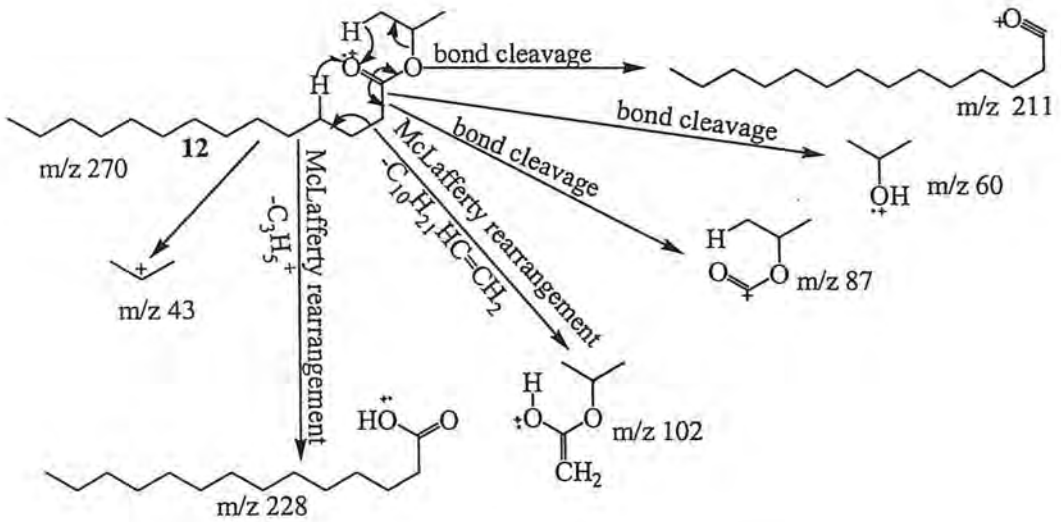


Figure 4.15. Mass Spectrum of 1-chlorotetradecane **(11)**

4.2.5 Isopropyl myristate (12)

The MS of compound **12** showed a molecular ion peak (M^+) at m/z 270 corresponding to the molecular formula $C_{17}H_{34}O_2$. The MS (Fig. 4.16 and scheme 4.2) exhibited prominent peaks at m/z 102 and 228 ascribable to $C_5H_{10}O_2^{\cdot+}$ and $C_{14}H_{28}O_2^{\cdot+}$, respectively, resulting to McLafferty rearrangement (Scheme 4.2).



Scheme 4.2. Proposed fragmentation pattern of isopropyl myristate **(12)**

The bond cleavage next to a C=O can give four ions; R-C(=O)-OR'. R^+, $R\text{-}C{\equiv}O^+$, $C(=O)OR^+$ and OR^+. In the MS profile of compound **12** the most prominent peak due to bond cleavage was m/z 211 and 60 corresponding to $R\text{-}C{\equiv}O^+$; $C_{14}H_{27}O^+$ and $OR^{+\cdot}$; $C_3H_8O^{+\cdot}$, respectively. A peak at m/z 87 due to $C(=O)OR^+$; $C_4H_7O_2^+$, was also visible. The peak due to bond cleavage at R^+ is not visible due to the long chain resulting after cleavage of compound **12**. The ion R is prominent in the short chain esters but diminishes rapidly with increasing chain length (Silverstein *et. al.*, 2005).

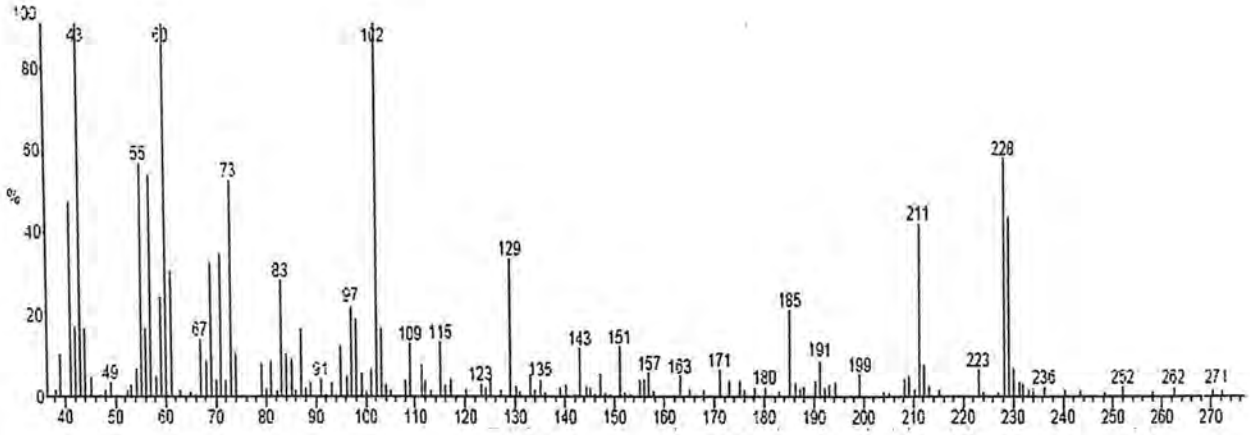
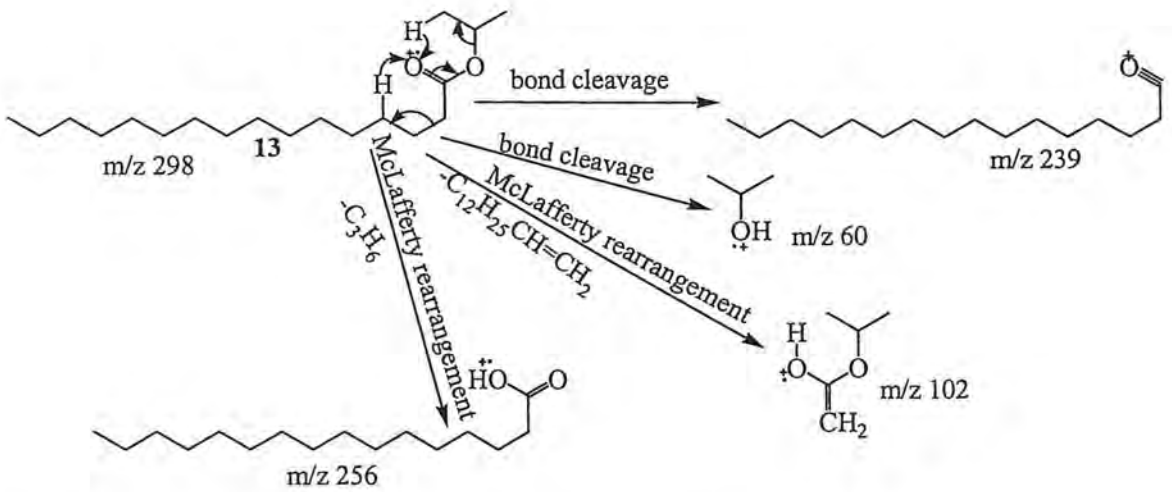


Figure 4.16. Mass Spectrum of isopropyl myristate **(12)**

4.2.6 Isopropyl palmitate (13)

The MS of compound **13** showed a molecular ion peak (M^+) at m/z 298 corresponding to the molecular formula $C_{19}H_{38}O_2$. The MS (Fig. 4.17 and Scheme 4.3) exhibited the base peak at m/z 256 and prominent peaks at m/z 102 ascribed to $C_{16}H_{32}O_2^{\cdot+}$ and $C_5H_{10}O_2^{\cdot+}$ respectively, being formed due to McLafferty rearrangement. The most prominent peaks due to bond cleavage were at m/z 239 and 60 corresponding to $R\text{-}C{\equiv}O^+$; $C_{16}H_{31}O^+$ and $OR^{\cdot+}$; $C_3H_8O^{\cdot+}$, respectively. The peak at m/z 87 was attributable to $C(=O)OR^+$; $C_4H_7O_2^+$.



Scheme 4.3. Proposed fragmentation pattern of isopropyl palmitate **(13)**

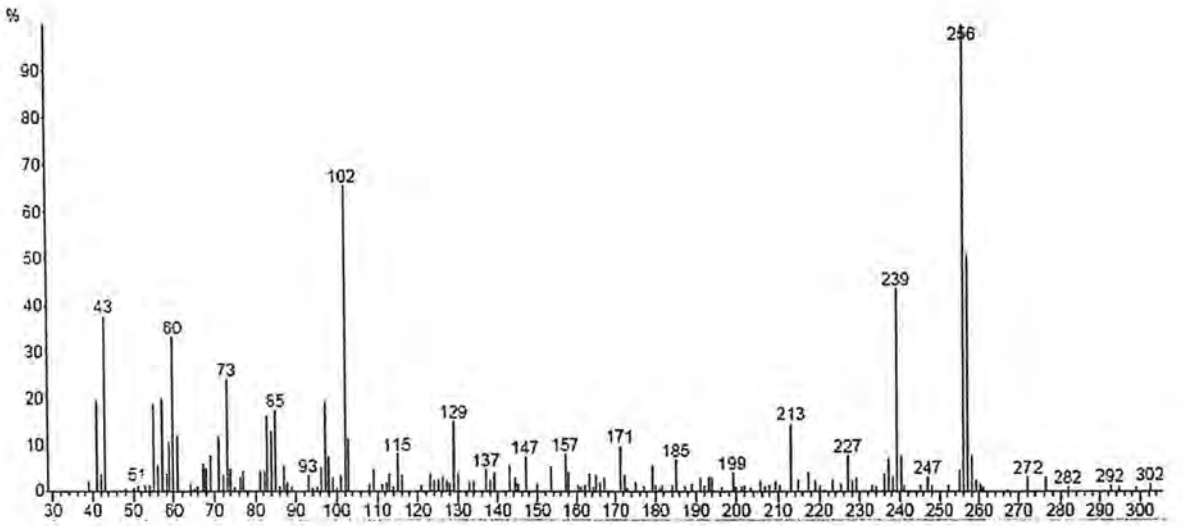
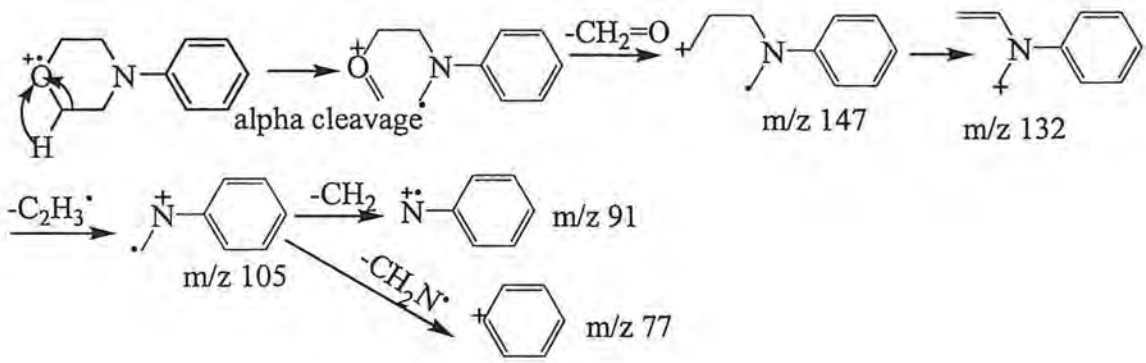


Figure 4.17. Mass Spectrum of isopropyl palmitate **(13)**

4.2.7 4-phenylmorpholine (14)

Compound **14** had a molecular formula $C_{10}H_{13}NO$ with molecular ion peak at m/z 163. Alpha cleavage at the oxygen position caused a small peak at m/z 147 ascribed to $C_{10}H_{13}N^{+\cdot}$ (Fig. 4.18 and Scheme 4.4). Further loss of a methyl group caused a peak at m/z

132, ($C_9H_{10}N^+$). The base peak at m/z 105 ($C_7H_7N^+$) was attributable to the loss of $CH_2CH_2OCH_2$. The peak at m/z 77 was ascribable to $C_6H_5^+$.



Scheme 4.4. Proposed fragmentation pattern of 4-phenylmorpholine **(14)**

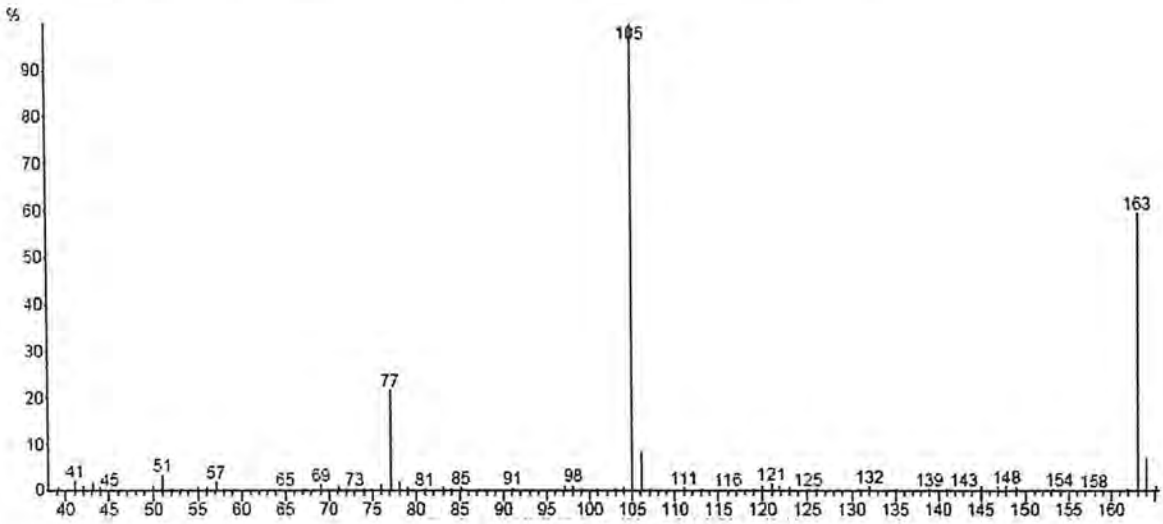


Figure 4.18. Mass Spectrum of 4-phenylmorpholine **(14)**

4.2.8 3-phenyl-1-azabicyclo[2.2.2]octane (15)

The mass spectrum of compound **15** gave a molecular ion peak as a base peak (m/z = 187) corresponding to molecular formula $C_{13}H_{17}N$ (Fig. 4.19). The peak at m/z 119 was

ascribable to $C_8H_9N^{+\cdot}$. Peak at m/z 104 was due to $C_8H_8^{+\cdot}$. The peak at 77 was due to benzyl ion, $C_6H_5^+$. The peak at 43 and 57 was due to cyclic amine ascribable to $C_2H_5N^{+\cdot}$ and $C_3H_7N^{+\cdot}$, respectively.

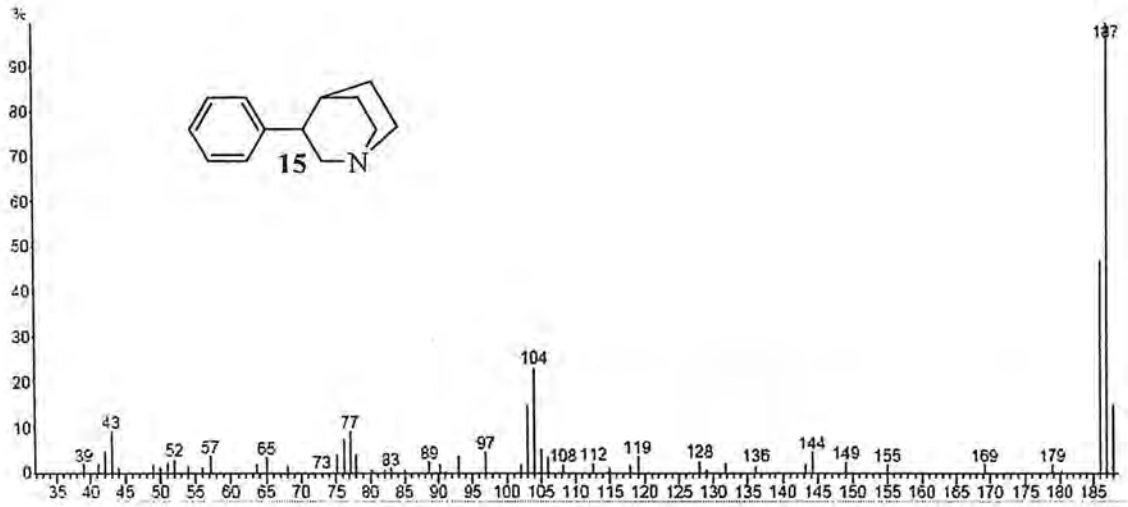


Figure 4.19. Mass Spectrum of 3-phenyl-1-azabicyclo[2.2.2]octane **(15)**

4.2.9 Eicosane (16)

The molecular ion peak of compound **16** is m/z 282 (Fig. 4.20) corresponding to the formula $C_{20}H_{42}$. Compound **16** was identified as a hydrocarbon due to its fragmentation pattern. The mass spectrum of a hydrocarbon has a fragmentation pattern characterized by clusters of peaks and corresponding cluster are 14 mass units (CH_2) apart. The largest peaks in each cluster represent C_nH_{2n+1} fragments (Silverstein *et. al.*, 2005). The most abundant fragments are at C_3 and C_4 and the fragment abundances decrease smoothly. The base peak at m/z 57 was due to $C_4H_9^+$ and the peak at m/z 43 corresponded $C_3H_9^+$. The peak at m/z 71 was due to $C_5H_{11}^+$.

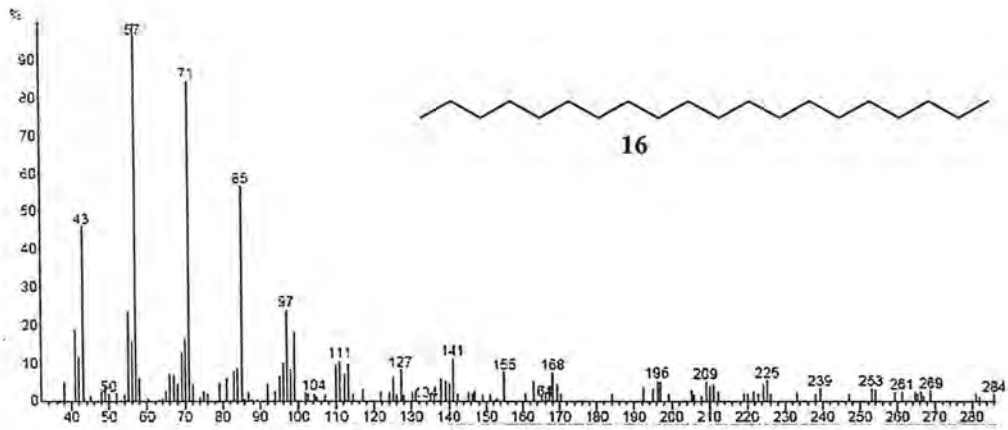


Figure 4.20. Mass Spectrum of eicosane **(16)**

4.2.10 2,4-bis(1-methyl-1-phenylethyl)phenol (17)

Compound **17** exhibited a molecular ion peak M^+ at m/z 330 (Fig. 4.21) corresponding to molecular formula $C_{24}H_{26}O$. The loss of a methyl group resulted to the peak at m/z 315 corresponding to $C_{23}H_{23}O^+$ which was the base peak. The peak at m/z 268 was due to $C_{19}H_{24}O^{\cdot}$, with loss of a methyl group from this radical giving a peak at m/z 253. A small peak at m/z 238 was ascribable to $C_{17}H_{18}O^{\cdot+}$. Loss of water molecule from m/z 238 gave a peak at 220 ($C_{17}H_{16}^{+}$). Other prominent peaks at m/z 135 and 91 were due to $C_9H_{11}O^+$ and $C_7H_7^+$, respectively.

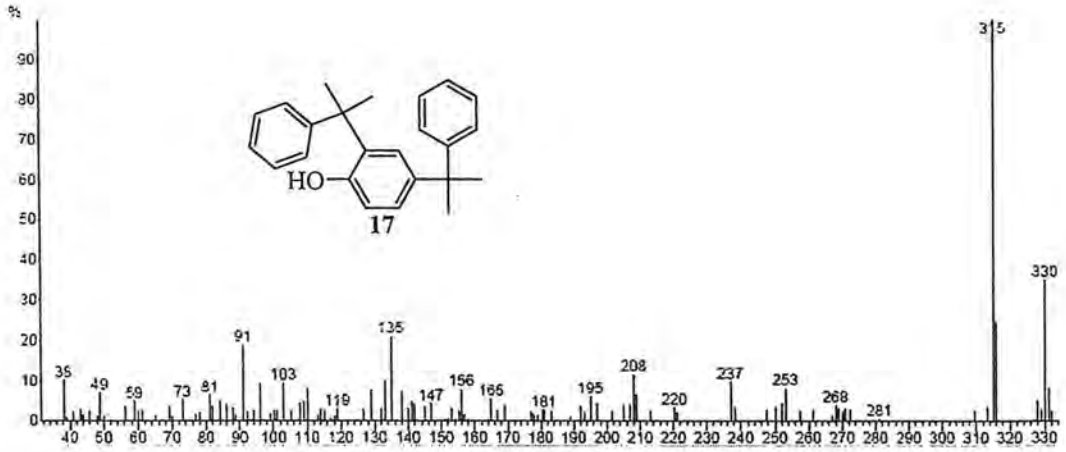


Figure 4.21. Mass Spectrum of 2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**

4.3 Compounds Identified from the Extract of Test Water containing *Culex quinquefasciatus* Larvae

Seven compounds were identified from the extract from test water containing *C. quinquifasciatus* larvae (Table 4.2, Fig. 4.22). These were, 4-methylphenol **(18)**, internal standard (ethyl nonanoate) **(IS)**, 4-(1,1,3,3-tetramethyl butyl)phenol **(19)**, 4-(1,1-di methylpropyl)phenol **(20)**, 2[(4-hydroxyphenyl)methyl]phenol **(21)**, N,N-dimethylthiocarb amoylphenyltrithiocarbonate **(22)**, 2,4-bis(1-methyl-1-phenylethyl)phenol **(17)** and (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-Tetracosahexane **(23)**. Other compounds; Z-2 -pentene, phenol, N-ethylbenzeneamine, dodecane, (E)-5-octadecene and vanillin were obtained in trace amounts.

Table 4.2. Compounds identified from the extract from test water containing *C. quinquefasciatus* larvae

Compound	Retention time (Min)	Concentration (ng)
4-methylphenol **(18)**	12.359	0.4559
4-(1,1,3,3-tetramethyl butyl)phenol **(19)**	20.922	0.6767
4-nonylphenol **(20)**	21.429	0.5204
2[(4-hydroxyphenyl)methyl]phenol **(21)**	23.961	1.0840
N,N-Dimethylthiocarbamoyl phenyl trithiocarbonate **(22)**	27.577	1.3769
2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**	28.396	0.6596
(all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-Tetracosahexane **(23)**	30.821	0.8966

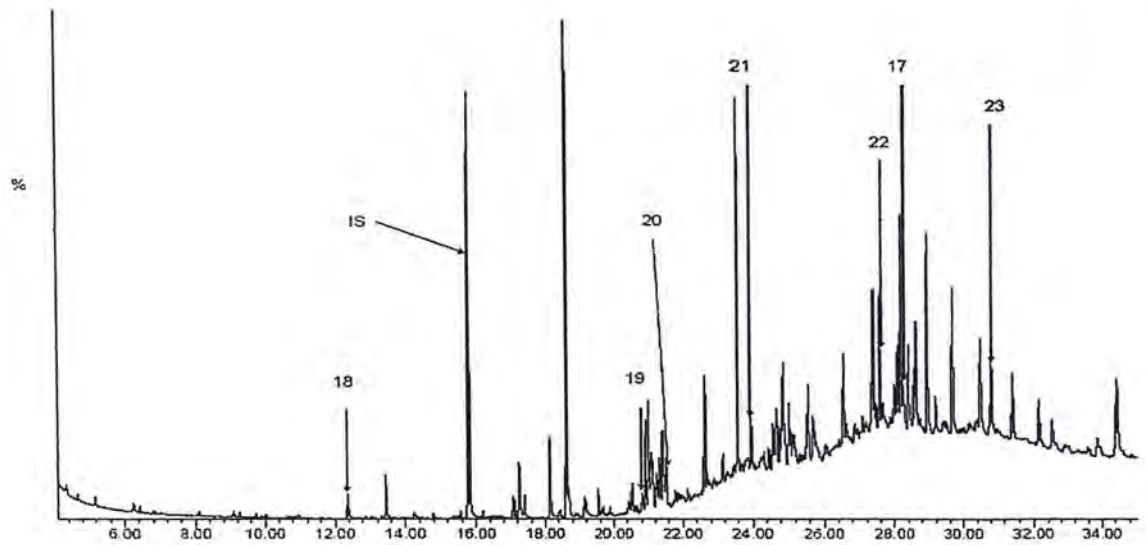
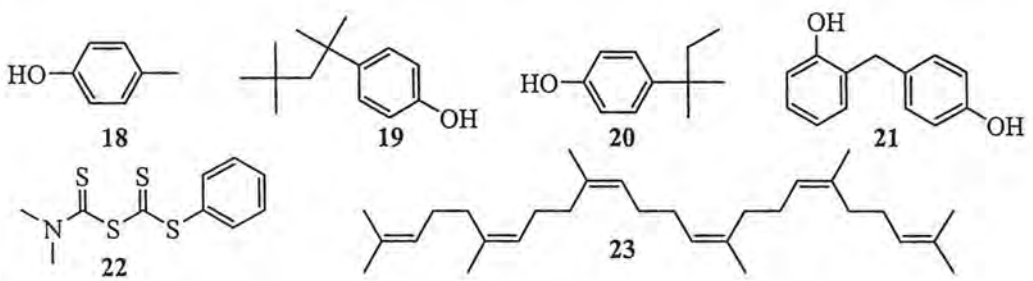


Figure 4.22. Gas chromatogram of extract from test water with *C. quinquefasciatus* larvae



4.3.1 4-methylphenol (18)

The MS of compound **18** (Fig. 4.23) gave an intense molecular ion peak M^+ at m/z 108 which agreed with the proposed structure C_7H_8O. The MS exhibited a base peak at m/z 107 due to the loss of hydrogen atom. The peak at m/z 77 was due to $C_6H_5^+$. The loss of formyl radicle (HCO·) gave a peak at m/z 79.

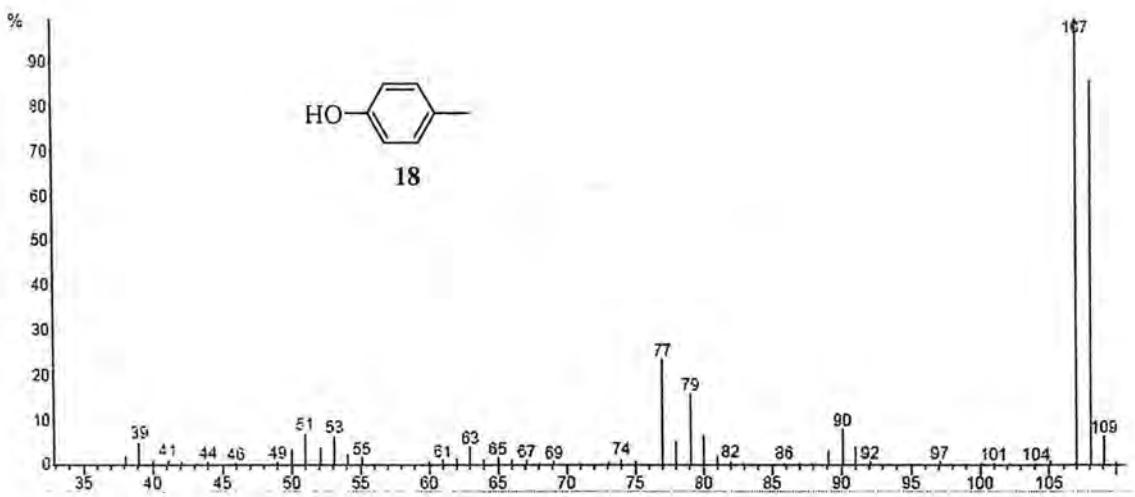


Figure 4.23. Mass Spectrum of 4-methylphenol **(18)**

4.3.2 4-(1,1,3,3-tetramethyl butyl)phenol (19)

The molecular ion M^+ peak of compound **19** was at m/z 206 corresponding to the molecular formula $C_{14}H_{22}O$ (Fig. 4.24). Loss of 3,3 dimethyl and one methyl group from butyl gave a small peak at m/z 149 ($C_{10}H_{13}O^{\cdot+}$). Loss of $(CH_3)_3CCH_2$ gave the base peak at m/z 135 ascribed to $C_9H_{11}O^{\cdot+}$. Further loss of CH_2 gave a peak at m/z 121. The peak at m/z 107 was due to $C_7H_7O^{+\cdot}$.

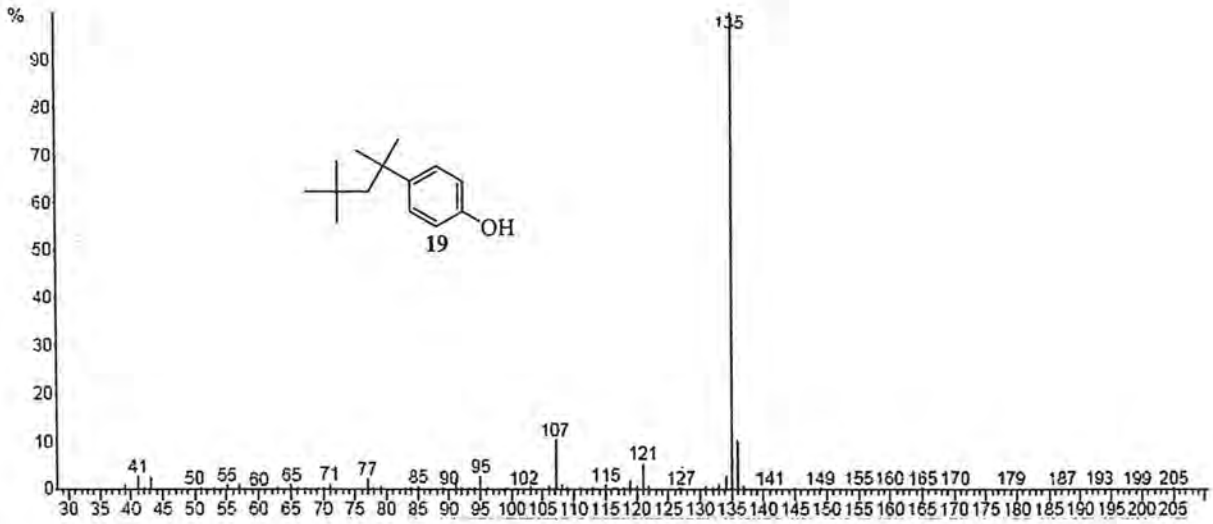
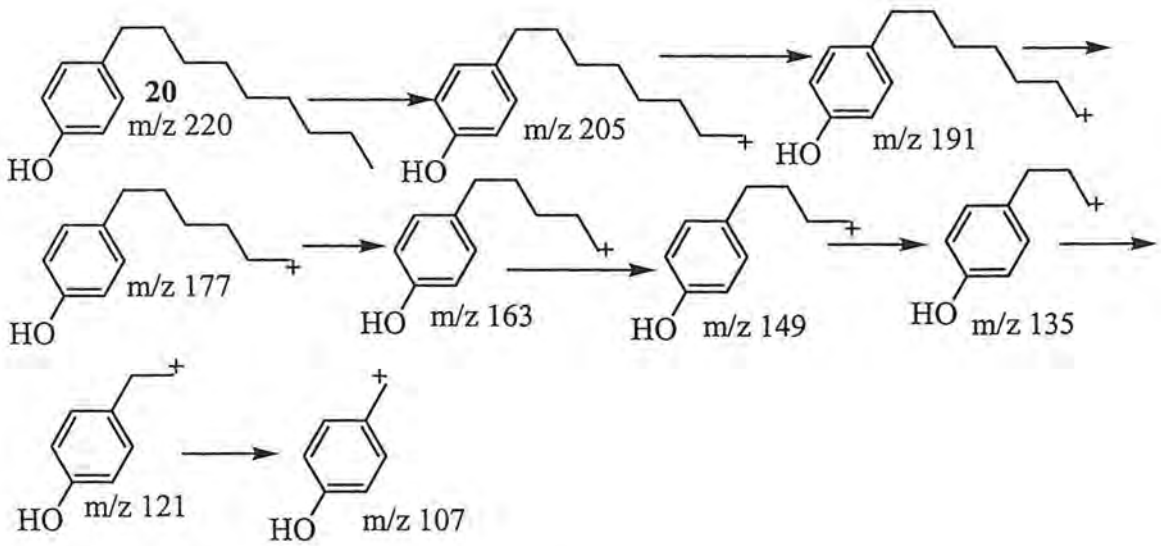


Figure 4.24. Mass Spectrum of 4-(1,1,3,3-tetramethyl butyl)phenol **(19)**

4.3.3 4-nonylphenol (20)

The molecular ion peak of compound **20** was at m/z 220 corresponding to molecular formula $C_{15}H_{24}O$. Loss of methyl group gave a peak at m/z 205 ($C_{14}H_{21}O^{\cdot+}$). Loss of pentyl group (M^{+}-C_5H_{11}) gave a small peak at m/z 149 corresponding to $C_{10}H_{13}O^{\cdot+}$ (Fig.4.25, Scheme 4.5). The loss of hexyl group (M^{+}-C_6H_{13}) gave the base peak at m/z 135 ($C_9H_{11}O^{+\cdot}$). The peaks at m/z 121 and 107, ascribable to $C_8H_9O^{\cdot+}$ and $C_7H_7O^{\cdot+}$, were due to loss of pentyl and octyl groups, respectively.



Scheme 4.5. Proposed fragmentation pattern of 4-nonylphenol **(20)**

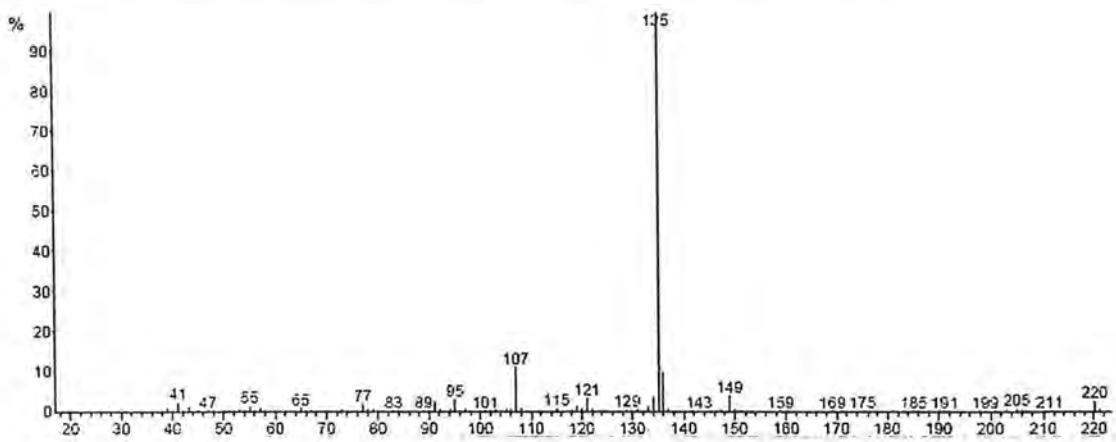


Figure 4.25. Mass Spectrum of 4-nonylphenol **(20)**

4.3.4 2[(4-hydroxyphenyl)methyl]phenol (21)

The MS (Fig. 4.26) gave a molecular ion peak as a base peak at m/z 200 corresponding to molecular formula $C_{13}H_{12}0_2$ and agreed with the proposed structure of compound **21**. Loss of CO gave M^+-28 peak at m/z 172, with the loss of formyl radicle ($HCO^{\cdot}$) giving a strong

peak at M^+-29 (m/z 171). The fragmentation of compound **21** at methelene group gave two peaks at m/z 107 and at 94 ascribable to $C_7H_7O^+$ and $C_6H_6O^{+\cdot}$, respectively.

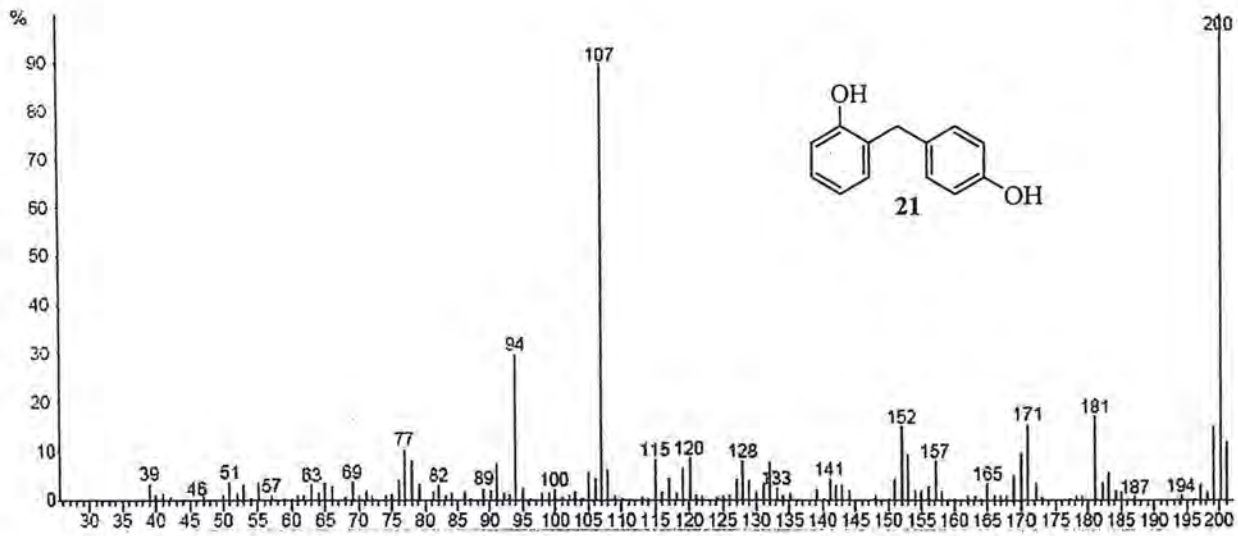
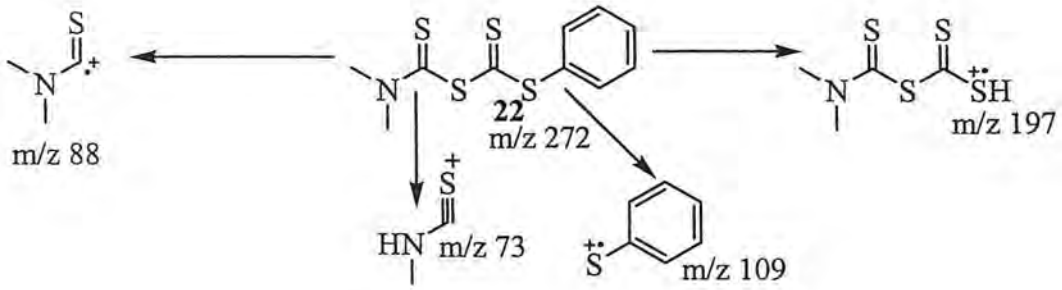


Figure 4.26. Mass Spectrum of 2[(4-hydroxyphenyl)methyl]phenol **(21)**

4.3.5 N,N-Dimethylthiocarbamoyl phenyl trithiocarbonate (22)

Mass spectrum of compound **22** (Fig. 4.27) gave molecular ion peak m/z 272 corresponding to the formula $C_{10}H_{11}NS_4$. The base peak at m/z 88 was due to $C_3H_6NS^{+\cdot}$ (Scheme 4.6). Loss of a methyl group from the base peak caused a peak at m/z 73 corresponding to molecular formula $C_3H_6NS^{+\cdot}$. The peak at m/z 109 corresponds to $C_6H_5S^{+\cdot}$ which after losing a hydrogen ion gave a more prominent peak at m/z 108.



Scheme 4.6. Proposed fragmentation pattern of N,N-Dimethylthiocarbamoyl phenyl trithiocarbonate **(22)**

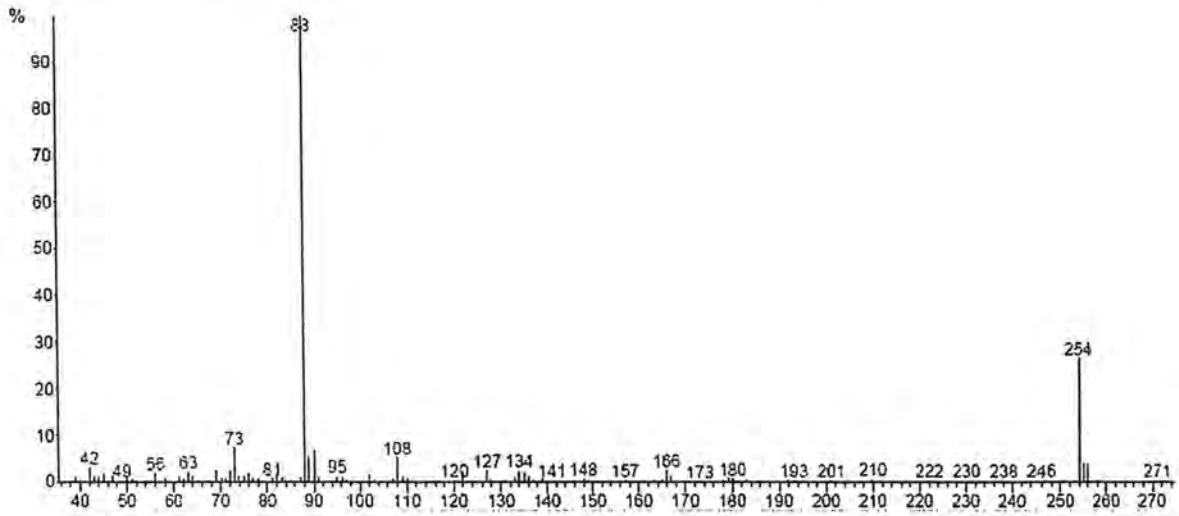
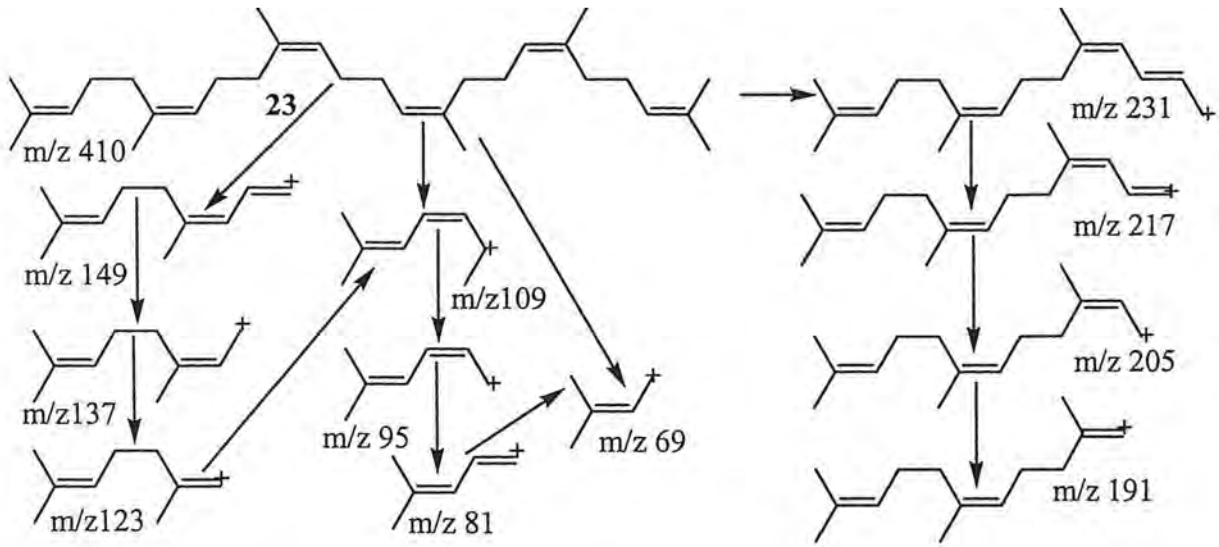


Figure 4.27. Mass Spectrum of N,N-Dimethylthiocarbamoyl phenyl trithiocarbonate **(22)**

4.3.6 (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexene (23)

The molecular ion peak of compound **23** at 410 was weak. The invisible peak corresponds to molecular ion peak $C_{30}H_{50}$ which is an unsaturated hydrocarbon. The compound has a base peak at m/z 69 which corresponds to $C_5H_9^+$, an isoprene unit. The peaks at m/z 149, 137, 123, 109, 95 and 81 correspond to (Fig 4.28, Scheme 4.7) $C_{11}H_{17}^+$, $C_{10}H_{17}^+$, $C_9H_{15}^+$, $C_8H_{13}^+$, $C_7H_{11}^+$ and $C_6H_9^+$, respectively.



Scheme 4.7. Proposed fragmentation pattern of (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexene **(23)**

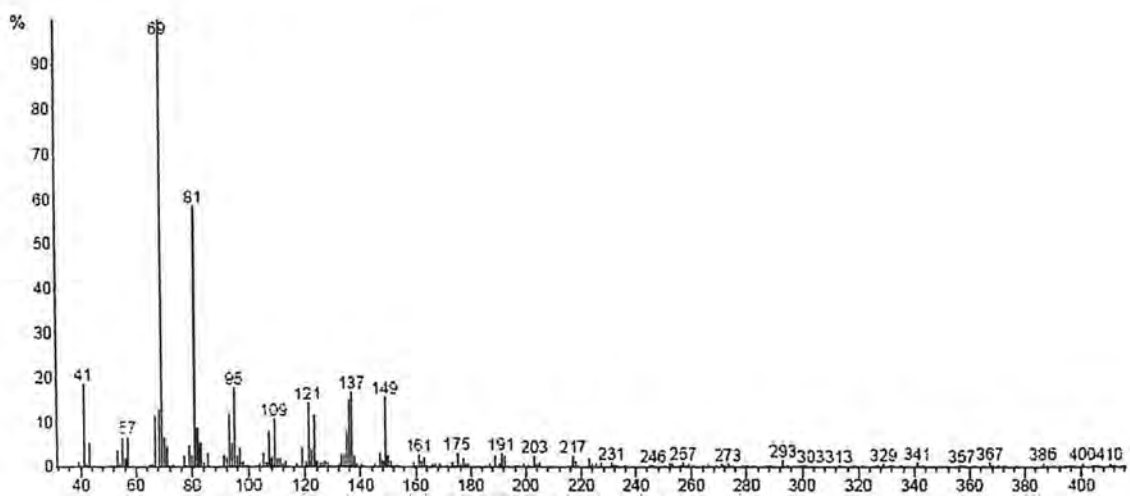


Figure 4.28. Mass Spectrum of (all-E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexene **(23)**

Four of the compounds found in the extract from test water with *C. quinquefasciatus* larvae were phenols. Compound **17** was also found in *C. quinquefasciatus* larvae volatiles.

4.4 Compounds Identified from the Extracts from Test Water

The test water used for bioassay was extracted using dichloromethane as described in section 5.2. 200 µl of organic portion was put in a vial in ice and dried by blowing nitrogen over it. The sample was diluted for GC-MS analysis using dichloromethane. Nine compounds were identified from the extract of test water (Table 4.3, Fig 4.29): internal standard (ethyl nonanoate) **(IS)**, 2,4-bis(1,1-dimethylethyl)phenol **(10)**, 4-(1,1-dimethylpropyl)phenol **(20)**, 6,10,14-trimethyl-2-pentadecanone **(24)**, 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenyl ethyl)phenol **(25)**, 2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol **(26)**, phytol **(27)**, 2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**, (all-E)-2,6,10,15,19,23-hexa methyl-2,6,10,14,18,22-tetracosahexaene **(23)** and 4-octyl-N-(4-octylphenyl)benzeneamine **(28)**.

Table 4.3. Compounds identified from the extract of test water

Compound	Retention time (Min)	Concentration (ng)
2,4-bis(1,1-dimethylethyl)phenol **(10)**	18.630	1.2169
4-(1,1-dimethylpropyl)phenol **(20)**	21.005	0.7736
6,10,14-trimethyl- 2-Pentadecanone **(24)**	22.348	0.9969
2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol **(25)***	24.275	0.9525
2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol **(26)***	24.723	1.5480
Phytol **(27)***	24.946	6.7742
2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**	28.396	1.4750
(all-E)- 2,6,10,15,19,23-hexamethyl- 2,6,10,14,18,22-Tetracosahexaene **(23)**	30.815	0.7984
4-octyl-N-(4-octylphenyl)benzenamine **(28)**	32.158	0.7134

*Compounds found only in test water but not in test water containing larvae

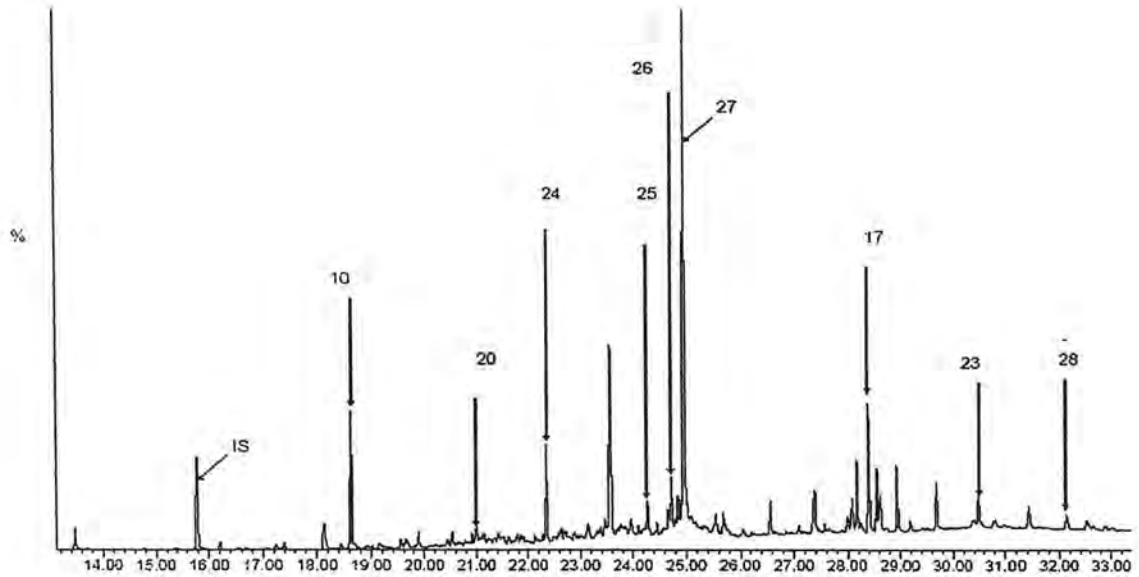
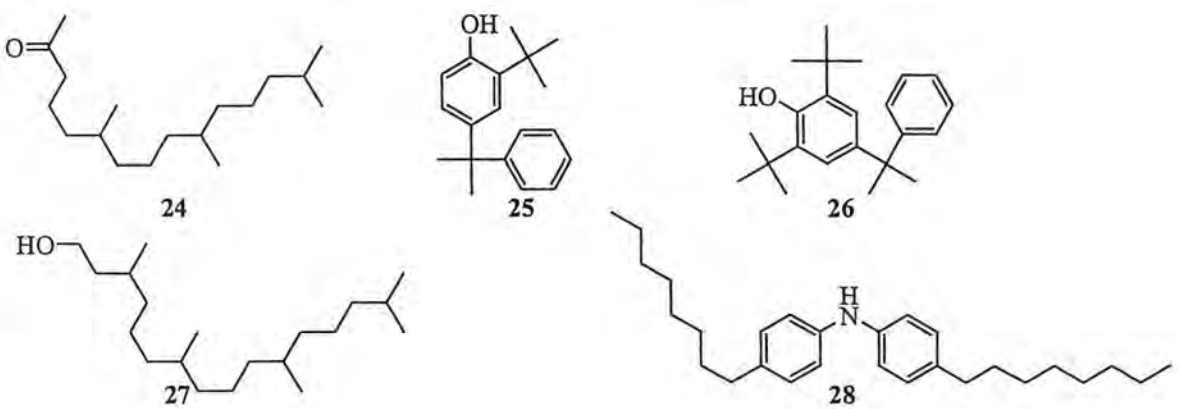


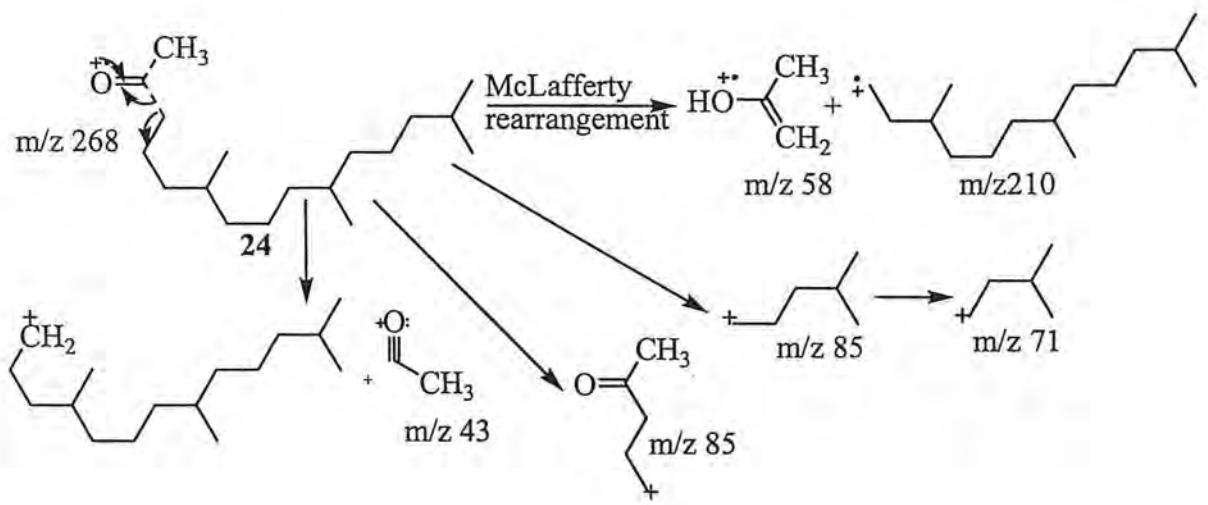
Figure 4.29. Gas chromatogram of extract of test water



4.4.1 6,10,14-trimethyl-2-pentadecanone (24)

The MS of **24** revealed the molecular ion M^+ peak at m/z 268 consistent with the molecular formula $C_{18}H_{36}O$. The peak at m/z 250 was ascribable to the fragment ion $C_{18}H_{34}^{\cdot+}$, being formed due to loss of water from the molecular ion. The α cleavage results in a loss of larger alkyl group giving a peak at m/z 43. The MS exhibited the base peak at m/z 58 and a peak at m/z 210 ascribable to $C_3H_6O^{\cdot+}$ and $C_{15}H_{30}^{\cdot+}$, respectively, being formed due to

McLafferty rearrangement (Fig. 4.30, Scheme 4.8.). The peak at m/z 85 can occur due to fragmentation ion $C_6H_{13}^+$ on the side of alkyl group or $C_5H_9O^+$ on the side of carbonyl group.



Scheme 4.8. Proposed fragmentation pattern of 6,10,14-trimethyl-2-pentadecanone **(24)**

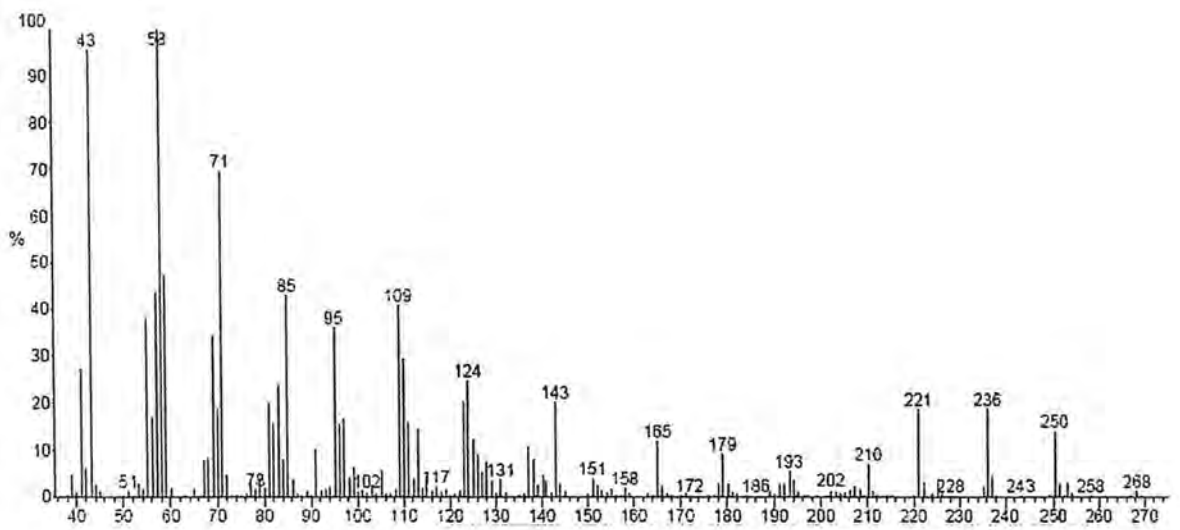


Figure 4.30. Mass Spectrum of 6,10,14-trimethyl-2-pentadecanone **(24)**

4.4.2 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol (25)

The mass spectrum of compound **25** (Fig. 4.31) gave a molecular ion peak at m/z 268 corresponding to molecular formula $C_{19}H_{24}O$. The loss of a methyl group gave the most prominent peak at m/z 253 corresponding to $C_{18}H_{21}O^+$. Loss of three methyl groups from the substitution on the phenol side gives a small peak at m/z 225 corresponding to the ion $C_{16}H_{17}O^+$. Peaks at m/z 119, 105 and 91 are ascribable to $C_9H_{11}^+$, $C_8H_9^+$ and $C_7H_7^+$, respectively.

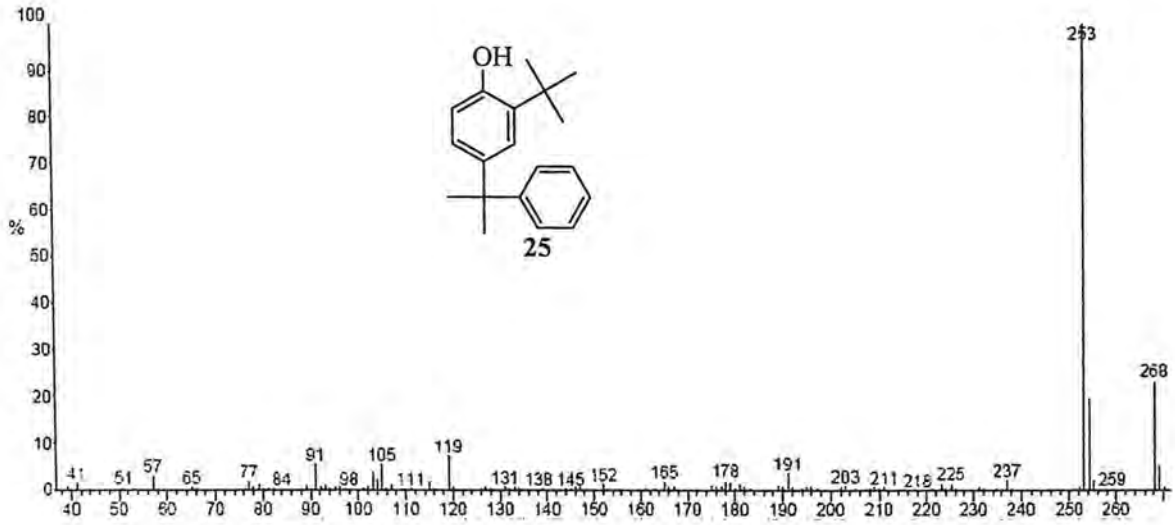


Figure 4.31. Mass Spectrum of 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol **(25)**

4.4.3 2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol (26)

The MS of compound **26** (Fig. 4.32) exhibited a molecular ion peak at m/z 324 which corresponds to molecular formula $C_{23}H_{32}O$. The base peak at m/z 309 was formed by the

loss of one of methyl groups from the substitution. Peaks at m/z 119 and 91 are ascribable to $C_9H_{11}^+$ and $C_7H_7^+$, respectively.

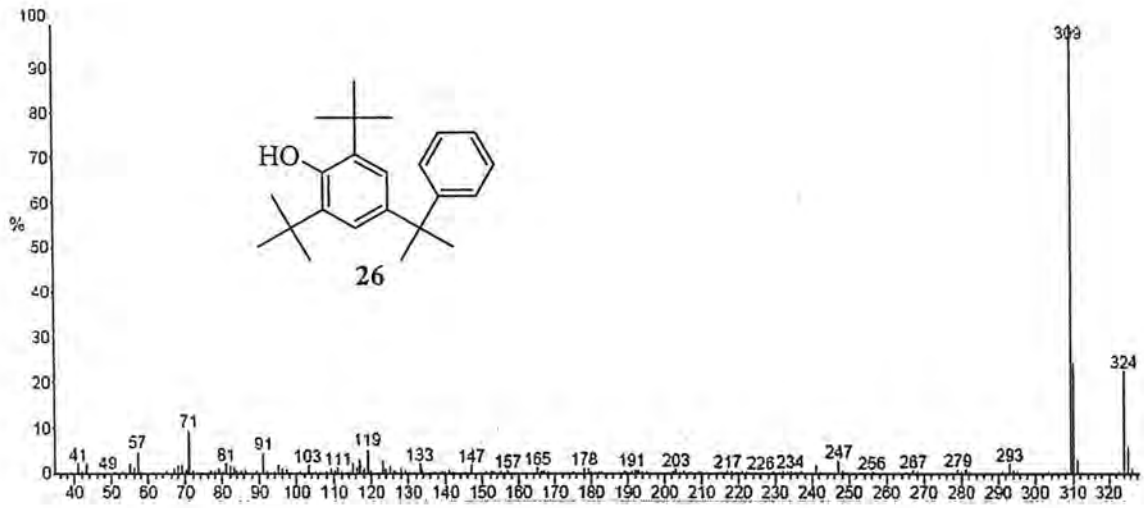


Figure 4.32. Mass Spectrum of 2,6-bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl) phenol **(26)**

4.4.4 Phytol (27)

In long chain primary alcohols (>C6) the fragmentation is dominated by the hydrocarbon pattern. The molecular ion peak of compound **27** at m/z 298 is missing and instead there is a weak M^+-2 peak at m/z 296 (Fig. 4.33) due to R-CH=O$^{\cdot+}$. The peak at m/z 85 is due to $C_6H_{13}^+$ while the base peak at m/z 71 is ascribed to $C_5H_{11}^+$. The peaks at m/z 57 and 43 were assigned to $C_4H_9^+$, $C_3H_7^+$, respectively.

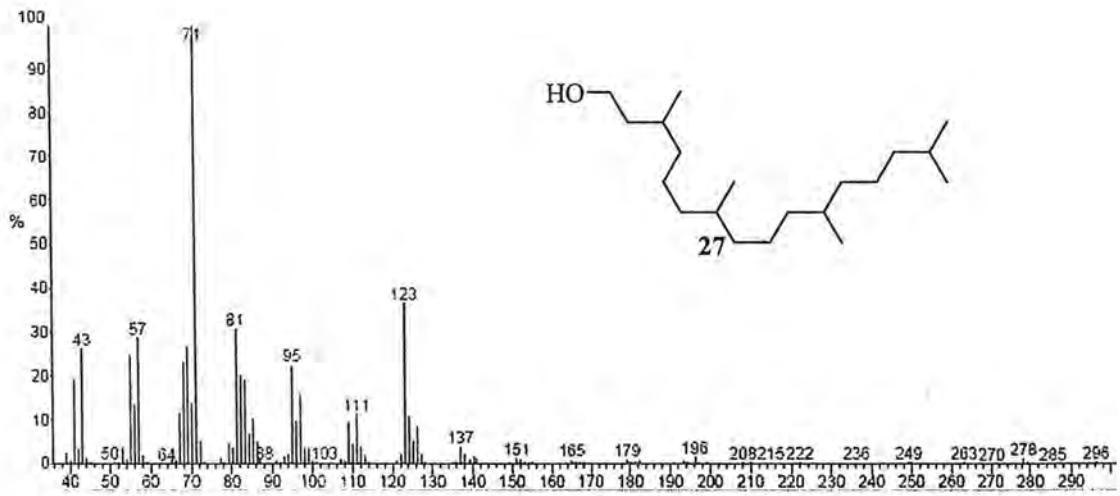
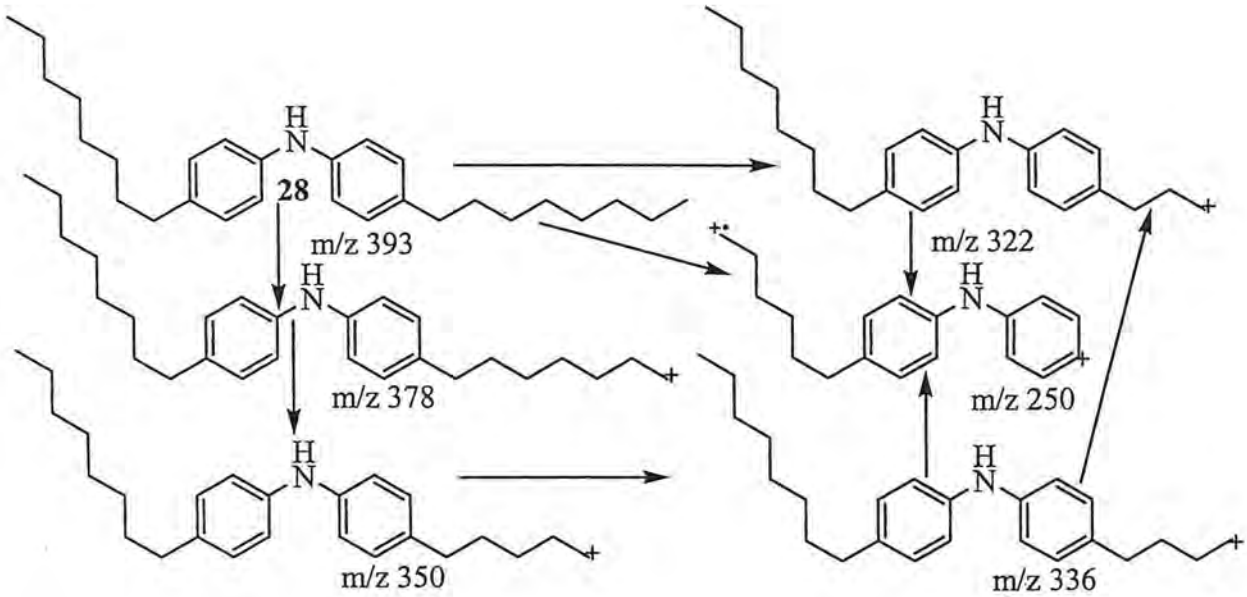


Figure 4.33. Mass Spectrum of Phytol **(27)**

4.4.5 4-octyl-N-(4-octylphenyl)benzenamine (28)

The mass spectrum of compound **28** (Fig. 4.34) exhibited a molecular ion peak at m/z 393 and was assigned to $C_{28}H_{43}N$. The loss of methyl group gives a low intense peak at m/z 378 due to the $C_{27}H_{40}N^+$ molecular ion. Further loss of a methyl group gave the peak at m/z 350 ascribable to $C_{25}H_{36}N^+$. Loss of butyl group (M^+-C_4H_9) from one side of octyl substitution gives a peak at 336 ascribed to $C_{24}H_{34}N^+$. The peak at m/z 250 ($C_{18}H_{20}N^+$) is due to loss of octyl group on one side and loss of ethyl group on the other side of octyl substitution (scheme 4.9).



Scheme 4.9. Proposed fragmentation pattern of 4-octyl-N-(4-octylphenyl)benzenamine **(28)**

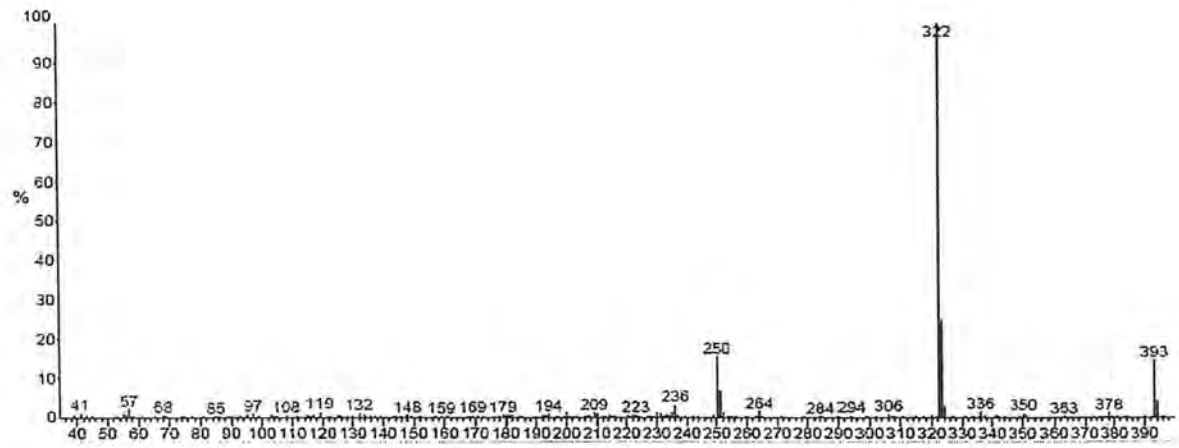


Figure 4.34. Mass Spectrum of 4-octyl-N-(4-octylphenyl)benzenamine **(28)**

Six of the compounds found in extract from test water were also present in test water with *C. quinquefasciatus* larvae, differing only in the amount in each sample. Compound **17** was also found in *C. quinquefasciatus* larvae volatiles, extract of test water with *C. quinquefasci atus* larvae and also in extract from test water.

4.5 Compounds Identified from the *An. gambiae* Egg Extract

Mosquito eggs, once placed on oviposition ponds by a gravid female mosquito, are vulnerable to attack by a variety of preditors. In addition eggs may also suffer from microbial diseases. Due to this it is postulated that insect eggs may possess certain characteristics that play a role in blunting the predation. These characteristics make the eggs less accessible to enemies (Monika and Torsten, 2002). The insects may also employ an oviposition deterrent pheromone on the egg which can obviate over-colonisation.

Seven compounds were identified from *An. gambiae* eggs extract (Table 4.4, Fig 4.35); internal standard (ethyl nonanoate) **(IS)**, tetradecanoic acid **(29)**, Z-11-hexadecenoic acid **(30)**, n-hexadecanoic acid **(31)**, (Z)-9-octadecenoic acid **(32)**, octadecanoic acid **(33)**, docosane **(34)**, (all-E)- 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene **(23)**. Other compounds found in trace amount were 3-hexanone, 4-hydroxy-4-methyl-2-pentanone, hexanoic acid, heptanol, 2-pentyl furan, nonanal, 2,6,6,6-tetramethyl-4-piperidone, 2-methyl-2-octen-4-one, 3-octanol, nonanoic acid, 4-hydroxy-benzaldehyde, cyclodecane, and dodecanoic acid.

Table 4.4. Compounds identified from extract of *An. gambiae* egg

Compound	Retention time (Min)	Concentration (ng)
Tetradecanoic acid **(29)**	21.391	1.9151
Z-11-hexadecenoic acid, **(30)**	23.293	2.3521
n-hexadecanoic acid **(31)**	23.476	4.3990
(Z)- 9-octadecenoic acid, **(32)**	25.136	2.5396
Octadecanoic acid **(33)**	25.325	1.6099
Docosane **(34)**	25.657	1.8020
(all-E)- 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene **(23)**	30.776	3.9031

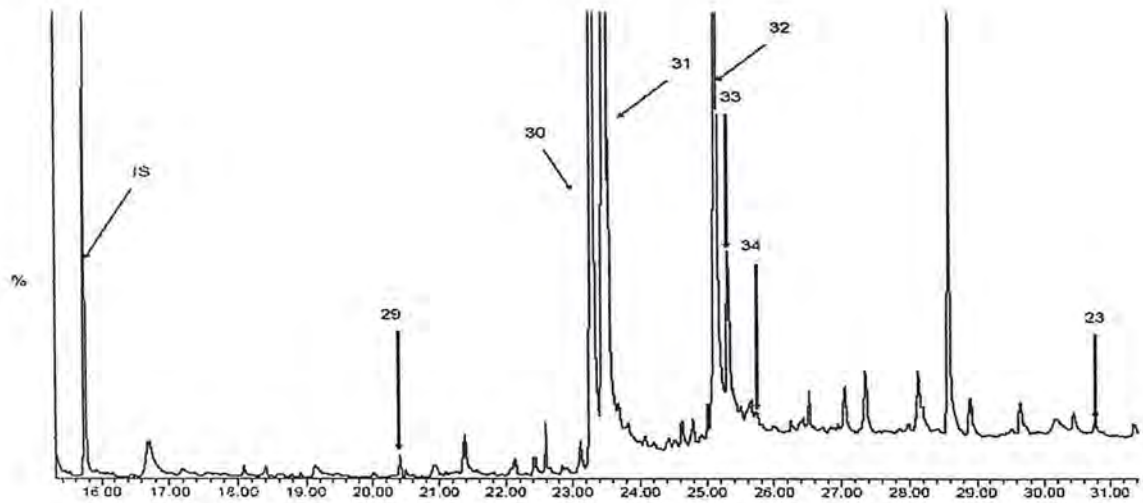
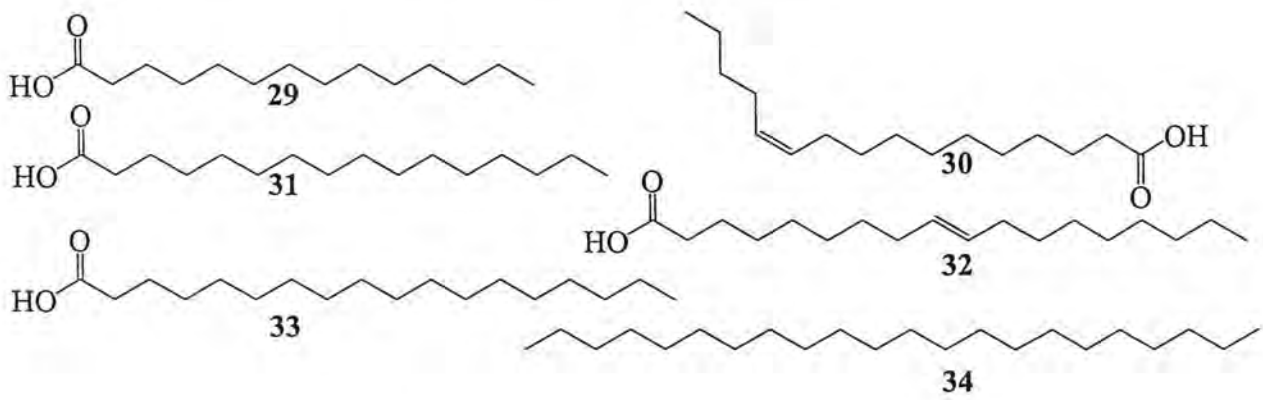


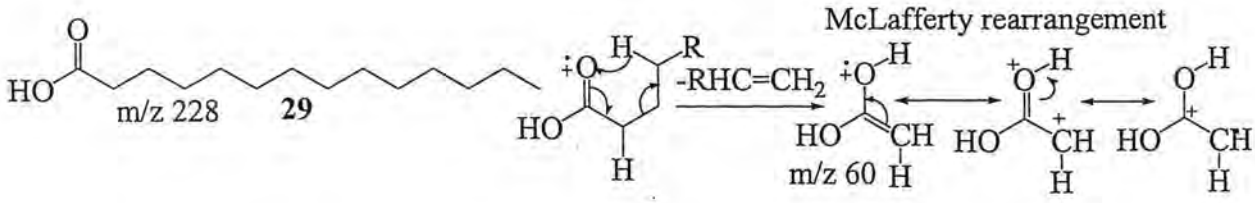
Figure 4.35. Gas chromatogram of extract from *An. gambiae* eggs



4.5.1 Tetradecanoic acid (29)

The most characteristic peak of a straight chain monocarboxylic acid results from McLafferty rearrangement (Fig. 4.36, Scheme 4.10). In long chain acids, spectrum consists of two series of peaks resulting from cleavage at each C-C bond with retention of charge either on the oxygen-containing fragment (m/z 45, 59, 73, 87,.....) or on the alkyl fragment (m/z 29, 43, 57, 71, 85,.....). Besides McLafferty rearrangement peaks, the spectrum of a long chain acid resembles the series of hydrocarbon clusters (Silverstein *et. al.*, 2005).

The molecular ion peak of compound **29** was at m/z 228 corresponding to the molecular formula $C_{14}H_{28}O_2$. The most prominent peak followed the fragmentation pattern of $C_nH_{2n-1}O_2^{+\cdot}$. The base peak at m/z 73 was ascribed to $C_3H_5O_2^{+\cdot}$. The peaks due to McLafferty rearrangement at m/z 60. 115, 129, 143, 157, 171, 185 and 199 are ascribable to $C_6H_{11}O_2^{+\cdot}$, $C_7H_{13}O_2^{+\cdot}$, $C_8H_{15}O_2^{+\cdot}$, $C_9H_{17}O_2^{+\cdot}$, $C_{10}H_{19}O_2^{+\cdot}$, $C_{11}H_{21}O_2^{+\cdot}$, $C_{12}H_{23}O_2^{+\cdot}$, respectively.



Scheme 4.10. McLafferty rearrangement of straight chain monocarboxylic acid (Silverstein et. al., 2005)

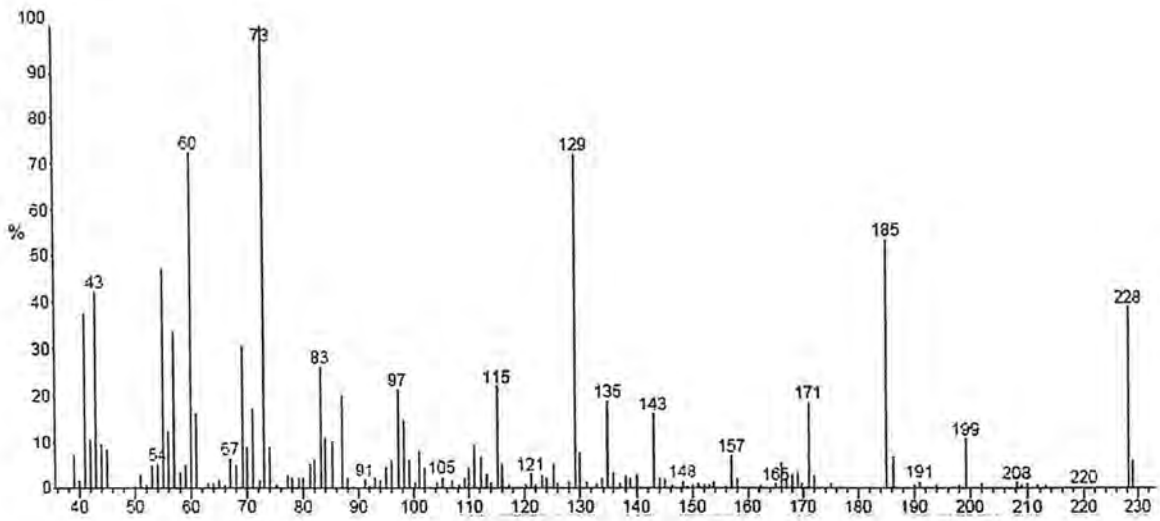


Figure 4.36. Mass Spectrum of tetradecanoic acid **(29)**

4.5.2 Z-11-hexadecenoic acid (30)

The MS for compound **30** (Fig. 4.37) exhibited a molecular ion peak at m/z 254 corresponding to $C_{16}H_{30}O_2$. Loss of water gave a peak at m/z 236. The base peak was due to fragmentation near a double bond at m/z 55 ($C_4H_7^{+\cdot}$). The alkyl fragment gave the most prominent peaks at m/z 69, 83, 97 and 111 corresponding to $C_5H_9^{+\cdot}$, $C_6H_{11}^{+\cdot}$, $C_7H_{13}^{+\cdot}$ and $C_8H_{15}^{+\cdot}$, respectively.

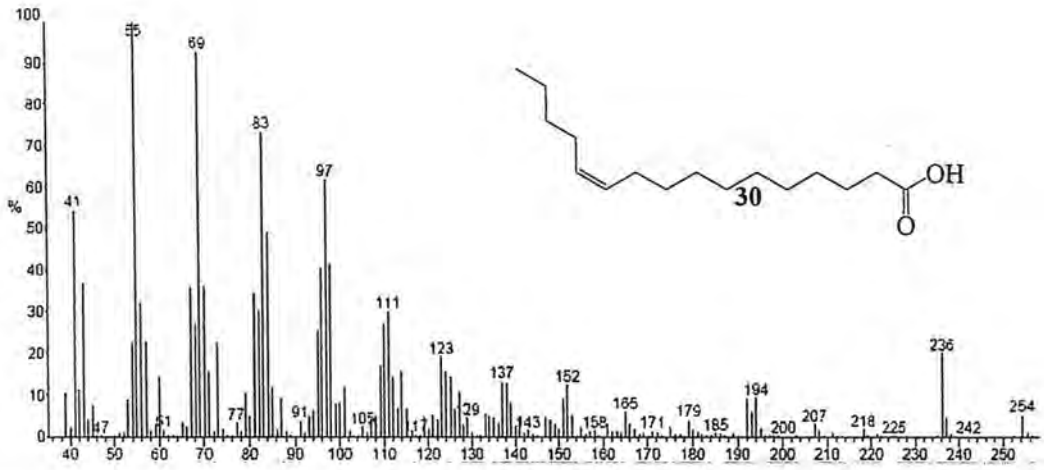


Figure 4.37. Mass Spectrum of Z-11-hexadecenoic acid **(30)**

4.5.3 n-Hexadecanoic acid (Palmitic acid) (31)

The mass spectrum of compound **31** (Fig. 4.38) revealed the molecular ion, M^+, peak at m/z 256 corresponding to $C_{16}H_{32}O_2$. The peak at m/z 60 was due to McLafferty rearrangement as shown in section 4.5.1. The peaks at m/z 73, 115, 129, 143, 157, 171, 185, 199, 213 and 227 follows fragmentation pattern of $C_nH_{2n-1}O_2^{+\cdot}$, corresponding to $C_3H_5O_2^{+\cdot}$, $C_6H_{11}O_2^{+\cdot}$, $C_7H_{13}O_2^{+\cdot}$, $C_8H_{15}O_2^{+\cdot}$, $C_9H_{17}O_2^{+\cdot}$, $C_{10}H_{19}O_2^{+\cdot}$, $C_{11}H_{21}O_2^{+\cdot}$, $C_{12}H_{23}O_2^{+\cdot}$, $C_{13}H_{25}O_2^{+\cdot}$ and $C_{14}H_{27}O_2^{+\cdot}$, respectively.

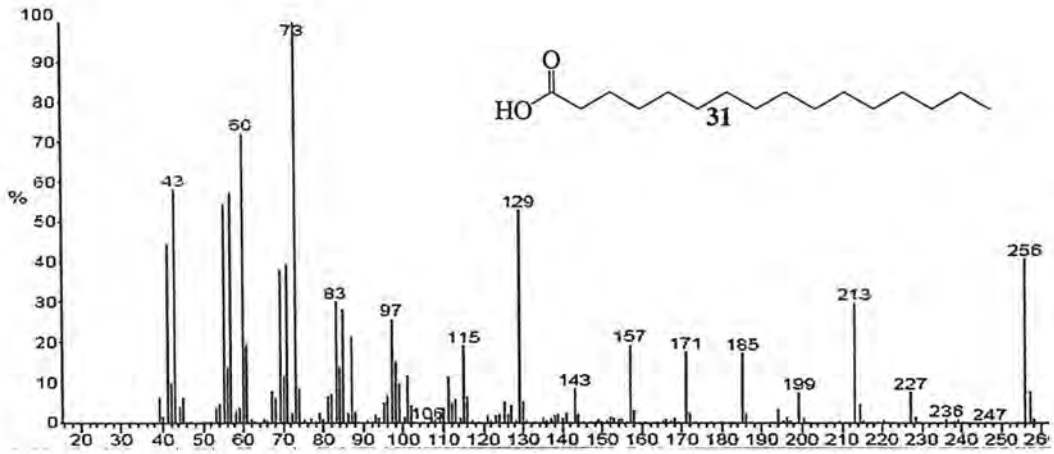


Figure 4.38. Mass Spectrum of n-Hexadecanoic acid (Palmitic acid) **(31)**

4.5.4 (Z)-9-Octadecenoic acid (32)

The MS profile of compound **32** (Fig. 4.39) exhibited a molecular ion peak at m/z 282 corresponding to $C_{18}H_{34}O_2$. Loss of water gave a peak at m/z 264. The base peak was due to fragmentation near a double bond at m/z 55 ascribed to $C_4H_7^{+\cdot}$. The alkyl fragment gave the most prominent peaks at m/z 69, 83, 97 and 111 corresponding to $C_5H_9^{+\cdot}$, $C_6H_{11}^{+\cdot}$, $C_7H_{13}^{+\cdot}$ and $C_8H_{15}^{+\cdot}$, respectively.

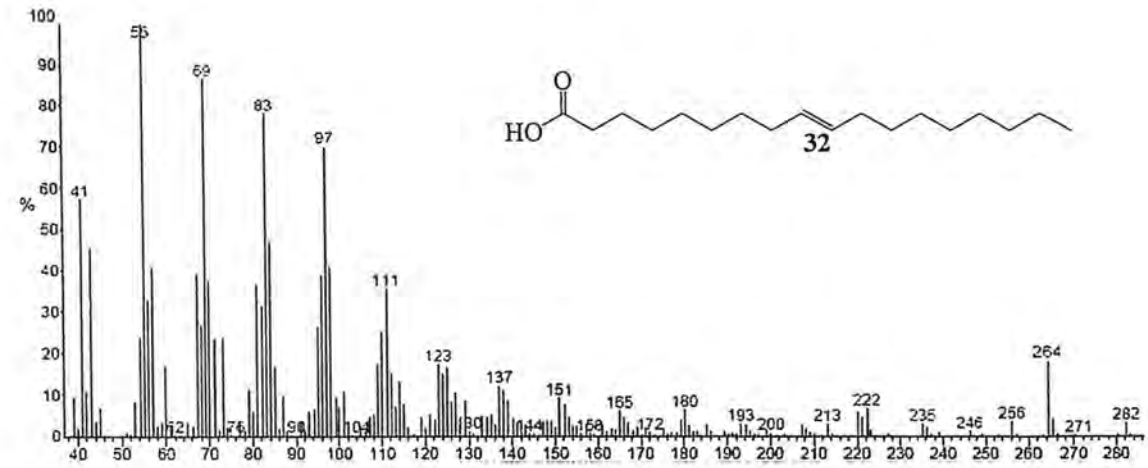


Figure 4.39. Mass Spectrum of (Z)-9-octadecenoic acid **(32)**

4.5.5 Octadecanoic acid (33)

The molecular ion peak of compound **33** occurred at m/z 284 corresponding to the molecular formula $C_{18}H_{36}O_2$. The most prominent peaks follow fragmentation pattern of $C_nH_{2n-1}O_2^{+\cdot}$ (Fig. 4.40). The base peak m/z 73 is ascribable to $C_3H_5O_2^{+\cdot}$.

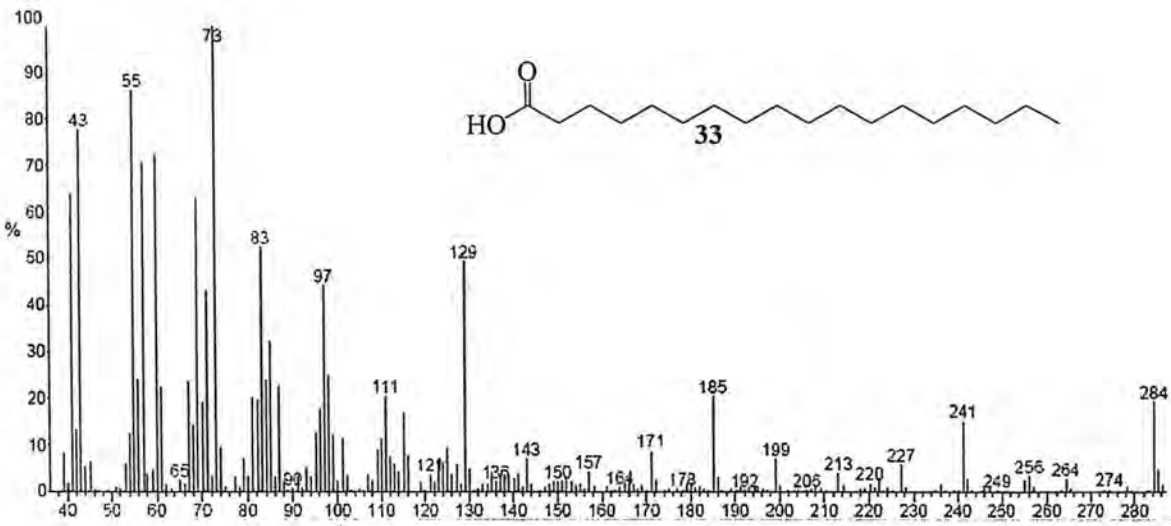


Figure 4.40. Mass Spectra of octadecanoic acid **(33)**

4.5.6 Docosane (34)

The molecular ion peak of compound **34** was at m/z 310 (Fig. 4.41) corresponding to the formula $C_{22}H_{46}$. **34** was identified as a hydrocarbon due to its fragmentation pattern characterized by clusters of peaks with corresponding clusters being 14 mass units (CH_2) apart. The base peak m/z 57 was due to $C_4H_9^+$, with peaks at m/z 43 and 71 and being due to $C_3H_9^+$ and $C_5H_{11}^+$, respectively.

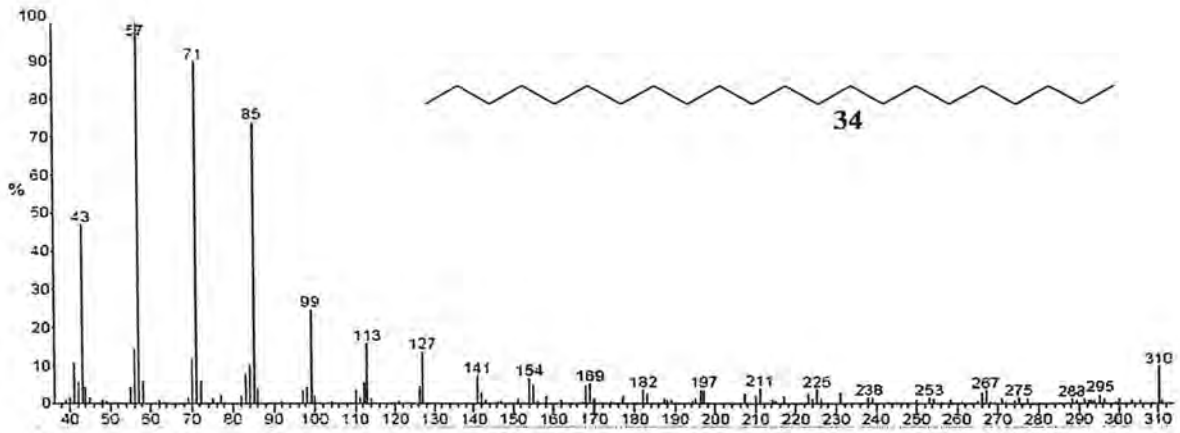


Figure 4.41. Mass Spectrum of docosane **(34)**

Most of the compounds found in the extract of the *An. gambiae* eggs were unsaturated and saturated fatty acids. The small fatty acids like heptanoic, nonanoic and dodecanoic acid were found in trace amounts. Compound **23** was also found in the extract from test water with *C. quinquefasciatus* larvae and also in extract from test water, but in differing amounts.

4.6 Compounds Identified from Extract from *C. quinquefasciatus* Egg Rafts

The egg rafts were extracted as described at section 3.10.3. The compounds internal standard (ethyl nonanoate) **(IS)**, Z-11- hexadecenoic acid **(30)**, n-hexadecanoic acid **(31)**, (Z)-9-octadecenoic acid **(32)**, octadecanoic acid **(33)**, N-butyl-4,9-decadien-2-amine **(35)**, arachidonic acid **(36)** and 1,2,3-propanetriyl ester hexanoic acid **(37)** were isolated in large amounts (Table 4.5 and Fig 4.42). Other compounds found in trace amount were sorbic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, undecanoic, dodecanoic, tetradecanoic acids and N-ethyl benzeneamine,.

Table 4.5. Compounds identified from extracts from *C. quinquefasciatus* egg rafts

Compound	Retention time (Min)	Concentration (ng)
Z-11-hexadecenoic acid **(30)***	23.424	129.9146
n-hexadecanoic acid **(31)***	23.603	103.9436
9-octadecenoic acid, (Z)- **(32)***	25.238	78.1937
octadecanoic acid **(33)***	25.395	44.2459
N-butyl-4,9-Decadien-2-amine **(35)**	26.470	20.5795
arachidonic acid **(36)**	26.649	21.0557
1,2,3-propanetriyl ester hexanoic acid **(37)**	27.545	35.0957

* Both in *Culex quinquefasciatus* egg rafts and *Anopheles gambiae* egg.

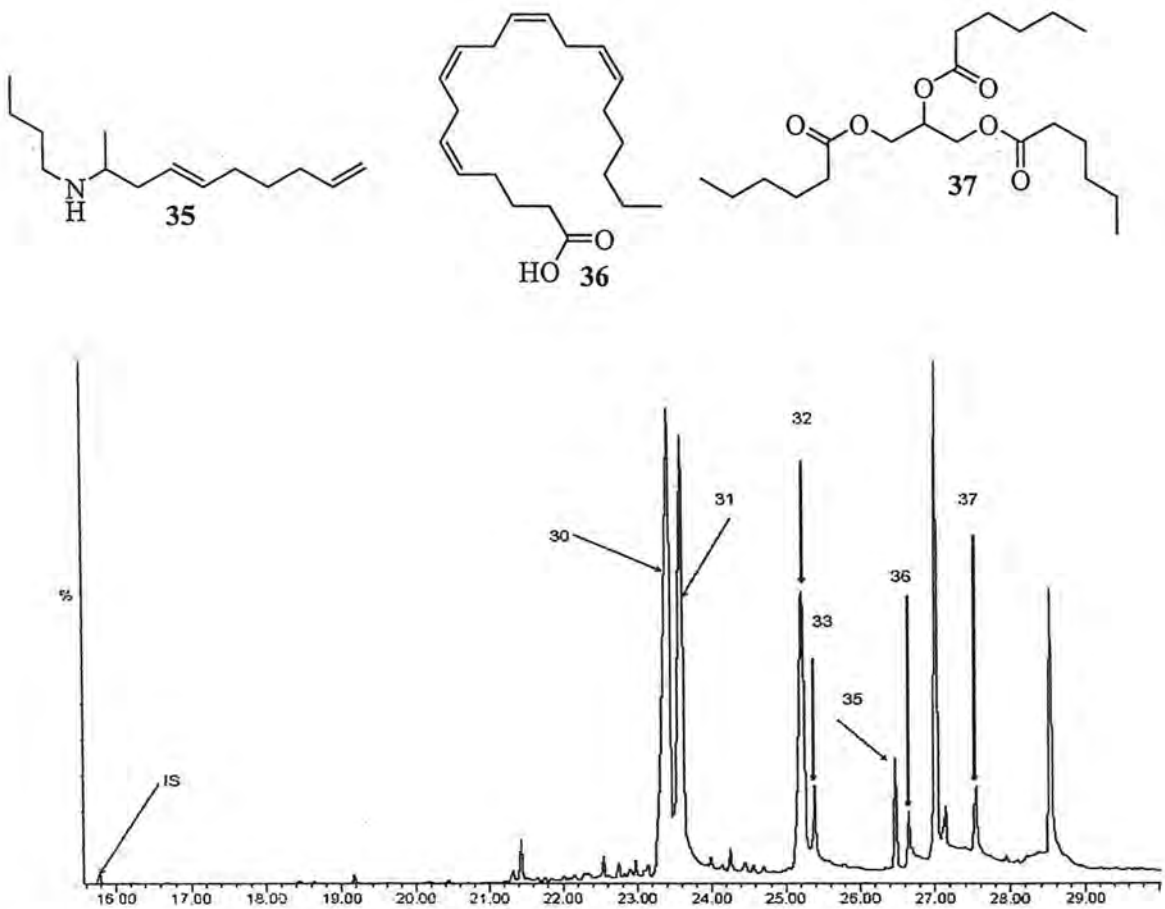
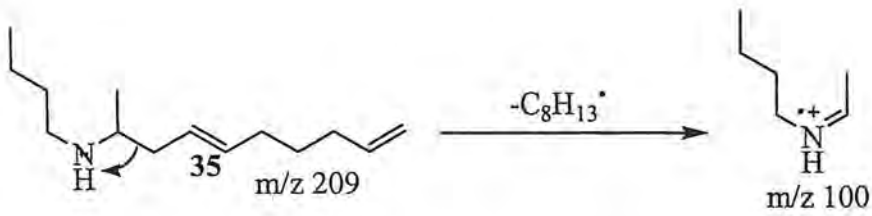


Figure 4.42. Gas chromatogram of extract from *C. quinquefasciatus* egg rafts

4.6.1 N-butyl-4,9-decadien-2-amine (35)

The MS profile of compound **35** (Fig. 4.43) exhibited a molecular ion peak at m/z 209 corresponding to molecular formula $C_{14}H_{27}N$. The base peak at m/z 100 was due to fragmentation at methyl substitution losing the long substitution and is ascribed to $C_6H_{14}N^{+\cdot}$ (Scheme 4.11). The peaks at m/z 55 and 43 were due to $C_4H_7^+$ and $C_3H_7^+$, respectively.



Scheme 4.11. Proposed formation of base peak at m/z 100

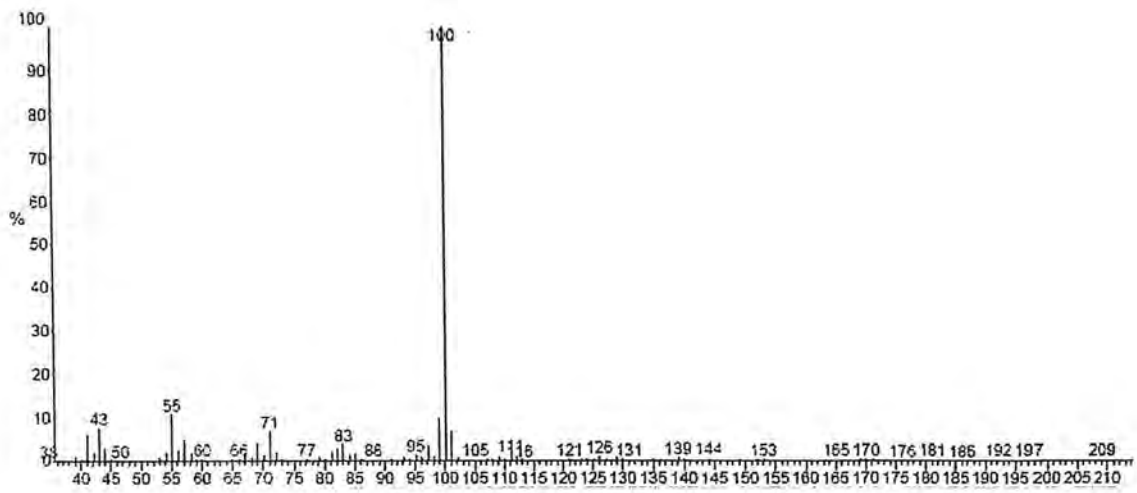


Figure 4.43. Mass Spectrum of N-butyl-4,9-decadien-2-amine **(35)**

4.6.2 Arachidonic acid (36)

The molecular ion peak of compound **36** occurred at m/z 304 corresponding to the molecular formula $C_{20}H_{32}O_2$. The peak at m/z 217, 177 and 150 (Fig. 4.44) correspond to $C_{16}H_{25}^+$, $C_{13}H_{21}^+$ and $C_{11}H_{18}^+$, respectively. The McLafferty rearrangement of compound **36** gave a peak at m/z 60 corresponding to $C_2H_4O_2^{+\cdot}$. After the McLafferty rearrangement the compound undergoes further fragmentation to give a peak at m/z 79 and 91 corresponding to $C_6H_7^{+\cdot}$ and $C_7H_7^{+\cdot}$, respectively.

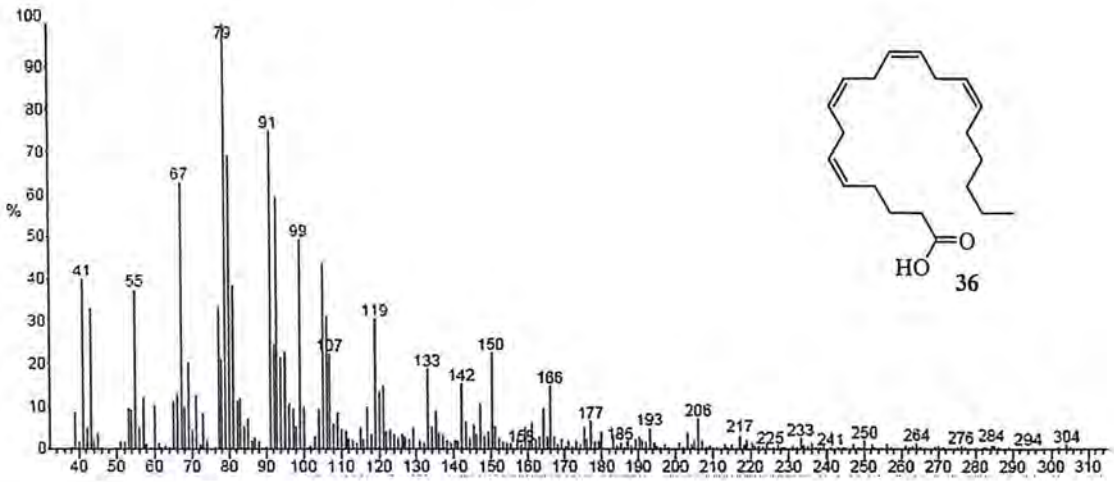
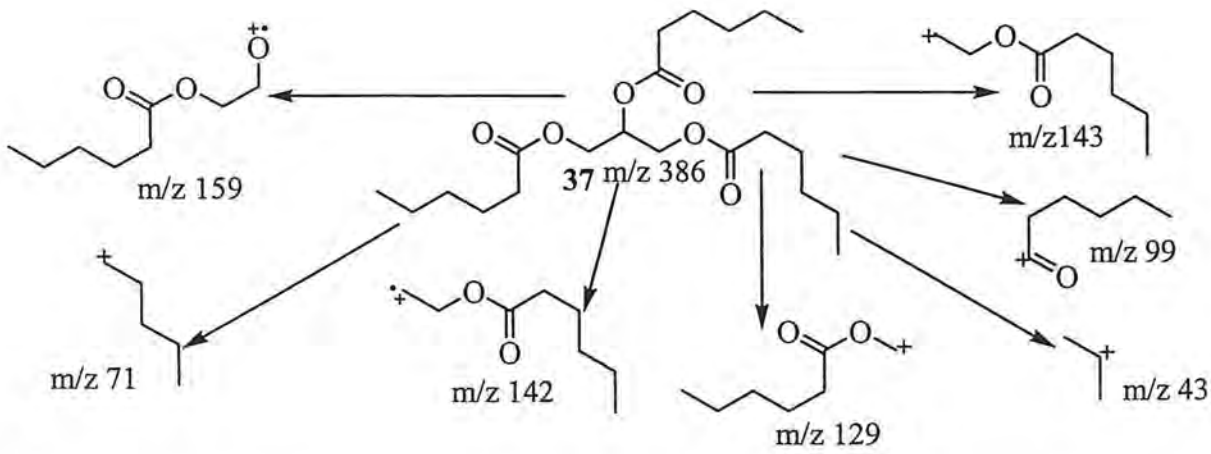


Figure 4.44. Mass Spectrum of arachidonic acid **(36)**

4.6.3 1,2,3-propanetriyl ester hexanoic acid (37)

The mass spectrum of compound **37** showed a molecular ion peak at m/z 386 corresponding to $C_{21}H_{38}O_6$. The base peak at m/z 99 was due to $C_6H_{11}O^+$ (Fig. 4.45, Scheme 4.12), while the peaks at m/z 159, 143, 142, 129, 71 and 43 corresponded to $C_8H_{15}O_3^{+\cdot}$, $C_8H_{15}O_2^+$, $C_8H_{15}O_2^{+\cdot}$, $C_7H_{13}O_2^+$, $C_5H_{11}^+$ and $C_3H_7^+$, respectively.



Scheme 4.12. Proposed fragmentation pattern of 1,2,3-propanetriyl ester hexanoic acid **(37)**

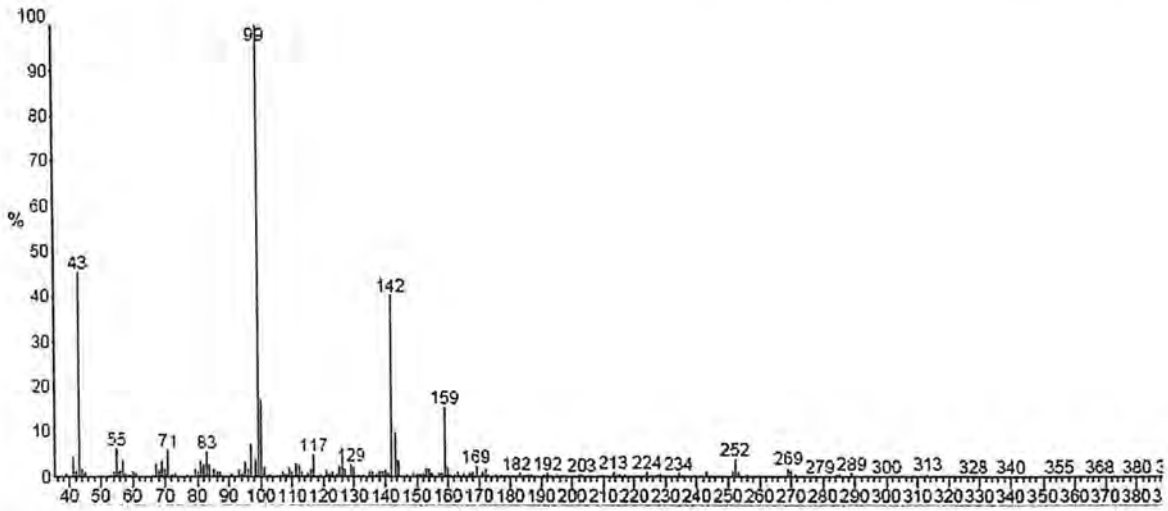


Figure 4.45. Mass Spectrum of 1,2,3-propanetriyl ester hexanoic acid **(37)**

The unsaturated and saturated fatty acids present in the extract of *C. quinquefasciatus* egg rafts were the same as those from extract of *An. gambiae* eggs.

4.7 Compounds Identified from Soil Volatiles

As discussed in Section 2.2.4, microbial activity plays a role in oviposition behaviour of gravid *An. gambiae.* In search of these compounds that cause effect to oviposition behaviour we trapped volatiles from the muddy soil obtained from Mbita. The trapping was done as discussed in section 3.10. Eleven compounds were identified (Table 4.6, Fig 4.46); d-limonene **(38)**, internal standard (ethyl nonanoate) **(IS)**, [3aR-(3a.alpha.,4.beta.,7.alpha.)] -2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-3H-3a,7-methano azulene **(39)**, 2,6-bis(1-methy lethyl)benzenamine **(40)**, [1S-(1.alpha.,7.alpha.,8a. alpha.)]-1,2,3,5,6,7,8,8a-octahydro-1,8a -dimethyl-7-(1-methylethenyl)naphthalene **(41)**, (1S-cis)-1,2,3,4-tetrahydro-1,6-dimethyl-4 -(1-methylethyl)naphthalene **(42)**, [R-[R*,R*(E)]]-3,7,11,15-tetramethyl-2-hexadecene **(43)**, 3,7,11,15-tetramethyl-2-hexadecen-1-ol **(44)**, (1-methyldodecyl)benzene **(45)**, 2[(4-hydroxy phenyl)methyl]phenol **(21)**, 2-phenyl-2-(phenylmethyl)-1,3-dioxolane **(46)** and 2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**.

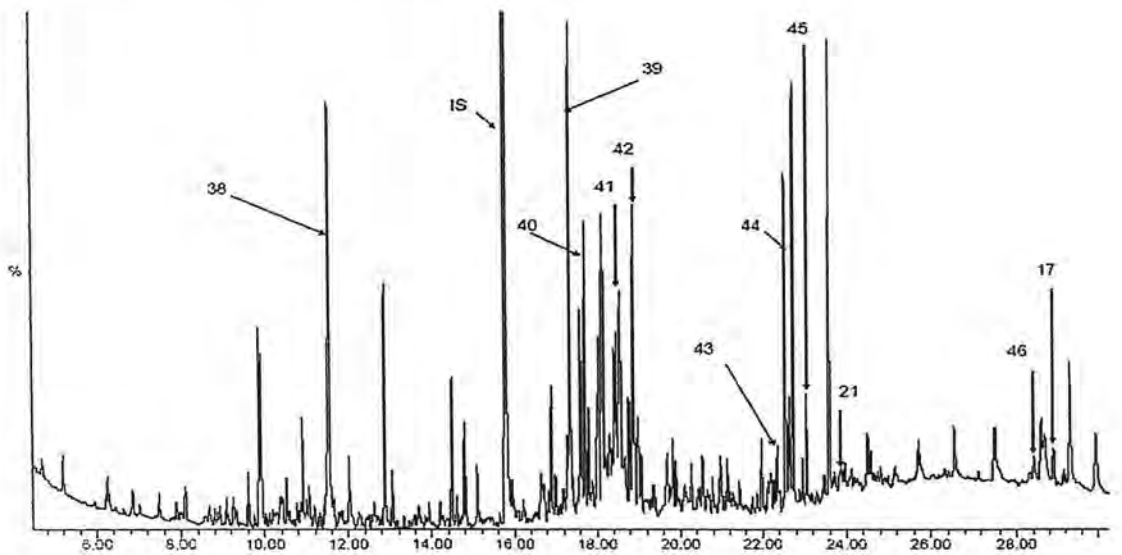
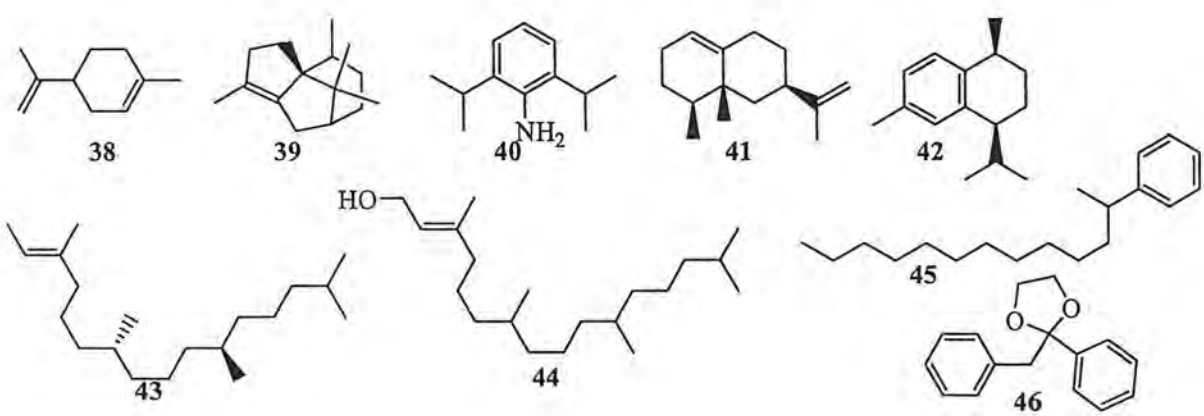


Figure 4.46. Gas chromatogram of soil volatiles

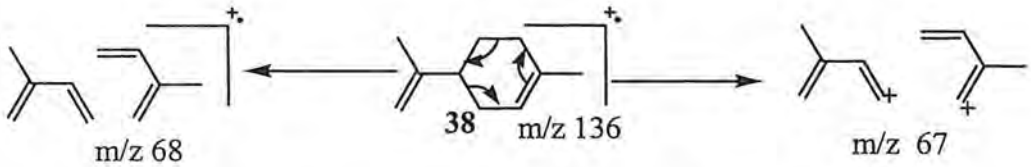
Table 4.6. Compounds identified from soil volatiles

Compound	Retention time (Min)	Concentration (ng)
D-Limonene **(38)**	11.553	
[3aR-(3a.alpha.,4.beta.,7.alpha.)]-2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-3H-3a,7-Methanoazulene **(39)**	17.354	0.1889
2,6-bis(1-methylethyl)benzenamine, **(40)**	17.712	0.2067
[1S-(1.alpha.,7.alpha.,8a.alpha.)]-1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)naphthalene **(41)**	18.541	0.3012
(1S-cis)-1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)naphthalene **(42)**	18.877	0.2510
[R-[R*,R*-(E)]]-3,7,11,15-tetramethyl-2-hexadecene **(43)**	22.348	0.1821
3,7,11,15-tetramethyl-2-hexadecen-1-ol **(44)**	22.550	0.3176
(1-methyldodecyl)benzene **(45)**	23.043	0.2007
2[(4-hydroxy phenyl)methyl] phenol **(21)**	23.967	0.1415
2-phenyl-2-(phenylmethyl)-1,3-dioxolane **(46)**	28.418	0.2396
2,4-bis(1-methyl-1-phenylethyl)phenol **(17)**	28.908	0.1886



4.7.1 D-limonene (38)

The mass spectrum of compound **38** (Fig 4.47) exhibited a molecular ion peak at m/z 136 corresponding to molecular formula $C_{10}H_{16}$. This compound undergoes a retro Diels Alder reaction to give a base peak at m/z 68 ($C_5H_8^{+\cdot}$) and another strong peak at m/z 67 corresponding to $C_5H_7^+$ (Scheme 4.13). The peaks at m/z 121, 107, 93 and 79 corresponded to $C_9H_{13}^+$, $C_8H_{11}^+$, $C_7H_9^+$ and $C_6H_7^+$, respectively.



Scheme 4.13. Proposed formation of base peak at m/z 68

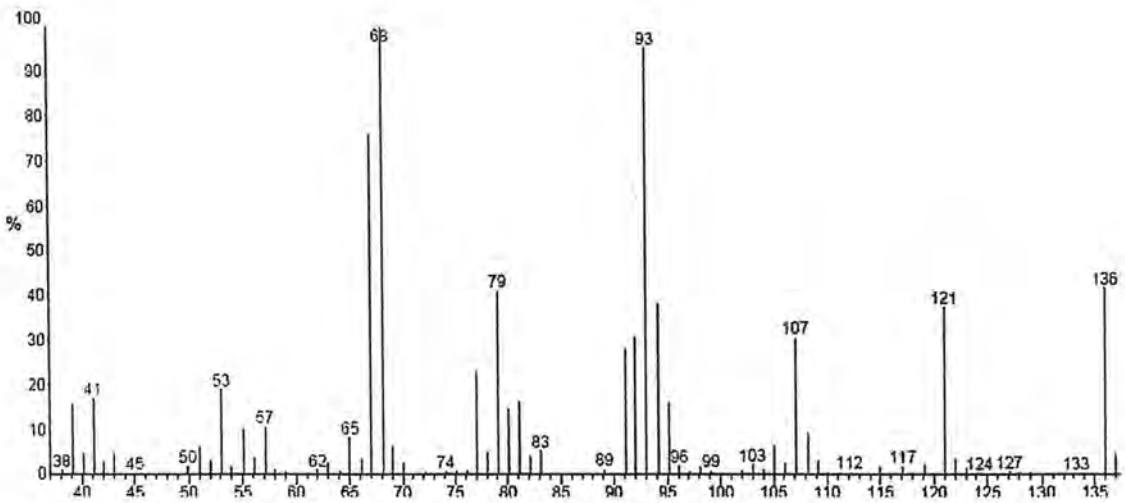


Figure 4.47. Mass Spectrum of D-limonene **(38)**

4.7.2 [3aR-(3a.alpha.,4.beta.,7.alpha.)]-2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-3H-3a,7-methanoazulene (39)

The mass spectrum of compound **39** (Fig. 4.48) revealed molecular ion M^+ peak which was also the base peak at m/z 204 corresponding to $C_{15}H_{24}$. The loss of a methyl group gave a peak at m/z 189 corresponding to $C_{14}H_{21}^+$. The fragmentation pattern follows the one for hydrocarbon with 14 mass units (CH_2) apart. The peaks at 175, 161, 147, 133 correspond to $C_{13}H_{19}^+$, $C_{12}H_{17}^+$, $C_{11}H_{15}^+$, $C_{10}H_{13}^+$, respectively.

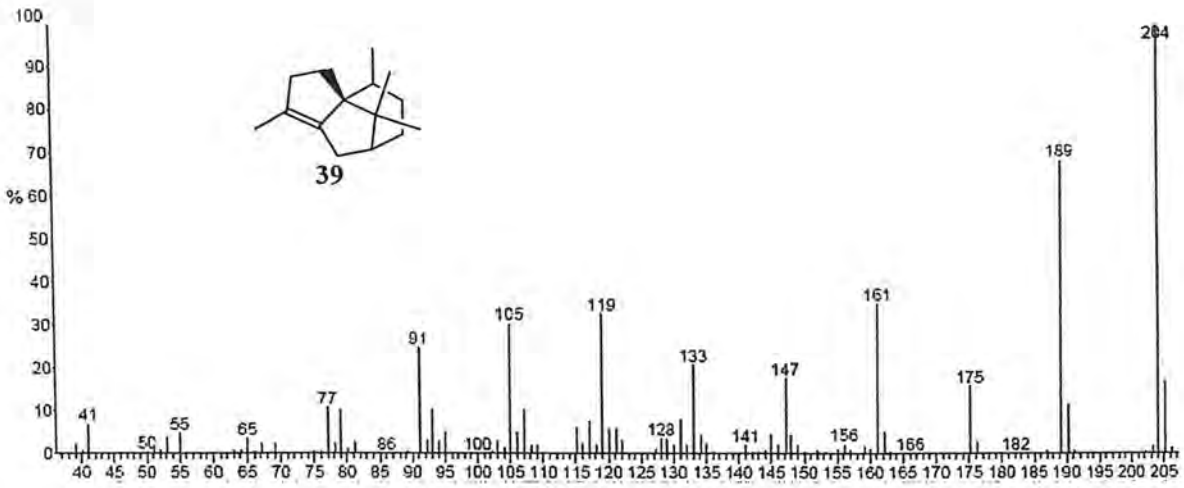


Figure 4.48. Mass Spectrum of [3aR-(3a.alpha.,4.beta.,7.alpha.)]-2,4,5,6,7,8-hexahydro-1,4,9,9-tetramethyl-3H-3a,7-methanoazulene **(39)**

4.7.3 2,6-bis(1-methylethyl)benzenamine (40)

The molecular ion peak of compound **40** at m/z 177 corresponded to molecular formula $C_{12}H_{19}N$ (Fig. 4.49). Loss of a methyl gave a base peak at m/z 162 ($C_{11}H_{16}N^+$). The peaks at m/z 120 and 91 corresponded to the ions $C_8H_{10}N^+$ and $C_6H_5N^+$, respectively.

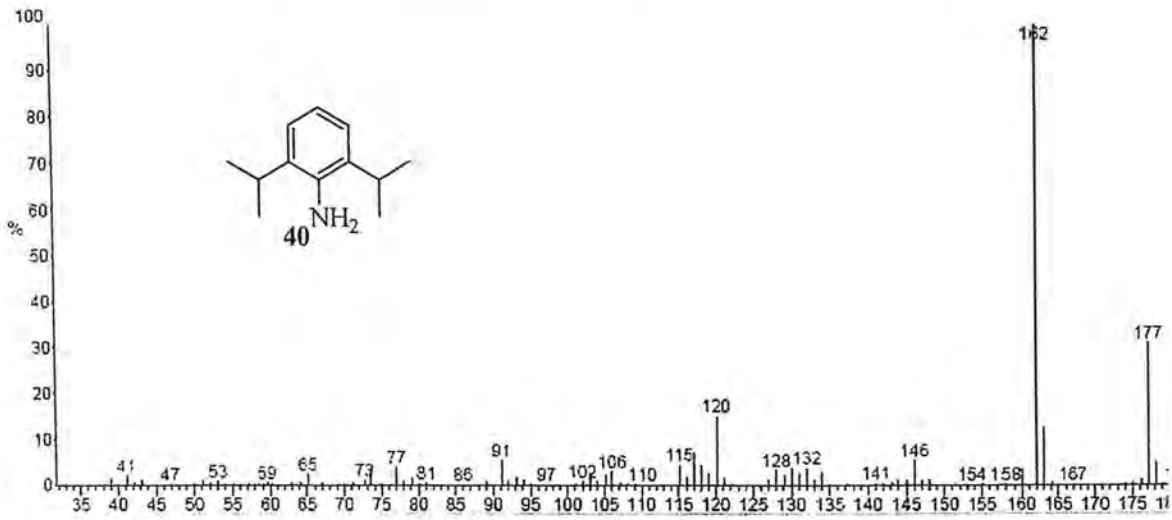


Figure 4.49. Mass Spectrum of 2,6-bis(1-methylethyl)benzenamine **(40)**

4.7.4 [1S-(1.alpha.,7.alpha.,8a.alpha.)]-1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)naphthalene (41)

The mass spectum of compound **41** exhibited a molecular ion peak at m/z 204 (Fig. 4.50) corresponding to molecular formula $C_{15}H_{24}$. The base peak at m/z 93 corresponding to formula $C_7H_9^+$ was formed by isomerization followed by cleavage. The second prominent peak at m/z 161 was due to $C_{12}H_{17}^+$. Peaks at m/z 189, 175, 133, 81 and 67 corresponded to $C_{14}H_{21}^+$, $C_{13}H_{19}^+$, $C_{10}H_{13}^+$, $C_6H_9^+$ and $C_5H_7^+$, respectively.

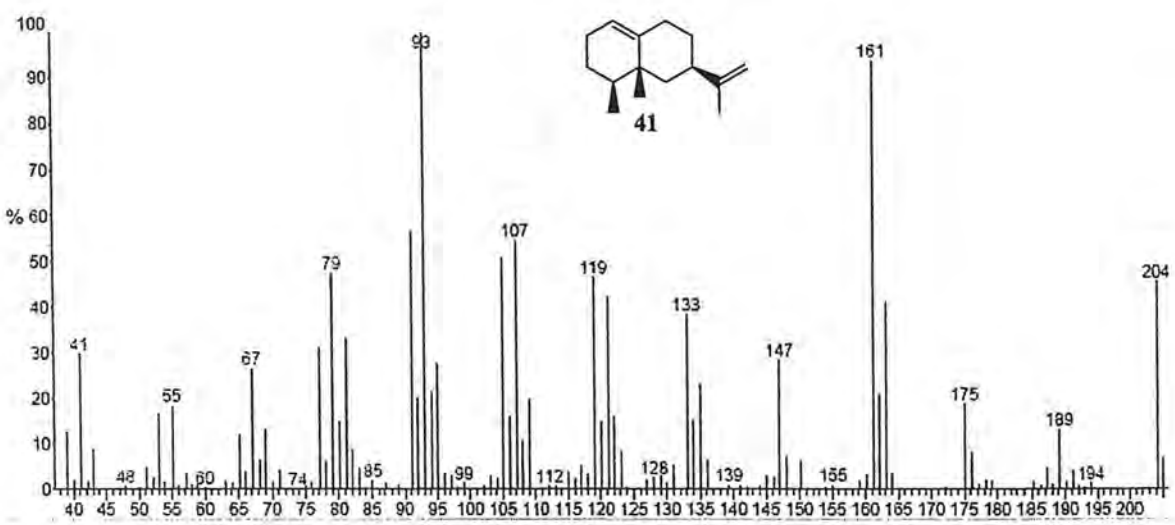


Figure 4.50. Mass Spectrum of [1S-(1.alpha.,7.alpha.,8a.alpha.)]-1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)naphthalene **(41)**

4.7.5 (1S-cis)-1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)naphthalene (42)

Mass spectrum of compound **42** (Fig. 4.51) showed a molecular ion peak at m/z 202 correponding to molecular formula $C_{15}H_{22}$. Loss of 1-methylethyl substitution gives a base peak at m/z 159 corresponding to molecular formula $C_{12}H_{15}^+$. The peaks at m/z 144 and 129 were due to $C_{11}H_{12}^{+\cdot}$ and $C_{10}H_9^{+\cdot}$, respectively.

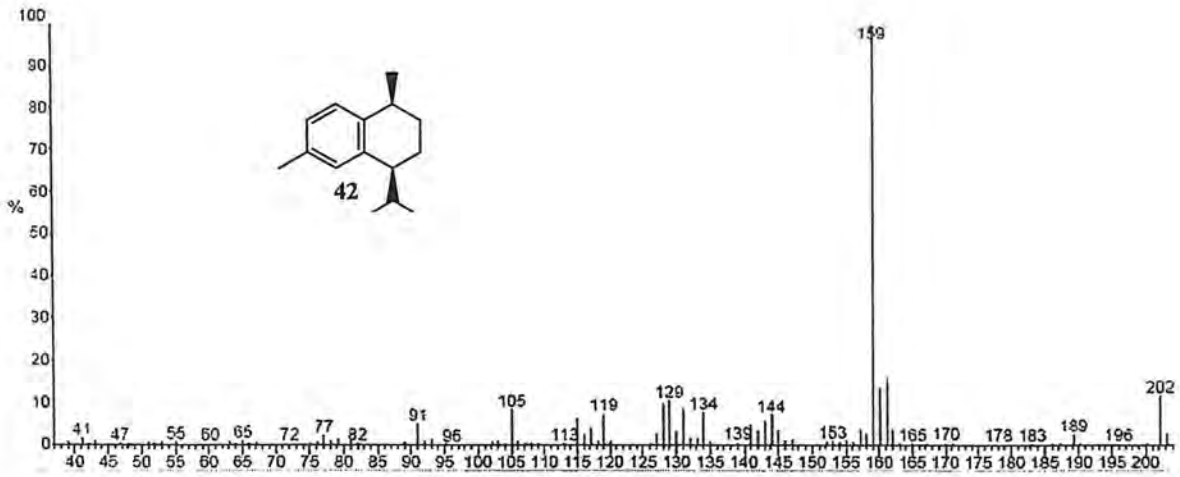


Figure 4.51. Mass Spectrum of compound **42**

4.7.6 [R-[R*,R*-(E)]]-3,7,11,15-tetramethyl-2-hexadecene (43)

The MS of compound **43** gave a molecular ion peak at m/z 280 corresponding to molecular formula $C_{20}H_{40}$ (Fig. 4.52). The base peak at m/z 70 was ascribed to $C_5H_{10}^{\cdot+}$. The peaks at m/z 140, 125, 111, 97, 83, 69, 57 and 43 were ascribed to $C_{10}H_{20}^{\cdot+}$, $C_9H_{17}^{+}$, $C_8H_{15}^{+}$, $C_7H_{13}^{+}$, $C_6H_{11}^{+}$, $C_5H_9^{+}$, $C_4H_9^{+}$ and $C_3H_7^{+}$, respectively.

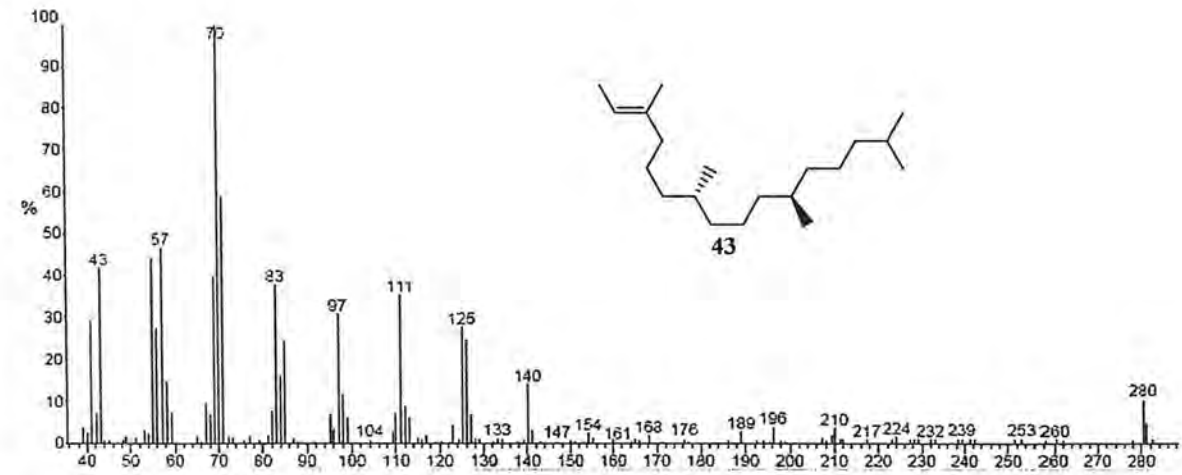


Figure 4.52. Mass Spectrum of [R-[R*,R*-(E)]]-3,7,11,15-tetramethyl-2-hexadecene **(43)**

4.7.7 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (44)

The mass spectrum of compound **44** (Fig. 4.53) gave M^+-18 peak at m/z 278 corresponding to molecular formula $C_{20}H_{38}$. Loss of all methyl substitutes gave a peak at m/z 236. The base peak at m/z 82 is due to $C_6H_{10}^+$, while the peaks at m/z 95 and 123 were ascribable to $C_7H_{11}^+$ and $C_9H_{15}^+$, respectively.

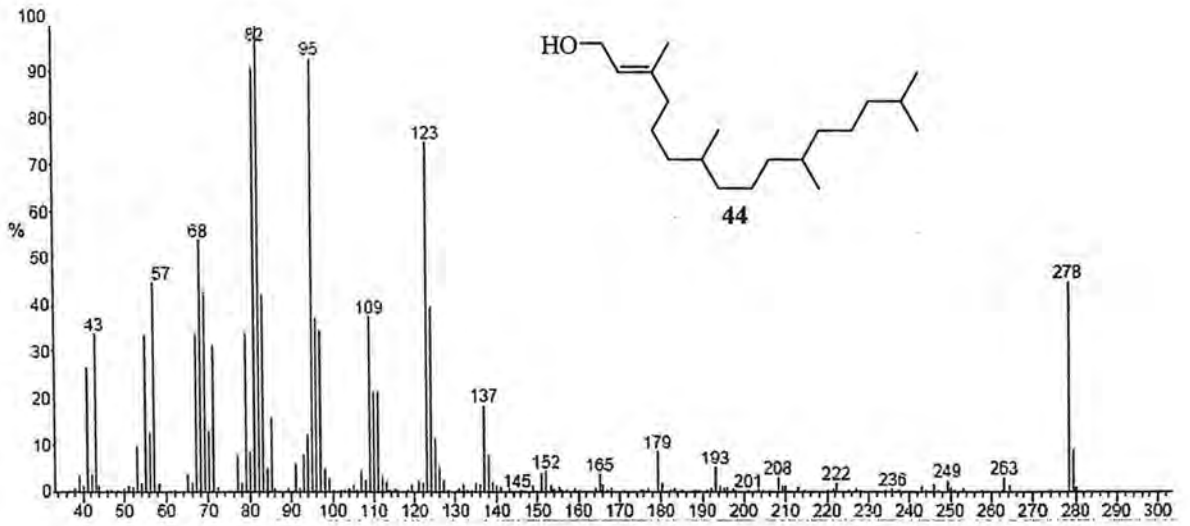


Figure 4.53. Mass Spectrum of 3,7,11,15-tetramethyl-2-hexadecen-1-ol **(44)**

4.7.8 (1-methyldodecyl)benzene (45)

The molecular ion peak of compound **45** was m/z 260 corresponding to molecular formula $C_{19}H_{32}$ (Fig. 4.54). The base peak at m/z 105 corresponded to $C_8H_9^+$. The peaks at m/z 91 and 77 correspond to $C_7H_7^+$ and $C_6H_5^+$, respectively.

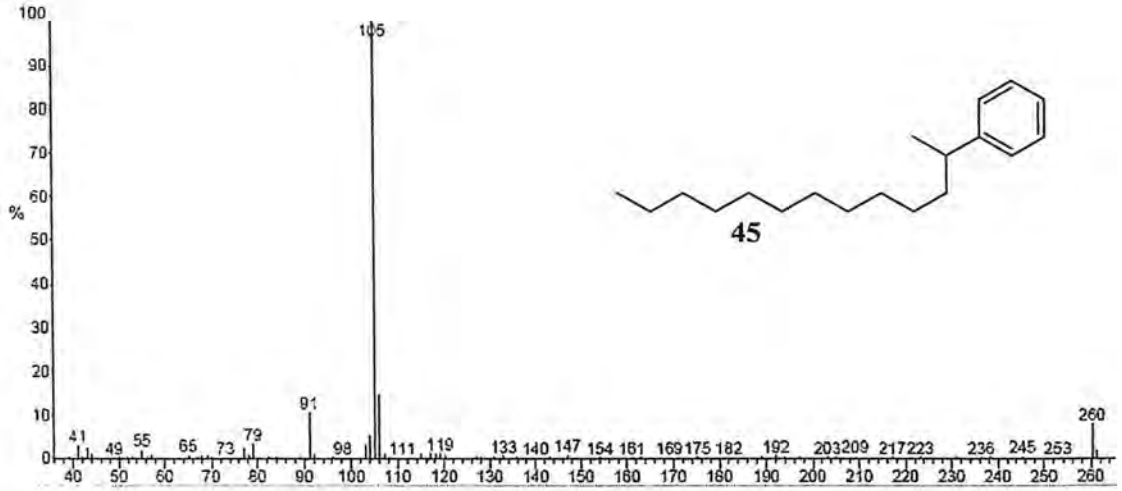
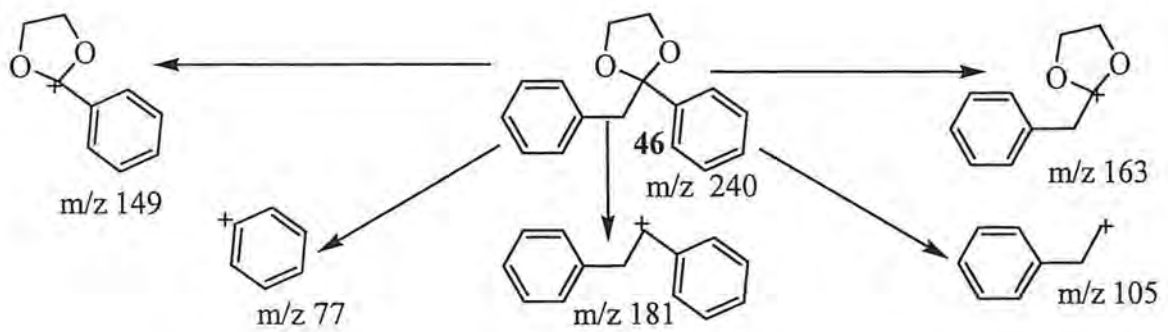


Figure 4.54. Mass Spectrum of (1-methyldodecyl)benzene **(45)**

4.7.9 2-phenyl-2-(phenylmethyl)-1,3-dioxolane (46)

The molecular ion peak of compound **46** at m/z 240 correspond to the formula $C_{16}H_{16}O_2$. The cleavage near oxygen gives peaks at m/z 163 and 149 corresponding to $C_{10}H_{11}O_2^+$ and $C_9H_9O_2^+$, respectively (Fig 4.55, Scheme 4.14). The base peak at m/z 105 was due to $C_8H_9^+$. The peaks at m/z 181 and 77 correspond to $C_{14}H_{13}^+$ and $C_7H_5^+$, respectively.



Scheme 4.14. Proposed fragmentation pattern of 2-phenyl-2-(phenylmethyl)-1,3-dioxolane **(46)**

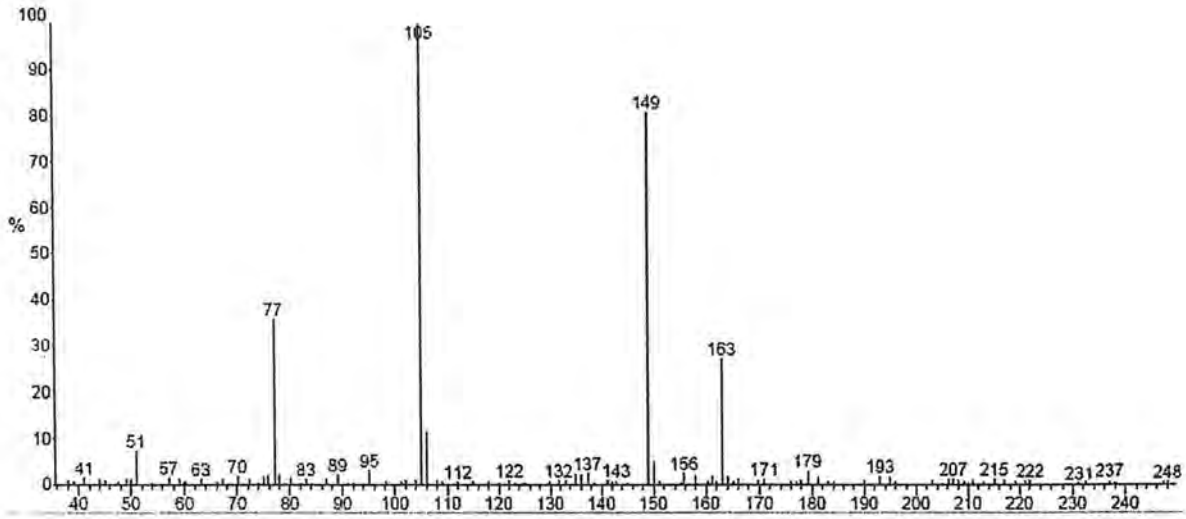


Figure 4.55. Mass Spectrum of 2-phenyl-2-(phenylmethyl)-1,3-dioxolane **(46)**

4.8 Compounds Identified from the Soil Bacteria Culture

Semiochemicals from microbial origin affect oviposition behaviour of gravid *An. gambiae* (Sumba et al., 2004a). The chromatogram obtained from volatiles of cultured bacteria (sterilized nutrient broth plus soil (Fig. 4.56, Fig.4.58)) was compared with the one for sterilized nutrient broth (control (Fig. 4.57, Fig 4.59)). The compounds; dimethyl disulfide **(7)**, dimethyl trisulfide **(8)**, 2-ethyl-1-hexanol **(47)**, 2-phenoxyethanol **(48)**, internal standard (ethyl nonanoate) **(IS)**, tetradecane **(49)**, 2,6-bis(1,1- dimethylethyl)- 2,5-cyclohexadiene-1,4-dione **(50)**, hexadecane **(51)**, octadecane **(52)**, isopropyl myristate **(12)**, 4-hydroxy-4-methyl-2-pentanone **(53)**, 1-undecene **(54)** and 4-phenylmorpholine **(14)** (Table 4.7, 4.8) were identified.

Table 4.7. Compounds identified from volatiles of cultured bacteria (dynamic trapping system)

Compound	Retention time (Min)	Concentration (ng)
Dimethyl disulfide **(7)**	4.527	0.3029
Dimethyl trisulfide **(8)**	10.356	1.4174
2-ethyl-1-hexanol **(47)**	11.563	0.8483
2-phenoxyethanol **(48)**	14.724	1.6203
Tetradecane **(49)**	17.186	1.7678
2,6-bis(1,1- dimethylethyl)- 2,5-Cyclohexadiene-1,4-dione **(50)**	18.154	0.7842
Hexadecane **(51)**	19.638	1.1202
Octadecane **(52)**	21.856	1.5268
Isopropyl myristate **(12)**	22.124	0.7274

Table 4.8. Compounds identified from volatiles of cultured bacteria (static trapping system)

Compound	Retention time (Min)	Concentration (ng)
Dimethyl disulfide **(7)**	4.520	3.0853
4-hydroxy-4-methyl-2-pentanone **(53)**	7.432	1.1342
Dimethyl trisulfide **(8)**	10.356	0.0462
1-Undecene **(54)**	12.650	1.1019
4-phenylmorpholine **(14)**	28.309	0.9663

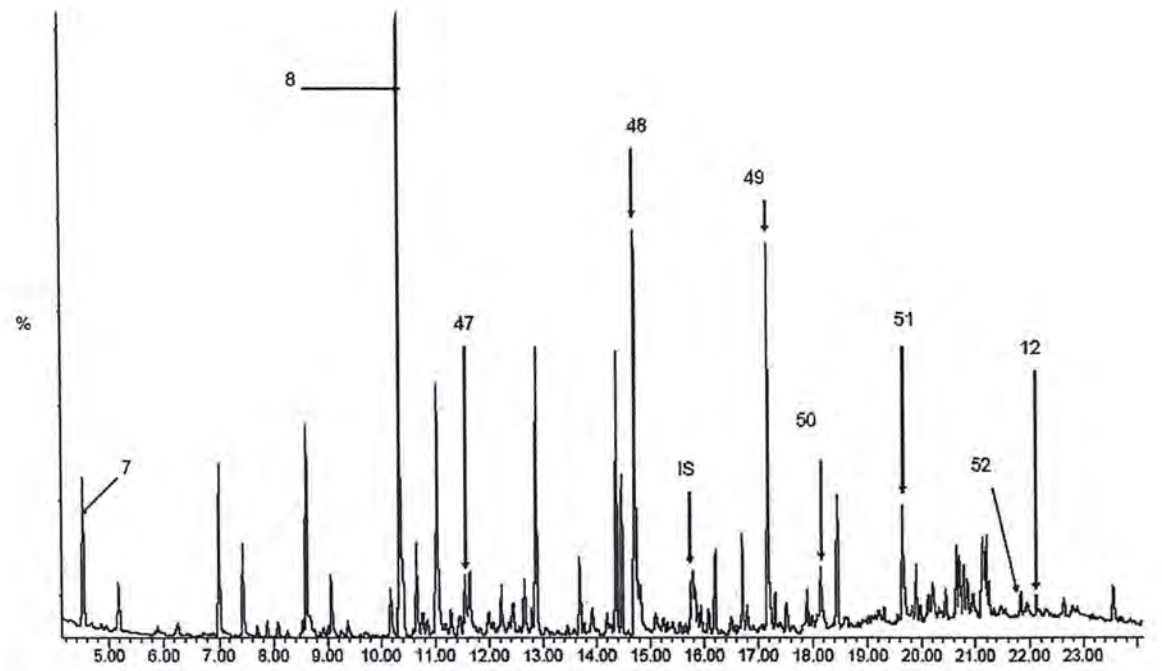


Figure 4.56. Gas chromatogram of volatiles obtained by dynamic trapping system of the soil bacteria volatiles

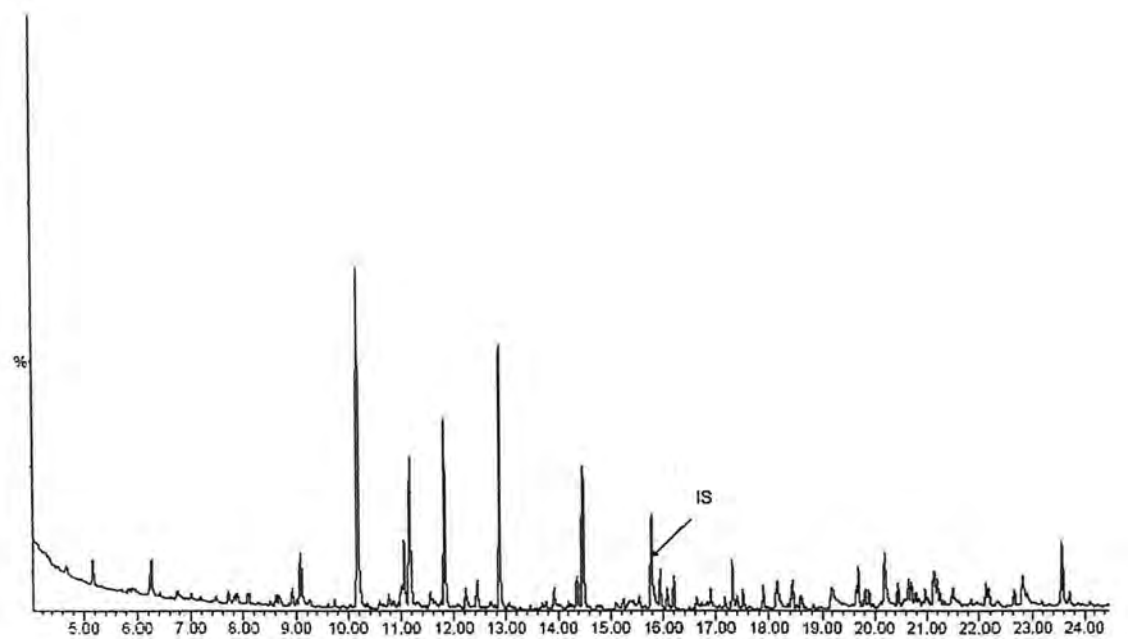


Figure 4.57. Gas chromatogram of volatiles obtained by dynamic trapping system of nutrient broth volatiles

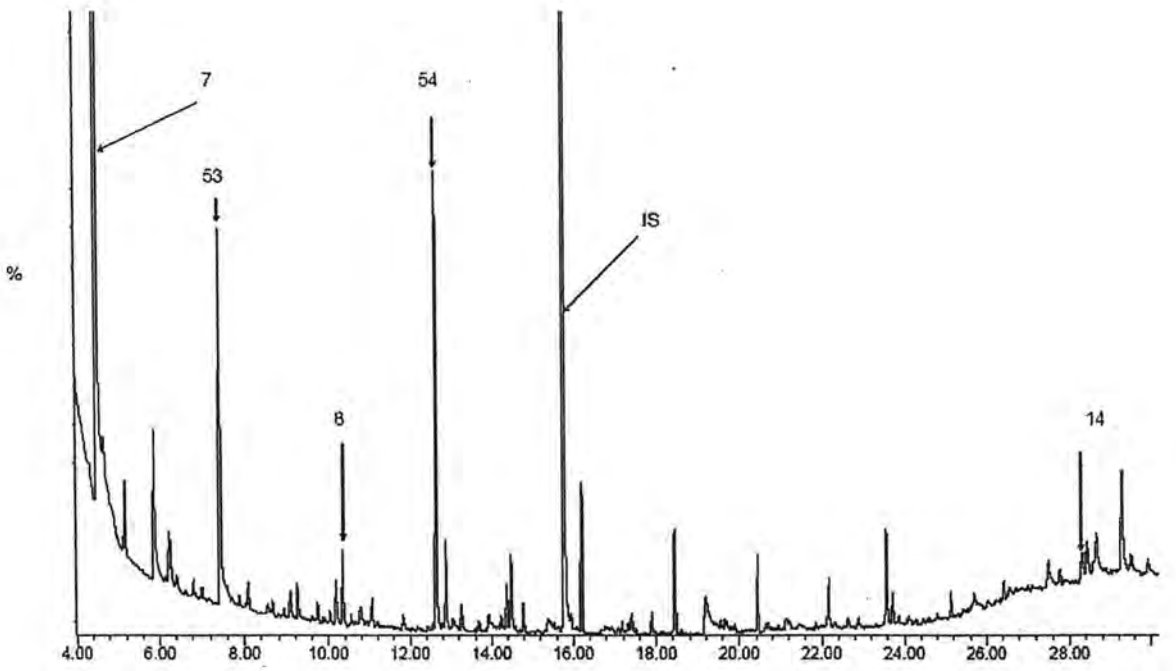


Figure 4.58. Gas chromatogram of volatiles obtained by static trapping system of the soil bacteria volatiles

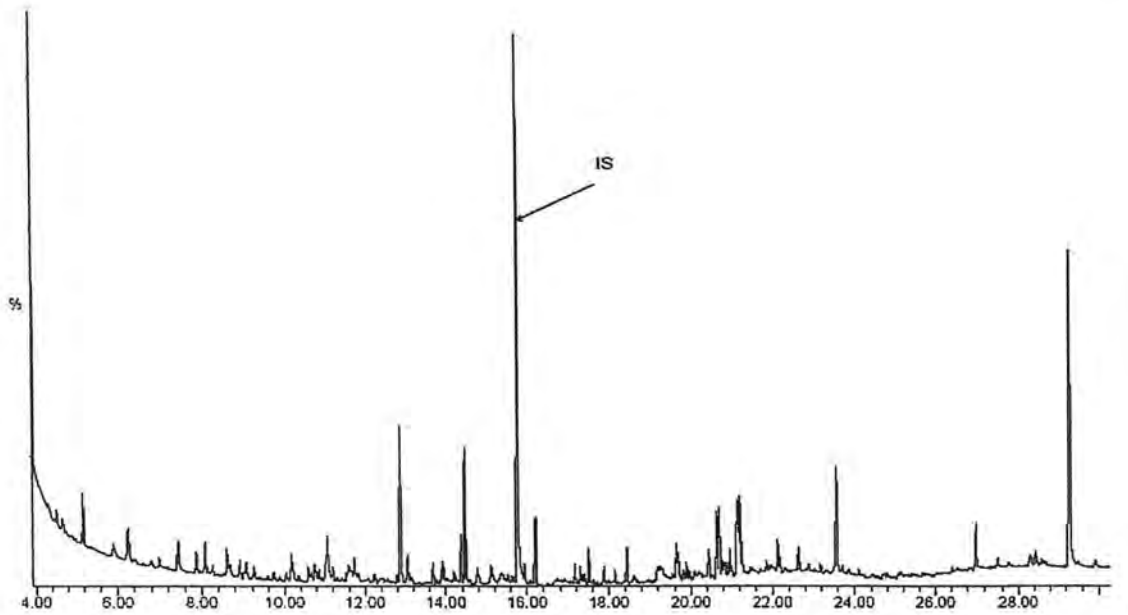
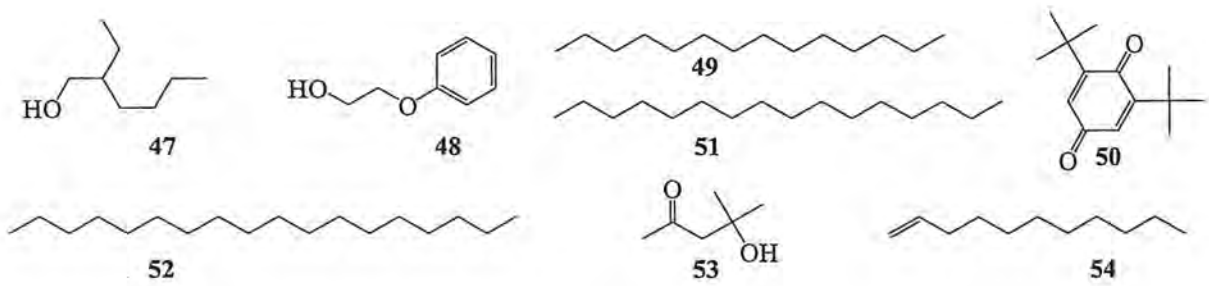
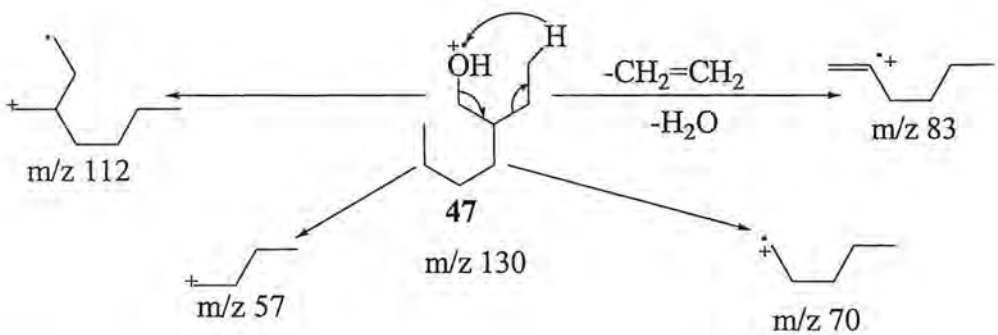


Figure 4.59. Gas chromatogram of volatiles obtained by static trapping system of the nutrient broth volatiles



4.8.1 2-ethyl-1-hexanol (47)

The molecular ion peak of compound **47** was m/z 130 which was weak and corresponds to molecular formula $C_8H_{18}O$. M^+-18 ($C_8H_{16}^{+\cdot}$) peak at m/z 112 was due to loss of water (Fig. 4.60). The base peak at m/z 57 was due to the fragment $C_4H_9^+$. Elimination of water plus an alkene, accounts for the presence of a peak at M-(CH_2=CH_2 + H_2O) m/z 83 corresponding to $C_6H_{11}^{+\cdot}$ (Scheme 4.15). The peak at m/z 70 corresponds to $C_5H_{10}^{+\cdot}$.



Scheme 4.15. Proposed fragmentation pattern of 2-ethyl-1-hexanol (**47**)

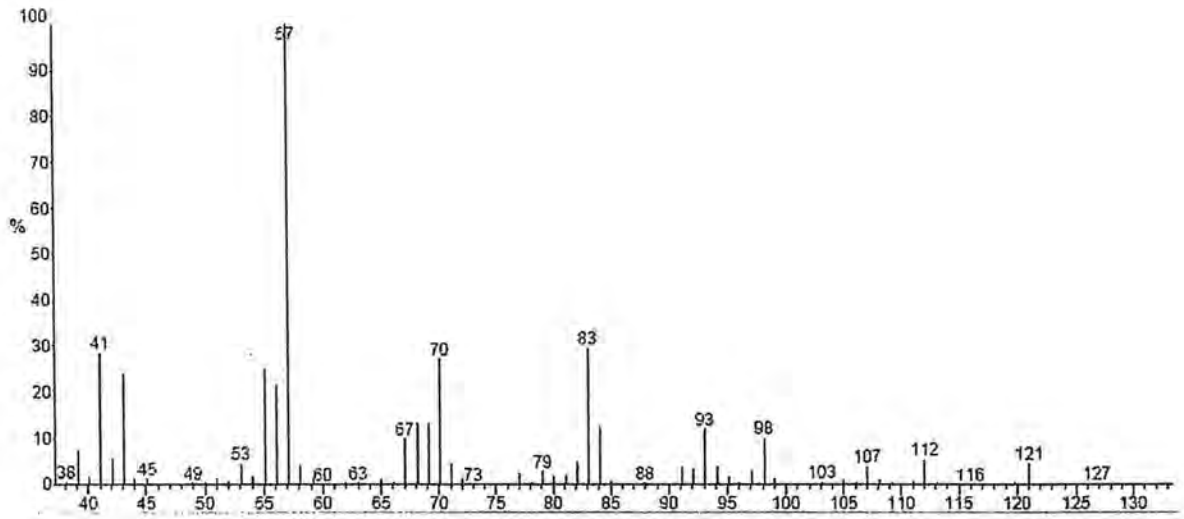
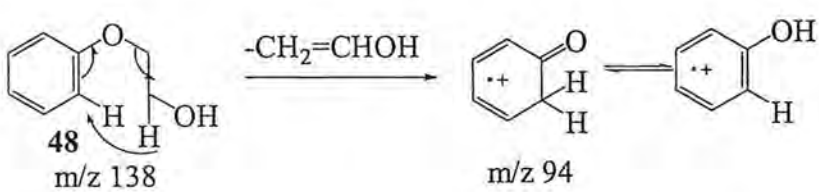


Figure 4.60. Mass Spectrum of 2-ethyl-1-hexanol **(47)**

4.8.2 2-phenoxyethanol (48)

Mass spectrum of compound **48** (Fig. 4.61) exhibited molecular ion peak at m/z 138 corresponding to molecular formula $C_8H_{10}O_2$. The base peak at m/z 94 ($C_6H_6O^{+\cdot}$) is due to β cleavage at the ring accompanied by hydrogen migration (Scheme 4.16). Further loss of CO from the base peak gave a peak at m/z 66 corresponding to $C_5H_6^{+\cdot}$. Cleavage at oxygen atom gave a peak at m/z 93 ascribable to $C_6H_5O^{+\cdot}$. The peak at m/z 77 was due to $C_6H_5^{+}$.



Scheme 4.16. Proposed formation of the base peak at m/z 94

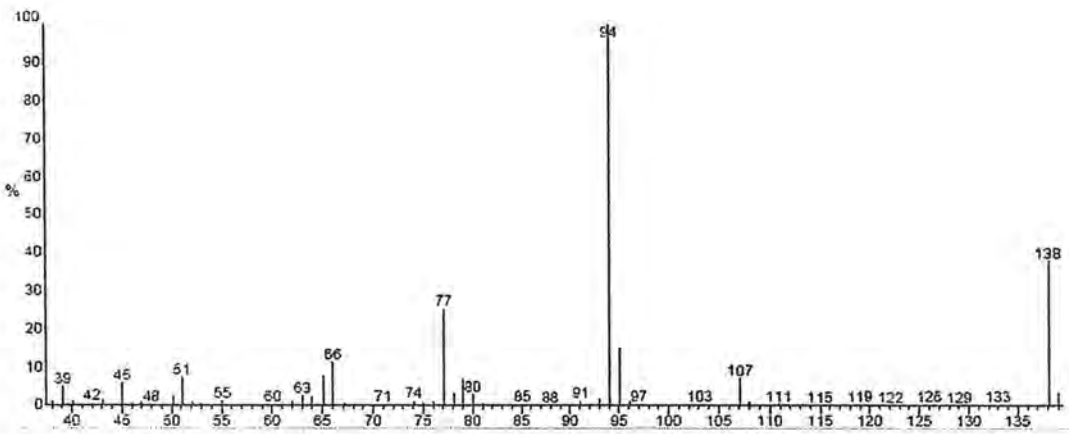


Figure 4.61. Mass Spectrum of 2-phenoxyethanol **(48)**

4.8.3 Tetradecane (49), Hexadecane (51), Octadecane (52)

The molecular ion peaks of compound **49, 51** and **52** are m/z 198, 226 and 254 corresponding to the formulae (Fig. 4.62, Fig. 4.63, Fig. 4.64) $C_{14}H_{30}$, $C_{16}H_{34}$ and $C_{18}H_{38}$ respectively. These were identified as a hydrocarbons due to their fragmentation pattern characterized by clusters of peaks 14 mass units (CH_2) apart. The base peak in all the three compounds was at m/z 57, which was due to $C_4H_9^+$, the peak at m/z 43 corresponded to $C_3H_9^+$, while the peak at m/z 71 was due to $C_5H_{11}^+$.

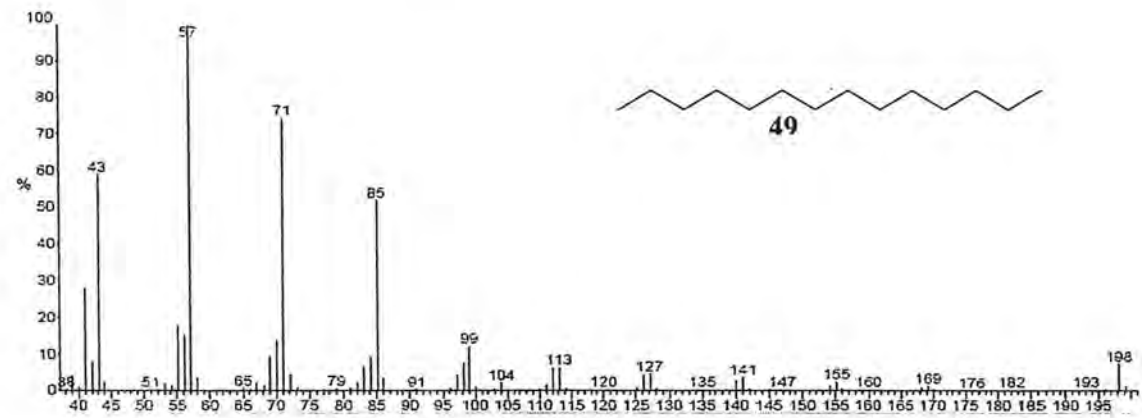


Figure 4.62. Mass Spectrum of tetradecane **(49)**

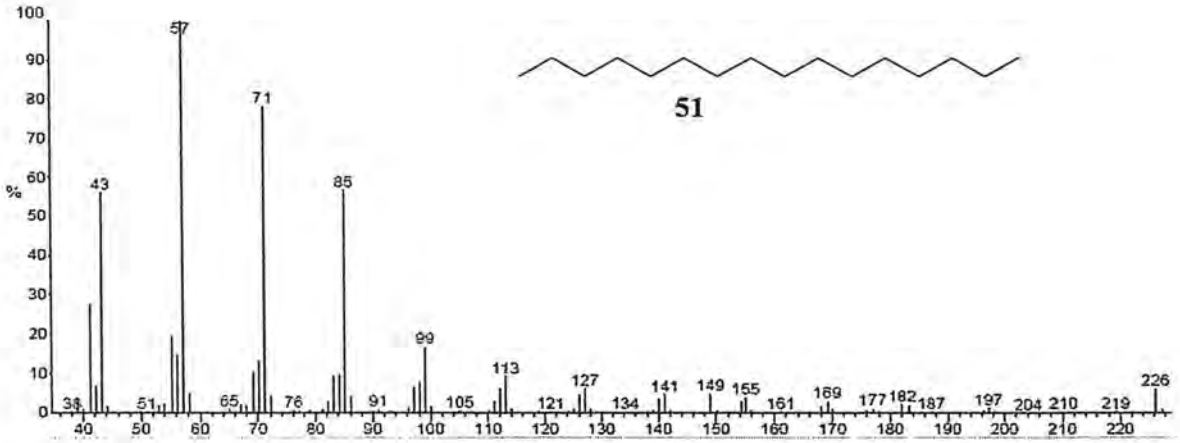


Figure 4.63. Mass Spectrum of hexadecane **(51)**

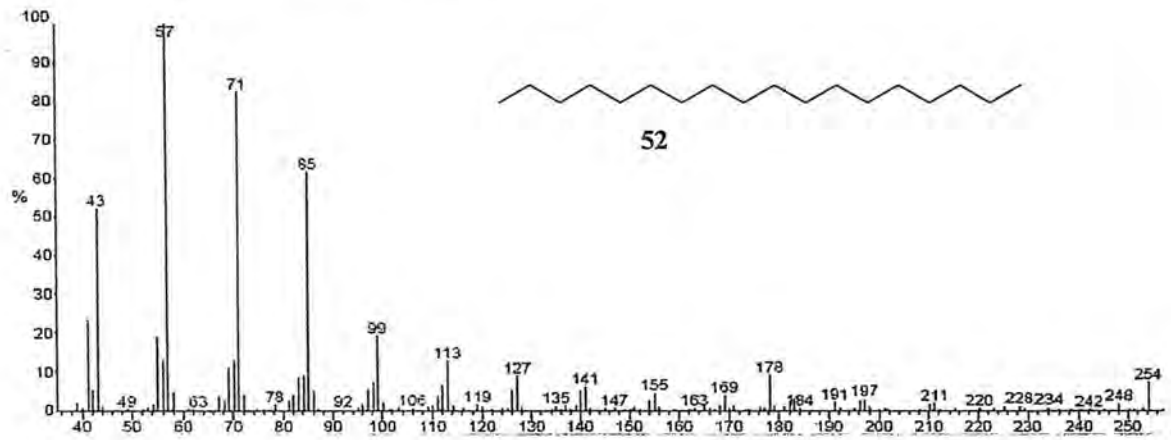


Figure 4.64. Mass Spectrum of octadecane **(52)**

4.8.4 2,6-bis(1,1- dimethylethyl)- 2,5-Cyclohexadiene-1,4-dione (50)

The molecular ion peak of compound **50** at m/z 220 corresponded to molecular formula $C_{14}H_{20}O_2$ (Fig. 4.65). The loss of a methyl group gave a peak at m/z 205 ($C_{13}H_{17}O_2^+$). The base peak at m/z 177 ($C_{11}H_{13}O_2^+$) was due to the loss of C_3H_7. The peaks at m/z 163, 149, 135, 121 and 107 corresponded to $C_{10}H_{11}O_2^+$, $C_9H_9O_2^{\cdot+}$, $C_8H_7O_2^{\cdot+}$, $C_7H_5O_2^{\cdot+}$ and $C_6H_3O_2^+$, respectively.

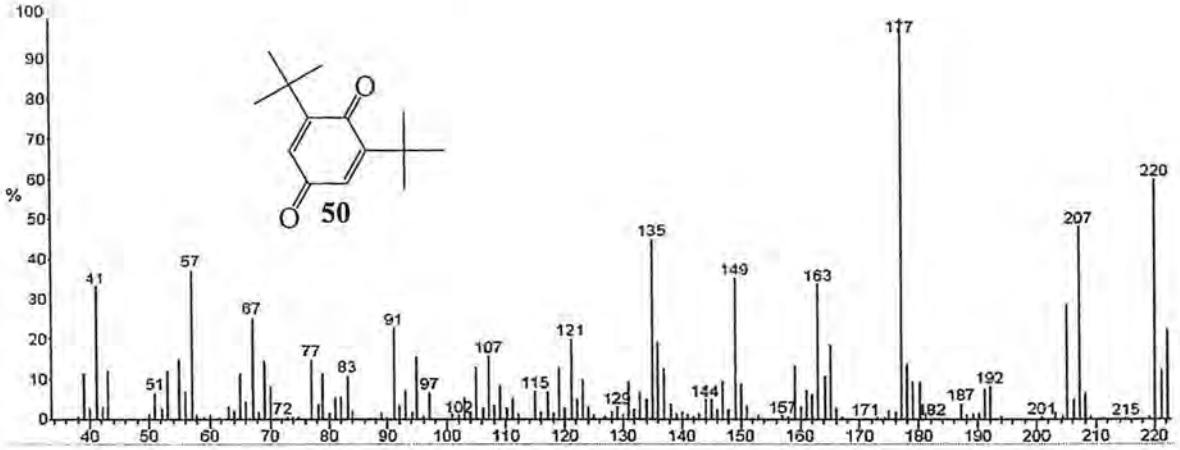
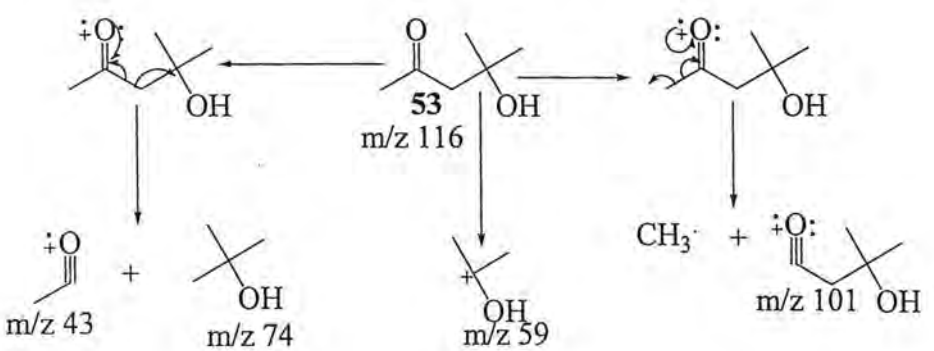


Figure 4.65. Mass Spectrum of 2,6-bis(1,1- dimethylethyl)- 2,5-Cyclohexadiene-1,4-dione **(50)**

4.8.5 4-hydroxy-4-methyl-2-pentanone (53)

The mass spectrum of compound **53** (Fig. 4.66) revealed a peak at m/z 98 consistent with the molecular formula $C_6H_{10}O$ due to loss of water molecule. The α cleavage of **53** resulted in the loss of a larger and smaller alkyl group giving peaks at 43 ($C_2H_3O^{+\cdot}$) and at 101 ($C_5H_9O_2^{+\cdot}$), respectively. Loss of the larger alkyl radical led to the more intense ion peak which was the base peak at m/z 43. The peak at m/z 59 corresponded to the ion $C_3H_7O^+$.



Scheme 4.17. Proposed fragmentation pattern of 4-hydroxy-4-methyl-2-pentanone **(53)**

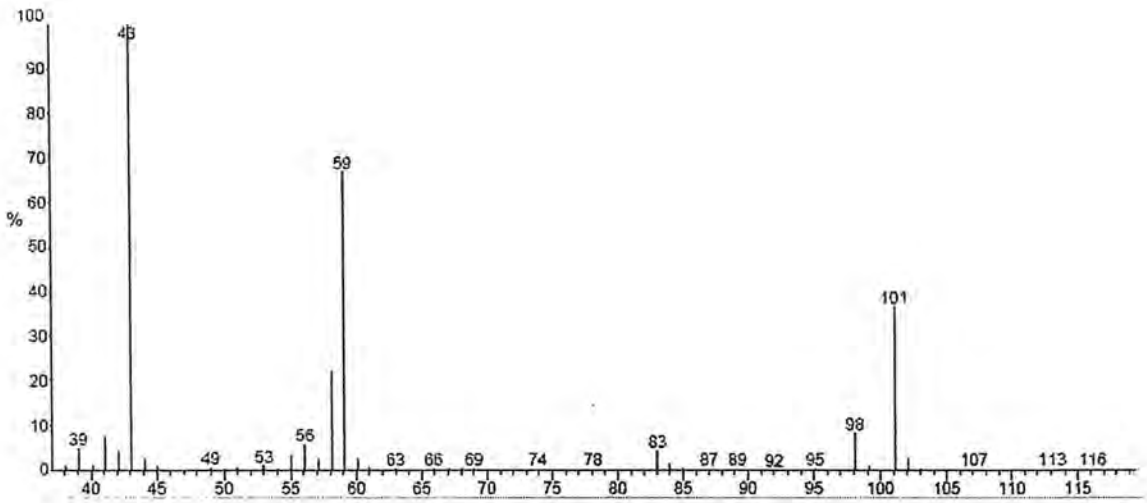


Figure 4.66. Mass Spectrum of 4-hydroxy-4-methyl-2-pentanone **(53)**

4.8.6 1-Undecene (54)

The molecular ion peak of compound **54** was at m/z 154 corresponding to the molecular formula $C_{11}H_{22}$. The C5-C6 bond cleavage in compound **54** gave a base peak at m/z 70 and a prominent peak at m/z 69 corresponding to $C_5H_{10}^+$ and $C_5H_9^+$ (Fig. 4.67). The breakage of allyl-positioned bond gave allyl cation at m/z 41 ($C_3H_5^+$). The peaks at m/z 111, 97, 83 and 55 correspond to $C_8H_{15}^+$, $C_7H_{13}^+$, $C_6H_{11}^+$ and $C_4H_7^+$, respectively.

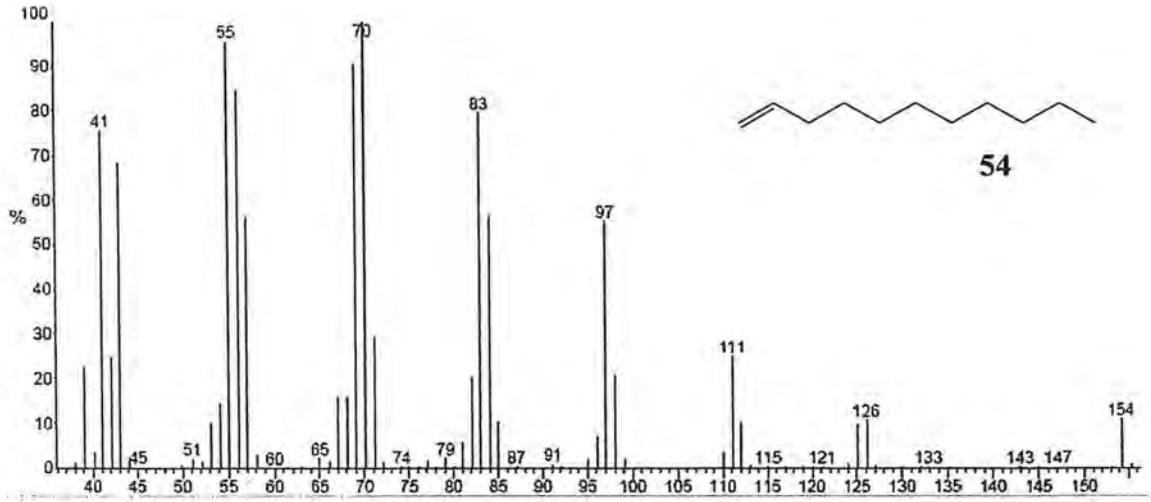


Figure 4.67. Mass Spectrum of 1-Undecene **(54)**

Compound **7, 8, 12** and **14** were also found in volatiles of water containing *C. quinquefasciatus* larvae. Studies by Schulz and Dickschat indicates that compound **7** was found in all 26 strains of *streptomyces spp.*, while compound **8** was present in 23 strains. These two compounds were also emitted by several actinomycetes from different genera (Schulz and Dickschat, 2007).

As earlier discussed in Section 2.2.5 semiochemicals play a role in selection of oviposition site by gravid mosquitoes. Some of the semiochemicals that alter the oviposition decision of gravid *An. gambiae* were identified. In search of their operational use some of the synthetic compounds were tested for their oviposition effect on gravid *An. gambiae* and these is reported Section 4.9 below.

4.9 Oviposition Response of Gravid *Anopheles gambiae* to Some Identified Synthetic Compounds

4.9.1 Dimethyl disulfide (7)

Dimethyl disulfide **(7)** was present in volatiles of *C. quinquifasciatus* larvae (Section 4.2) and from cultured soil bacteria in relatively large amount (Section 4.8). The presence of compound **7** in both samples together with other compounds may have contributed to the oviposition response of gravid *An. gambiae* to *C. quinquifasciatus larvae* (Section 4.1.2). Commercial dimethyl disulfide **(7)** [Aldrich, 99% purity]. This compound, which was available in ICIPE laboratory, was evaluated for its effect on oviposition of gravid *An. gambiae* as described on Section 3.7.3.

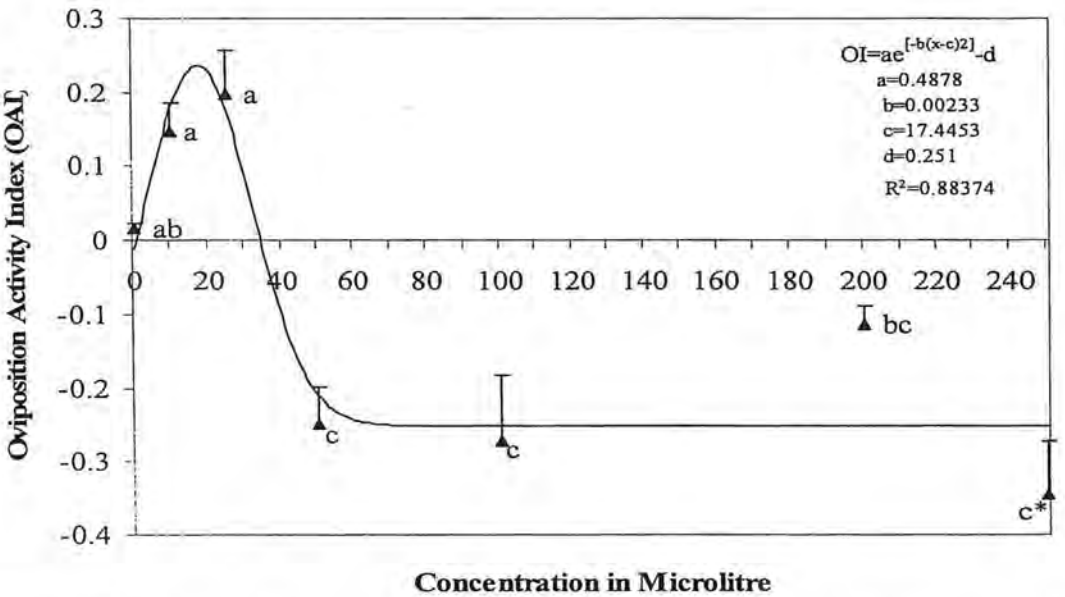


Figure 4.68. Oviposition response of *An. gambiae* to different concentrations of dimethyl disulfide **(7)**

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

Gravid *An. gambiae* laid more eggs in the cup containing dimethyl disulfide **(7)** than in the control cup, when the concentration was low (10 µl to 25 µl equivalent to 10.6 and 26 µg respectively (Fig. 4.68)). Oviposition indices <+0.3 meaning that there was no significant difference from zero. There was significant difference from zero when the amount of dimethyl disulfide was 250 µl corresponding to 265 µg with OAI of -0.34 indicating fewer eggs laid on the cup containing compound **7**. Dimethyl disulfide has not been evaluated before as a stimulant or deterrent of oviposition of *An. gambiae*. Further work on electrophysiological and behavioural assays of the compound mixed with other candidate compounds identified needs to be done in order to evaluate fully the role of this compound in oviposition behaviour of gravid *An. gambiae*.

4.9.2 N-hexadecanoic acid (31) and octadecanoic acid (33)

These two compounds were present in dichrolomethane extracts of *An. gambiae* eggs and that of *C. quinquifasciatus* egg rafts. The compounds were dissolved separately in hexane to make a concentration of 1 ppm. The two compounds were mixed together in the ratio of 1:1. From the mixture, 10, 25 and 50 microlitre were pipetted on to 1 cm x 1 cm glass microfibre filter disc. The procedure described above (Section 3.7.3) was repeated.

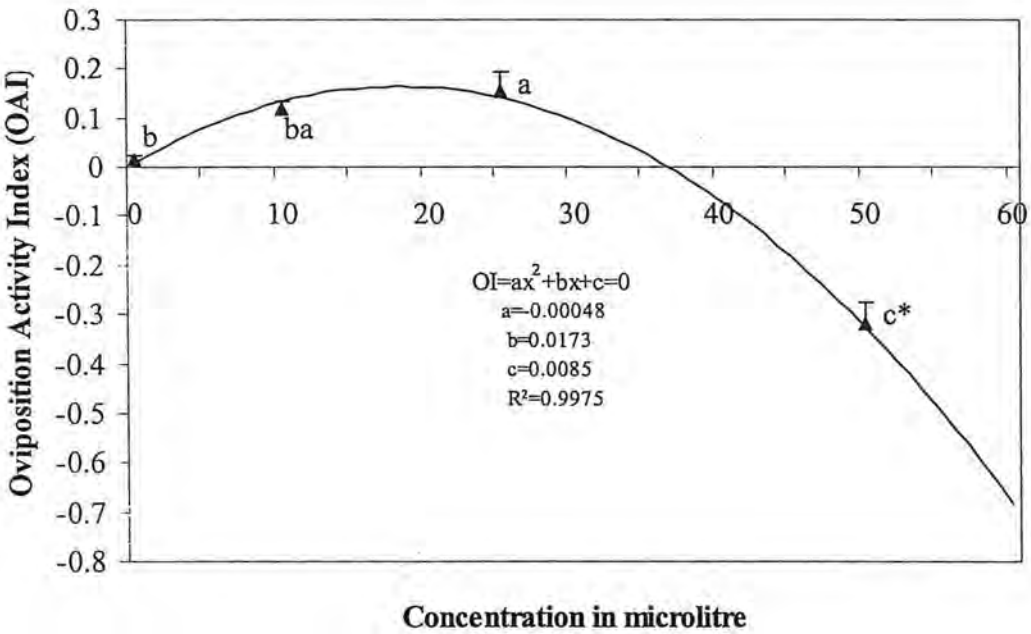


Figure 4.69. Oviposition response of gravid *An. gambiae* to a mixture of two fatty aids (n-hexadecanoic acid **(31)** and octadecanoic acid **(33)**

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

Gravid *An. gambiae* laid more eggs in the cup containing the mixture of the two acids at low concentrations than in control but statistically there was no significant difference from zero. As the concentration increase the gravid *An. gambiae* laid more eggs on control cup than in the cups with the acids.

4.9.3 *Erythro*-6-acetoxy-5-hexadecanolide isomeric mixture

An isomeric mixture of compound **(1)** was obtained from Rothamstead (mixture of +erythro-6-acetoxy-5-hexadecanolide and - erythro-6-acetoxy-5-hexadecanolide). One microlitre of the isomer was dissolved in 1 ml of hexane to make a solution of 1 ppm. Ten, 25, 50, 100, 200 and 250 microlitres were pipetted onto 1 cm x 1 cm glass microfibre filter disc. The procedure described above (Section 3.7.3) was repeated.

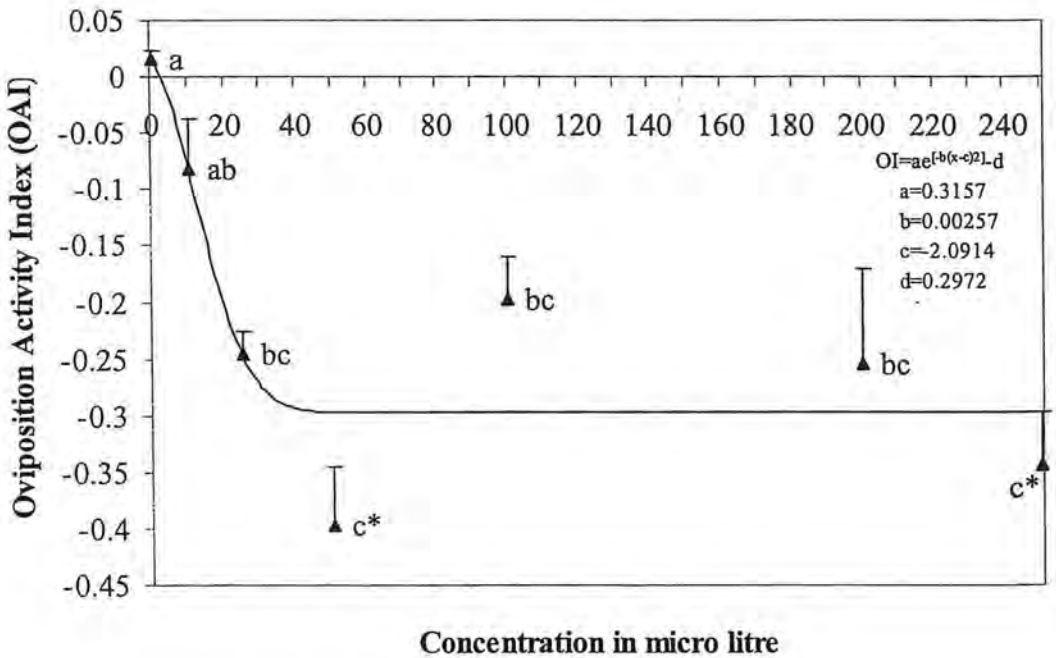


Figure 4.70. Oviposition response of gravid *An. gambiae* to erythro-6-acetoxy-5-hexadecanolide active isomer

*$p<0.05$ significantly differ from zero (t-test); OAIs with different letters at different doses are significantly different from one another (Student-Newman-Keuls Test, $p<0.05$)

Though not detected in the egg extract of *C. quinquifasciatus* the oviposition pheromone of *C. quinquifasciatus* compound **(1)** was previously isolated from the egg rafts of *C.*

quinquifasciatus (Laurence and Pickett, 1982) and has been shown to increase oviposition of *C. quinquifasciatus*. This study shows that at low concentration of the active isomer the gravid *An. gambiae* laid more eggs on control cup than the cup with the isomer although this was not statistically significant. As the concentration increased to 50µl equivalent to 50µg the ($p<0.05$) OAI was -0.39 which was significantly different from zero.

Compound **(1)** together with others identified in *C. quinquifasciatus* egg raft extracts may play a significant role in influencing the oviposition response of *An. gambiae* to different densities of this mosquito's egg rafts in water (Section 4.5.1). Further work involving GC-EAD is necessary to identify other active compounds that may also be involved.

5.0 CONCLUSIONS AND RECOMMENDATIONS

1. Oviposition of *An. gambiae* females was affected by the presence of *C. quinquifasciatus* larvae and/or egg rafts in a dose dependent manner.
2. Eleven compounds were identified and characterized from *C. quinquefasciatus* larval volatiles. This is the first time volatiles from water containing larvae of *C. quinquefasciatus* have been identified.
3. Seven compounds were identified from the extract of test water with the larvae of *C. quinquifasciatus*, five of which were phenols.
4. Nine compounds were identified from the extract from test water.
5. Seven compounds were identified from *An. gambiae* eggs extract of which five were fatty acids, one saturated hydrocarbon and one unsaturated hydrocarbon. Thirteen more compounds were found in trace amounts. This is the first time the compounds from *An. gambiae* eggs have been reported.
6. Seven compounds were identified from *C. quinquifasciatus* egg rafts extract. Six of the compounds were fatty acids while one was an amine. Ten more compounds were found in trace amounts.
7. Eleven compounds were identified from soil volatiles.
8. Twelve compounds were identified after culturing the soil bacteria. These included two sulfides, two phenols, three hydrocarbons, one alkene, two ketones, one ester and morpholine.
9. Ovipositing gravid *An. gambiae* were sensitive to the synthetic compounds tested.

Recommendations for further work

1. Carry out GC-EAD studies on identified compounds and investigate oviposition responses on *An. gambiae* so as to identify the semiochemicals.
2. Further work needs to be done on electrophysiological activity to allow GC-EAD location of potential behaviour-exciting candidates. GC-EAD active compounds should be identified.
3. Bioassay of identified GC-EAD active compounds should be done relative to the concentration present in the sample.
4. The GC-EAD active compounds can be used in combination to establish the most effective blend and a more effective push-pull strategy to manipulate the oviposition behaviour of *An. gambiae*.
5. Bacteria should be identified to the species level and compounds identified and tested for their contribution to behavioural effects on gravid *An. gambiae*.

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